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Detection of 14 alleles derived from the MHC class I *A* locus in cynomolgus monkeys

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Abstract A basic understanding of the major histocompatibility complex (MHC) class I, which, together with T-cell receptors, is a key player in antigen recognition by cytotoxic T lymphocytes, is necessary to study the cellular immune response to intracellular pathogens. The MHC has hardly been reported in cynomolgus monkeys (*Macaca facicularis*), although cynomolgus monkeys have been frequently used as the surrogate animal model. We attempted to determine the nucleotide sequences of the MHC class I *A* locus of cynomolgus monkeys (*Mafa-A*) and eventually 34 independent sequences of *Mafa-A* were obtained from 29 cynomolgus monkeys. These 34 sequences were classified into 14 *Mafa-A* alleles according to the results of phylogenetic analyses using the neighbor-joining method. One to three *Mafa-A* alleles were obtained from a single animal. We also tried to establish a multiplex PCR-SSP method for convenient typing of *Mafa-A* alleles. cDNA from a family of cynomolgus monkeys, which is composed of four sirs and four dams, were examined by multiplex PCR-SSP. The result of multiplex PCR-SSP showed that an individual cynomolgus monkey had two or three *Mafa-A* alleles, suggesting that the *A* locus of cynomolgus monkeys might be duplicated.

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Introduction

The major histocompatibility complex (MHC) class I consists of heavy chain, β_2-microglobulin (β_2m), and antigen peptide (Hennecke et al. 2001). Human cells are known to express three highly polymorphic MHC heavy chains (*HLA-A*, *-B*, and *-C*) and three conserved MHC heavy chains (*HLA-E*, *-F*, and *-G*). *HLA-A*, *-B*, and *-C* present antigen peptides to cytotoxic T lymphocytes (CTL) and the CTL are then activated (Flynn et al. 1992; Hou et al. 1992; York and Rock 1996). These classical molecules, especially *HLA-C*, also provide both stimulatory and inhibitory signals to natural killer (NK) cells through killer cell immunoglobulin-like receptors (KIR) (Valiante et al. 1997).

The gene encoding the class I heavy chain is composed of eight exons. Exon 1 encodes the signal peptide, exons 2–4 specify the extracellular domains α_1–α_3, exon 5 codes for the transmembrane domain, and exons 6–8 code for the cytoplasmic domain. The α_1 and α_2 domains are the most polymorphic, while the α_3 domain contributes to the β_2m association (Hebert et al. 2001) and interaction with the CD8 molecule (Salter et al. 1990).

The rhesus MHC has been extensively studied among non-human primates because rhesus monkeys are most frequently used as the surrogate animal model (Allen et al. 2001; Horton et al. 2001; Mothe et al. 2002) for HIV infection in human. Rhesus MHC (*Mamu*) class I *A* (Boyson et al. 1996b; Miller et al.1991; Urvater et al. 2000a; Voss and Letvin 1996; Watanabe et al. 1994), *B* (Boyson et al. 1996b; Voss and Letvin 1996; Yasutomi et al. 1995), *E* (Boyson et al. 1995), *F* (Otting and Bontrop 1993), *G* (Boyson et al. 1996a), *AG* (Slukvin et al. 1999), and *I* (Urvater et al. 2000b) have already been reported. Rhesus monkeys were shown to carry at least one *A* and two *B* loci, because three *Mamu-A* and five *Mamu-B* alleles have been identified in a single animal (Boyson et

al. 1996b). *HLA-C* homologues have been identified in the common chimpanzee, bonobo, gorilla, and orangutan (Adams et al. 1999, 2000; Cooper et al. 1998; de Groot et al. 2000; Lawlor et al. 1990, 1991), while no evidence of an *HLA-C* homologue was observed in old and new world monkeys (Adams and Parham 2001). Although SIV infection in cynomolgus monkeys is also used as the animal model for human HIV infection (McClure et al. 1990; Putkonen et al. 1992), there are few reports about cynomolgus MHC (*Mafa*) except for class II loci (Gaur and Nepom 1996; Kriener et al. 2000; Otting et al. 1992), class I *E* (Alvarez et al. 1997; Boyson et al. 1995), and *I* loci (Urvater et al. 2000b).

In this study, we have determined the nucleotide sequences of the genes coding for the cynomolgus MHC class I *A* molecules and found 14 *Mafa-A* alleles. In addition, we established a convenient method to detect the *Mafa-A* alleles.

Materials and methods

Animals

All the cynomolgus monkeys were raised and reared in the Tsukuba Primate Center for Medical Science, National Institute of Infectious Diseases. Both genders were involved and the cynomolgus monkeys were between 2 and 23 years old. This study was conducted in accordance with the Guide for Animal Experiments Performed at the National Institute of Infectious Diseases.

RT-PCR and nucleotide sequencing

Peripheral blood mononuclear cells (PBMC) were isolated from the fresh blood of 29 cynomolgus monkeys by a standard Ficoll-Hypaque gradient method. PBMC were washed twice with PBS and suspended in 5 ml of RPMI-1640 (Sigma, St. Louis, Mo.) containing 100 U/ml penicillin (Meiji Seika Kaisha, Tokyo, Japan), 10% FCS (GIBCO-BRL, Grand Island, N.Y.), and 5 μg/ml concanavalin A (ConA; Pharmacia, Cleveland, Ohio) at a concentration of 10^5 cells/ml. PBMC were cultured at 37 °C for 3–4 days. Messenger RNA extracted from the cultured PBMC (2–

Table 1 Primers used for the amplification and sequencing of MHC class I cDNAs from cynomolgus monkeys

Primer	Sequence	Concentration (pmol/sample)	Annealing temperature (°C)
Primers used for RT-PCR			60
Mafa-A-s	5′-GCAGGATCCGAATCTCCCCAGACGCGCA-3′	10	
Mafa-A-a	5′-GCTCTAGACCTCACAAGGCAGCTGTC-3′	10	
Mafa-A13-s	5′-CGAACCCTCCTCCTGG-3′	10	
Mafa-A1013-a	5′-CTGAGAGTAGCTCCCTCCTTTTCTAT-3′	10	
Primers used for multiplex PCR			
Primer set 1			72
IA01-s	5′-GCAGCGGGATGGAGAGGAA-3′	20	
IA02-s	5′-GCTGTGGTTGTGCCTTCTGGAAAA-3′	10	
IA03-s	5′-ACGCTGCAGCGCGCA-3′	2	
IA04-s	5′-GCGGCGGATGTGGCGGAGAG-3′	2	
IA05-s	5′-CTGCGACCTGGGGCCG-3′	2	
IA-a	5′-CCTGGGCACTGTCACTGCTT-3′	20	
Primer set 2			72
IA06-s	5′-GGGCCTGTGCGTGGAGTCCCTG-3′	10	
IA07-s	5′-CACACTGACCTGGCAGCGT-3′	10	
IA08-s	5′-CTGCGACCTGGGGCCA-3′	10	
IA09-s	5′-CTACAACCAGAGCGAGGCCA-3′	10	
IA10-s	5′-GCAGCCCCGCTTCATCT-3′	10	
IA-a	5′-CCTGGGCACTGTCACTGCTT-3′	20	
Primer set 3			70
IA11-s	5′-ACACATGTGACCCATCACCCT-3′	5	
IA12-s	5′-GCCGGAGTATTGGGACCA-3′	20	
IA13-s	5′-GGCCTGCAGGAGATGGAAA-3′	20	
IA14-s	5′-CGGACCTGGGGGCTCAA-3′	15	
IA-a	5′-CCTGGGCACTGTCACTGCTT-3′	20	
Primers used for sequencing			
T7 primer	5′-TAATACGACTCACTATAGGG-3′	3	55
SP6 primer	5′-ATTTAGGTGACACTATAG-3′	3	55
Ia698	5′-TAGAAGCCCAGGGCCCAGGC-3′	3	55
Is437	5′-ATTACATCGCCCTGAACGAG-3′	3	55

$10{\times}10^6$ cells) using a Quick Prep Micro mRNA Purification kit (Pharmacia Biotech, Uppsala, Sweden) were converted into cDNA using a High Fidelity RNA PCR kit (Takara, Shiga, Japan). The amplification of *A* locus was carried out by using specific primer sets, either Mafa-As/Mafa-Aa or Mafa-A13-s/Mafa-A1013-a (Table 1). Forty cycles of amplification were carried out at 94 °C for 30 s, at 60 °C for 30 s, and at 72 °C for 30 s, followed by an additional extension at 72 °C for 7 min using GeneAmp PCR System 9700 (Applied Biosystem, Norwalk, Conn.). The PCR products were cloned into pCR4-Blunt-TOPO plasmid using Zero Blunt TOPO PCR Cloning kit (Invitrogen, Carlsbad, Calif.). The clones were sequenced with sequencing primers T7, SP6, Ia698, and Is437 (Table 1) by an ABI model 310 DNA Sequencer (Applied Biosystem, Foster City, Calif.).

The multiplex PCR-SSP

The multiplex PCR-SSP was carried out using cDNA from cynomolgus monkeys as the template. The primers used are listed in Table 1. Primer set 1 was a mixture of IA-01s, IA-02s, IA-03s, IA-04s, IA-05s, and IA-a, primer set 2 IA-06s, IA-07s, IA-08s, IA-09s, IA-10s, and IA-a, and primer set 3 IA-11s, IA-12s, IA-13s, IA-

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Leader Peptide
                -20        -10
Mafa-A*01  MAVM APRTLLLVLS GALALTQTRA
Mafa-A*02  .... .......... .V........
Mafa-A*03  .... .......... ..........
Mafa-A*04  .... .......... ..........
Mafa-A*05  .... .......... ..........
Mafa-A*06  .... .......... .F......L.
Mafa-A*07  .... .......... .........W
Mafa-A*08  ..I. .......... .........W
Mafa-A*09  .... .......... .........W
Mafa-A*10  ..I. .......... .........W
Mafa-A*11  ..I. .......... ..........
Mafa-A*12  .... .......... .........W
Mafa-A*13  .P.. .......... .........W
Mafa-A*14  .... .......... .V........
Mamu-A*01       .......... ...V......
Mamu-A*02            ..... ..........
Mamu-A*03               .. .........W
Mamu-A*04               .. .V........
Mamu-A*05               .. .........W
Mamu-A*06     . .......... .........W
Mamu-A*07  .... .......... .V.....E.W
Mamu-A*08            ..... .........W
Mamu-A*12  .... .......... .V........
Mamu-B*02  .R.. .......L.. .......E.W
Mamu-B*03  .R.. ....F..L.. .......E.W
Mamu-B*04  .R.. ....F..L.. .......E.W
Mamu-B*05     . ....F..L.. .......E.W
Mamu-B*06               .. .V.....E.W
Mamu-B*07  .R.. .......L.. .........W
Mamu-B*08  .R.. ....F..L.. .......E.W
Mamu-B*09  .R.. ..G....L.. .......E.W
Mafa-E*01           ...L.. —...K.W
Mafa-E*02           ...L.. —...K.W

Alpha 1 domain
                   10         20         30         40         50         60         70         80         90
Mafa-A*01  GSHSMSYFYT SVSRPGRGQP RFIAVGYVDD TQFVRFDSDA ASQRMEPRAP WVEQEGPEYW DRETRNMKTE TQMAPVDLQN LRGYYNQSEA
Mafa-A*02  .......... YM......E. ..V....... .......... .......... .......... ..N..I..A. ..N...N.R. ..........
Mafa-A*03  .....R.... YM.G....E. .......... .......... .......... .......... ..N..I..A. ..N...G.R. ..........
Mafa-A*04  .......... .M.....WE. .......... .......... E......... .......... ..S..Y.... ..N...N.R. ..........
Mafa-A*05  .....R.... .M.....WE. .......... .......... .......... .......... ..N.....A. ..N...N.R. ..........
Mafa-A*06  .....R.... A.......E. .......... .......... .......... .......... .........A ..N...N.R. ..........
Mafa-A*07  .....R..H. CM......E. .......... .......... E.P....... .....E.... ....QK.... ......A.R. ..........
Mafa-A*08  ....LR..H. A.......E. ..FT...... .........H. ..P.K..... .......... .....IS.AN ..NYR.N.R. ..........
Mafa-A*09  ....LR.... .......... .......... .......... E.P.E..... .......... ..N..IY.A. ..NYRES.R. ..........
Mafa-A*10  .....R.... .......... ...S...... .......... E.P....... .......... ....QK.... ..TYRGN.GT .LR.......
Mafa-A*11  .....R..H. A.......E. ..FT...... .......... ..P.K..... .......... EE...IS.AN ..TYREN.RT ALR.......
Mafa-A*12  ....LR.... .M........ .......... .......... E.P.E..... .......... .QN..IY.AA ..TYRGS... ..........
Mafa-A*13  .....R.... .......... .......... .......... .......... .......... EE...IS.AN ..TYRES.R. ..........
Mafa-A*14  ....LR.... .M........ .......... .......... ......S... .......... ...AQ....A .LTYREN.RT ALR.....QG
Mamu-A*01  .....K.... .M........ .......... .......... .......... .......... .......... ..N...N.RT .LR.......
Mamu-A*02  .....R.... .M.....WE. .......... .......... .......... .......... .........A ..N...N.R. ..........
Mamu-A*03  .....R.... .......... ..MS...... .......... E.P.E..... .......... ..N..IY.A. ..N...A.R. ..........
Mamu-A*04  .......... YM......E. ..V....... .......... .......... .......... .....I.... ..N...A.R. ..........
Mamu-A*05  ....LR.... T......... ...S...... .......... E.P.E..... .......... .QN..IC.AD ..TLREN.RT .LR.......
Mamu-A*06  .....R.... .......... ..MS...... .......... E.P.E..... .......... .QN..IC.AD ..TYRES.R. ..........
Mamu-A*07  .....R.... .M........ ...S...... .......... E.P.E..... .......... ..N..IC.AN ..TYRES.R. .LR.......
Mamu-A*08  ....LR.... A......... ...S...... .......... E.P.E..... .......... ..N..IY.AA ..NYREG... ..........
Mamu-A*12  .....R.... .M........ .......... .......... .......... .......... .........A ..TYRES.R. ..........
Mamu-B*02  ...F.R..S. A......RE. WYLE...... .......... E.P....... .......... ..N...S.VT A.TFR.G.G. ........K.
Mamu-B*03  .....R..S. ........E. ...S...... .......... E.P.E..... .M........ EE....A.GH A.TDRA..G. ..........
Mamu-B*04  .....R..SA A.......E. .YLE...... .......... ..P....... .......... EE...RA.GN A.TFR.G.G. ..........
Mamu-B*05  ....LG.... A.......E. L......... .......... E.P...A... .I........ EEQ..RV.GH A.TFR.S.R. ........K.
Mamu-B*06  ....LR..H. A......RE. WYL....... .......... E.P....... .M........ EE...IA.GH A.TERGN.RT .LR......G
Mamu-B*07  ....LR..S. A......RE. WYFE...... .......... E.P....... .M........ EEA..RA.GN A.THRGN.RT ALR......G
Mamu-B*08  .....R..S. ........E. ...S...... .......... E.P.E..... .M........ EE...RA.GH A.TDRA..GT ..........
Mamu-B*09  ....LR..G. T.......E. .......... .......... E.P....... .M........ EE...IA.AR A.TERGN.RT ALR......G
Mafa-E*01  ....LK..H. ........G. ...S...... .....Y.... .......... .......... .Q...SARDT A.TFR.N.ET ..........
Mafa-E*02  ....LK..H. ........G. ...S...... .....Y.... .......... .......... .Q...SARDT A.TFR.N.ET ..........

Alpha 3 domain
                190        200        210        220        230        240        250        260        270
Mafa-A*01  DPPKTHVT HHPVSDYEAT LRCWALGFYP AGITLTWQRD GEEQTQDTEL VETRPAGDGT FQKWAAVVVP SGEEQRYTCH VQHEGLPEPL TLRW
Mafa-A*02  ........ .......... .......... .E........ ..D....... .......... .......... ..K.H..... .......... ....
Mafa-A*03  ........ ......H... .......... EE........ .......... .......... .......... ..K....... .......... ....
Mafa-A*04  ........ ......Q... .......... .E........ ..D....... .......... .......... ..K....... ......R... ....
Mafa-A*05  ........ ......Q... .......... .E........ ..D....... .......... .......... ..K....... ......R... ....
Mafa-A*06  ........ ......H... .....V.... .E........ ..D....... .......... .......... ..K....... .......K.. ..K.
Mafa-A*07  ........ ......H... .......... .E........ .......... .......... .......... ..K....... .......K.. ....
Mafa-A*08  ........ ......H... .......... .E........ ..D....... .......... .......... ..K....... .......... ....
Mafa-A*09  ........ ......H... .......... .E........ ..D....... .......... .......... ..K....... .......K.. ....
Mafa-A*10  ........ ......H... .......... .E........ ..D....... .......... .......... ..K....... .......... ....
Mafa-A*11  ........ ......H... .......... .E........ ..D....... .......... .......... ..K....... .......K.. ....
Mafa-A*12  ........ ......H... .......... .E........ ..D....... .......... .......... ..K....... .......K.. ....
Mafa-A*13  ........ ...I..H... .......... .E....R... ..D....... .........N .......... .......... .......K.. ....
Mafa-A*14  E....... ......H.T. .......... .E........ .......... .......... .......... ..K....... ......TK.. ....
Mamu-A*01  ........ ......H... .......... .E........ ..D....... .......... .......... .......... .......K.H ..K.
Mamu-A*02  ........ ......Q... .......... .E........ ..D....... .......... .......... ..K....... ......R... ....
Mamu-A*03  ........ ......H... .......... .E........ ..D....... .......... .......... .......... .......K.. ....
Mamu-A*04  ........ .......... .......... .E........ ..D....... .......... .......... ..K.H..... .......... ....
Mamu-A*05  .S...... ......H... .......... .E....R... ..D....... .......... .......... ..K....... .......... ....
Mamu-A*06  ........ ......H... .......... .E........ ..D....... .......... .......... .E........ .......K.. ....
Mamu-A*07  ........ ......H... .......... .E........ ..D....... .......... .......... ..K....... .......K.. ....
Mamu-A*08  ........ ......H... .......... .E........ ..D....... .......... .......... ..K....... .......... ....
Mamu-A*12  ........ ......H... .......... .E........ ..D....... .......... .......... ..K....... .......... ....
Mamu-B*02  ........ ......H... .....V.... .E........ ..D....... .......... ....G..... .......... .......... ....
Mamu-B*03  ........ ...I..H... .......... .E........ .......... .....G.... ....G..... ....H..... .......... ....
Mamu-B*04  KR...D.. ......H... .......... .E........ ..D....... .....G.... ....G..... .......... ...Q...... ....
Mamu-B*05  .T...... ...I..H... .......... .E........ .......... .....G.... ....G..... .......... ...K..T..R ....
Mamu-B*06  ........ ...I..H... .......... .E.S...... .......... .....G.... ....G..... .....K.... .......... ....
Mamu-B*07  ........ ......H... .......... .E........ ..D....... .....G.... .......... .......... .......... ....
Mamu-B*08  ........ ......H... .......... .E........ ..D....... .....G.... ....G..... .......... .......... ....
Mamu-B*09  ........ ......H... .......... .E........ .......... .....G...N .......... .......... .......L.. ....
Mafa-E*01  E....... ......H... .......... .E..V..... ..D....... .......... .......... .......... .........R A...
Mafa-E*02  E....... ......H... .......... .E..V..... ..D....... L......... .......... .......... .......... ....

Transmembrane domain
              280        290        300        310
Mafa-A*01  EPSSQS TIPIVGIIAG LVLLGAVVTG AVVAAVMWRR KSS
Mafa-A*02  ...... .......... .......... ....V..... ...
Mafa-A*03  ...... .......... .....T.... .......... ...
Mafa-A*04  ...... .......... .....I..I. ..I...I... ...
Mafa-A*05  ...... ..L....... .....I..I. ..I...I... ...
Mafa-A*06  ...... .......... .......... ...T...... ...
Mafa-A*07  ...... .......L.. .I........ ...S...... ...
Mafa-A*08  ...... .......... .......... ........K. ...
Mafa-A*09  ...... .......... .....T.... ....T...K. ...
Mafa-A*10  ...... .......... .......... ........K. ...
Mafa-A*11  ...... ...M...... .......... .......... ...
Mafa-A*12  ...... ...M...... .......... .......... ...
Mafa-A*13  ...... .......... .......... ........K. ...
Mafa-A*14  ...... .......... .........I ...T.LI... ...
Mamu-A*01  ..F... ...M...... .......... .......... ...
Mamu-A*02  ...... ..L....... .....I..I. ..I...I... ...
Mamu-A*03  ...... ...M...... .......... .......... ...
Mamu-A*04  ...... .......... .......... ....V..... ...
Mamu-A*05  ...... .......... .....T.... .......... ...
Mamu-A*06  ...... ...M...... .......... .......... ...
Mamu-A*07  ....E. .......... .......... ........W. ...
Mamu-A*08  ...... ...M...... .......... .......... ...
Mamu-A*12  ...... ...M...... .......... .......... ...
Mamu-B*02  ...... .......V.. .AV.AV.... .......... ...
Mamu-B*03  ...... .......V.. .AV.AV.... .......... ...
Mamu-B*04  ...... .......V-. .AV.AV.... .......... ...
Mamu-B*05  ...... .......V.. .AV.AV.-.. .......... -..
Mamu-B*06  .....P .......V.. .AV.....I. .......... ...
Mamu-B*07  ...... .......V.. .AV.AV.... ....V..... ...
Mamu-B*08  ...... .......V.. .AV....FI. .......... ...
Mamu-B*09  ...... ....M..V.. .AV.AV.... .......... ...
Mafa-E*01  ....R. .......... .......... ...V...... ...
Mafa-E*02  ...... .......... .......... ...V...... ...
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Fig. 1 Alignment of predicted amino acid sequences of *Mafa-A* with previously reported *Mamu-A*, *Mamu-B*, and *Mafa-E* sequences. Identity to predicted amino acid sequence of *Mafa-A*01* is indicated by *dots*, whereas amino acid replacements are depicted by the conventional *one-letter code*. The deletions of amino acid are indicated by *hyphens*

Cytoplasmic domain

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                   320       330        340
Mafa-A*01    DRKGGSY SQAASNDSAQ GSDVSLTACK V*
Mafa-A*02    ....... .....S.... .......... .*
Mafa-A*03    ....... .....S.... .......... .*
Mafa-A*04    ....... .....S.... .......... .*
Mafa-A*05    ....... .....S.... .......... .*
Mafa-A*06    ....... .....S.... .......... .*
Mafa-A*07    ....... .....S.... .......... .*
Mafa-A*08    ....... .....S.... .......... .*
Mafa-A*09    ....... .....S.... .......... .*
Mafa-A*10    ....... .....S.... .......... .*
Mafa-A*11    ....... .....S.... .......... .*
Mafa-A*12    ....... .....S.... .......... .*
Mafa-A*13    ....... .....S.... .......... .*
Mafa-A*14    ....... .....S.... .......... .*
Mamu-A*01    ....... .....S.... .......... .*
Mamu-A*02    ....... .....S.... .......... .*
Mamu-A*03    ....... .....S.... .......... .*
Mamu-A*04    ....... .....S.... .......... .*
Mamu-A*05    ....... .....S.... .......... .*
Mamu-A*06    ....... .....S.... .......... .*
Mamu-A*07    ....... .....S.... .......... .*
Mamu-A*08    ....... .....S.... .......... .*
Mamu-A*12    ....... .....S.... .......... .*
Mamu-B*02    GG..... .....S.... ........*
Mamu-B*03    GG..... .......... ........*
Mamu-B*04    GG..... .....S.... ........*
Mamu-B*05    GG..... ....WS.... ........*
Mamu-B*06    GG..... .....S.... ........*
Mamu-B*07    GG..... F......... ...E....*
Mamu-B*08    GG..... .......... ........*
Mamu-B*09    GG..... .....SN... ........*
Mafa-E*01    G...... ...SCS..T. ...E...... A*
Mafa-E*02    G...... ...LCS..T. ...E...... A*
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Fig. 1 (continued)

14s, and IA-a. The concentrations of these primers were optimized after preliminary experiments (Table 1). The reactions were heated at 95 °C for 5 min, and then 25 cycles of amplification consisting of denaturation at 95 °C for 30 s, annealing either at 72 °C (for primer set 1 and set 2) or 70 °C (for primer set 3) for 30 s, and extension at 72 °C for 30 s. The amplicons were separated by 1.3% agarose gel electrophoresis and excised bands were purified using Freeze 'N Squeeze Spin Columns (Bio-Rad, Hercules, Calif.). The nucleotide sequences were determined by an ABI model 310 DNA sequencer (Applied Biosystem).

GenBank accession numbers

The *Mafa-A* sequences described in this manuscript have been deposited at GenBank and were assigned accession numbers AB154760–AB154773. The GenBank accession numbers for all sequences used in this studies are as follows: *Gogo-B*0401*, AF157407; *Gogo-B*0501*, AF157408; *Gogo-B*0502*, AF157409; *Gogo-C*0103*, AF157410; *Gogo-C*0204*, AF157411; *HLA-A*0201*, AY365426; *HLA-A*0301*, L77702; *HLA-A*2402*, L47206; *HLA-A*2602*, M98453; *HLA-A*2901*, U83415; *HLA-A*8001*, L18898; *HLA-B*0801*, D83956; *HLA-B*1301*, D50290; *HLA-B*1802*, D25275; *HLA-B*2702*, L38504; *HLA-B*4201*, L76225; *HLA-B*57*, M32318; *HLA-B*7301*, U04787; *Mamu-A*01*, MMU50836; *Mamu-A*02*, MMU50837; *Mamu-A*03*, MMU41379; *Mamu-A*04*, MMU41380; *Mamu-A*05*, MMU41831; *Mamu-A*06*, MMU41834; *Mamu-A*07*, MMU41832; *Mamu-A*08*, AF243179; *Mamu-A*12*, AF157398; *Mamu-B*02*, MMU41833; *Mamu-B*03*, MMU41825; *Mamu-B*04*, MMU41826; *Mamu-B*05*, MMU41827; *Mamu-B*06*, MMU41828; *Mamu-B*07*, MMU41829; *Mamu-B*08*, MMU41830; *Mamu-B*09*, MMU41835; *Patr-A*0301*, AF500288; *Patr-A*0501*, AF500289; *Patr-A*0601*, AF500290; *Patr-A*11*, L47291; *Patr-A*14*, L47292; *Patr-B*01*, L47293; *Patr-B*16*, L47296; *Patr-B*17*, L47348; *Patr-C*03*, L47294; *Patr-C*04*, L47347; *Patr-C*05*, L47298; *Patr-C*06*, L47299; *Popy-A*0302*, AY034115; *Popy-A*0401*, AY034116; *Popy-A*0402*, AY034117; *Popy-A*0501*, AY034114; *Popy-B*0701*, AF118895; *Popy-B*0601*, AF118894; *Popy-B*0801*, AF118896; *Popy-B*0302*, AF118891; *Popy-B*0401*, AF118892; *Popy-B*0501*, AF118893; *Popy-C*0201*, AF118898; *Popy-C*0203*, AF470378; *Popy-C*0301*, AF470379; and *Popy-C*01, 012* AF470376.

Results

Detection of 14 MHC class I *A* locus alleles in cynomolgus monkeys

As there was no report on genes coding for class I MHC molecules in cynomolgus monkeys, we attempted to determine the nucleotide sequences of the alleles of the *A* locus in cynomolgus monkeys (*Mafa-A*). The amplicons obtained using *A*-locus-specific primers were cloned into pCR4-Blunt-TOPO plasmid and 8–48 independent clones were sequenced for each animal. When more than two clones from each animal showed the identical nucleotide sequences, the sequence was regarded as a consensus sequence representing a particular allele. Since PCR amplification and cloning procedures as well as sequencing are prone to misincorporation of nucleotides, amino acid sequences deduced from the nucleotide sequences were assessed by phylogenetic analysis using the neighbor-joining method (Saitou and Nei 1987) (data not shown). When the nucleotide sequence variation of the clone was negligible ($d<0.025$) compared with the consensus sequence, this particular clone was not considered to represent an independent allele. On the other hand, clones showing sequence variation of $d\geq 0.025$ were considered to represent independent alleles.

The deduced amino acid sequences of *Mafa-A* alleles are shown in Fig. 1, together with those published for *Mamu-A* and *-B*. All *Mafa-A* alleles encoded proteins of 365 amino acids. The putative glycosylation site was located at residue 86. In addition, conserved cysteine residues occurred at positions 101 and 164 in α_2 and at positions 203 and 259 in α_3. Other areas of similarity with *Mamu* included a region of variability at residues 77–83 near the C terminus of the α_1 helix, analogous to the site of the Bw4/Bw6 motif in human.

To ascertain that these alleles were indeed derived from cynomolgus MHC class I *A* locus, they were compiled with other primate MHC class I loci (Fig. 2) and a phylogenetic tree was constructed using the amino acid sequences of α_1 and α_2 domains, since both domains were the most polymorphic regions of the molecule. The result showed that the sequences from cynomolgus monkeys aligned within the same cluster as those from other non-human primates, indicating that these cDNA clones were derived from distinct alleles of the MHC class I *A* locus of cynomolgus monkeys, *Mafa-A*.

A family consisting of eight animals was subjected to genetic analysis for better understanding of inheritance of *Mafa-A* alleles (Fig. 3, Table 2). By nucleotide sequence analysis, four *Mafa-A* alleles (*Mafa-A*03*, *-A*07*, *-A*11*, and *-A*14*) were found in this family. *Mafa-A*03* was detected in monkeys 2010 and 3005, whereas *Mafa-A*07* was detected in monkeys 8225 and 2010. *Mafa-A*11* was carried by monkeys 9234, 5117, 4045, 5076, and 1102,

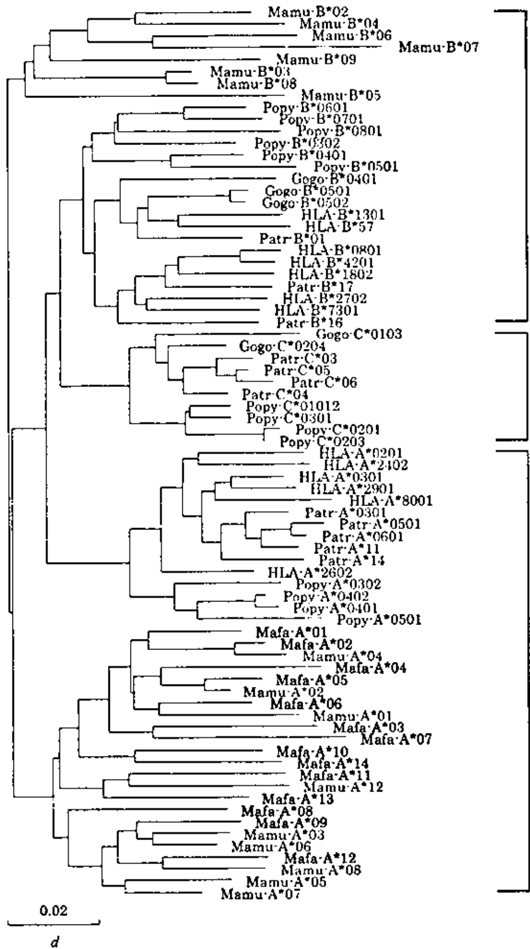

Fig. 2 Phylogenetic analysis of primate class I MHC molecules. The phylogenetic tree of α_1–α_2 was constructed using nucleotide sequences with the neighbor-joining method by DNASIS pro (Hitachi software, Yokohama, Japan)

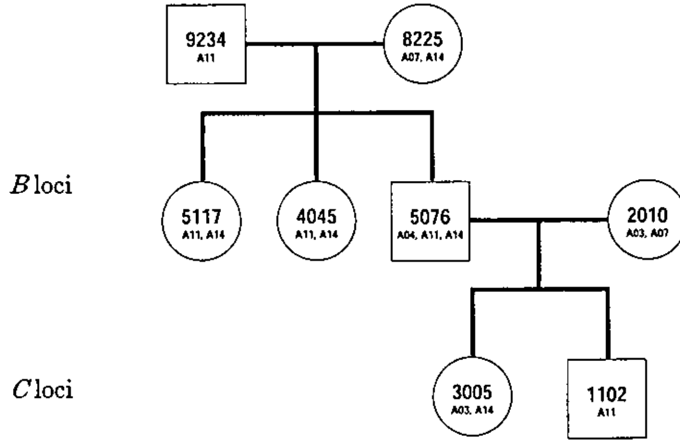

Fig. 3 The family pedigree demonstrating the inheritance of alleles of the MHC class I *A* locus in cynomolgus monkeys. Male or female is denoted by a *square* or *circle*, respectively. The animal number and allele assigned to the animal are shown

while *Mafa-A**14* was shared by monkeys 8225, 5117, 4045, 5076, and 3005. By nucleotide sequence analysis, only one allele (*Mafa-A**11*) was detected in 1102.

The establishment of the multiplex PCR-SSP method

We tried to develop a convenient method for MHC typing among relatively large numbers of animals. We have modified an existing PCR-SSP for detection of multiple allele simultaneously. The concentrations of primers and the annealing temperatures, which appeared critical, were described in Materials and methods in detail. To know whether this method was actually able to detect the *Mafa-A* alleles, plasmids harboring cDNA inserts for respective *Mafa-A* alleles were subjected to multiplex PCR-SSP. As shown in Fig. 4, distinct bands were amplified using appropriate primer sets, although the presence of non-specific bands was also noticed. The cDNAs obtained from members of the family mentioned above were analyzed by multiplex PCR-SSP. *Mafa-A**03* was found in monkeys 2010 and 3005. *Mafa-A**04* was detected in monkeys 5117, 4045, 5076, and 1102, while *Mafa-A**07* was detected in three monkeys: 8225, 2010 and 1102. *Mafa-A**08* was detected only in monkey 8225, while *Mafa-A**11* was shared by monkeys 5117, 4045, 5076, and 1102. *Mafa-A**14* was carried by monkeys 8225, 5117, 4045, 5076, and 3005. The results are summarized in

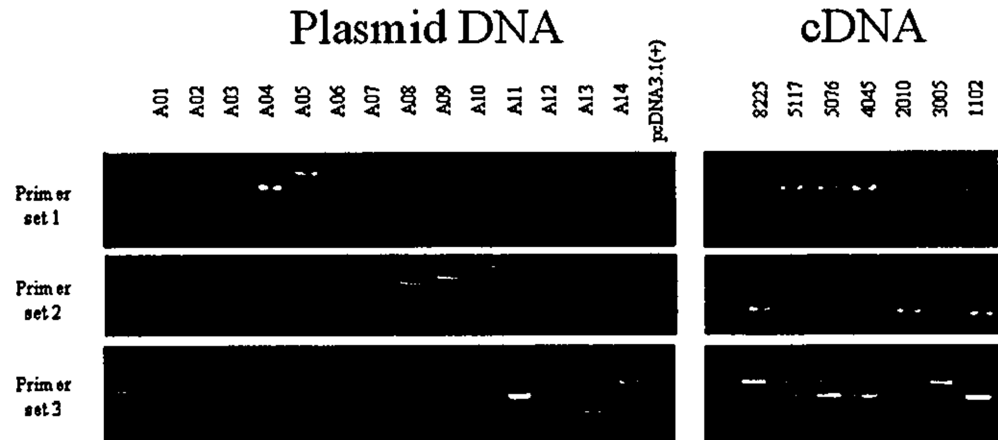

Fig. 4 Multiplex PCR-SSP analyses of *Mafa-A* alleles. Identification of *Mafa-A**01* through *A**14* was conducted using cloned genes inserted into pcDNA3.1(−) vector (*left panel*). Copy DNAs obtained from seven cynomolgus monkeys were similarly subjected to multiplex PCR-SSP (*right panel*)

Table 2 Summary of MHC class I cDNAs isolated from cynomolgus monkeys with RT-PCR and sequencing

Animal	Allele	Number of copies	Primer pair/s
9234	*A11*	8	Mafa-A-s/Mafa-A-a
8225	*A07*	5	Mafa-A-s/Mafa-A-a
	A14	31	Mafa-A-s/Mafa-A-a
5117	*A11*	20	Mafa-A-s/Mafa-A-a
	A14	11	Mafa-A-s/Mafa-A-a
4045	*A11*	35	Mafa-A-s/Mafa-A-a
	A14	7	Mafa-A-s/Mafa-A-a
5076	*A04*	6	Mafa-A13-s/Mafa-A1013-a
	A11	30	Mafa-A-s/Mafa-A-a, Mafa-A13-s/Mafa-A1013-a
	A14	10	Mafa-A-s/Mafa-A-a, Mafa-A13-s/Mafa-A1013-a
2010	*A03*	37	Mafa-A-s/Mafa-A-a, Mafa-A13-s/Mafa-A1013-a
	A07	9	Mafa-A13-s/Mafa-A1013-a
3005	*A03*	39	Mafa-A-s/Mafa-A-a, Mafa-A13-s/Mafa-A1013-a
	A14	15	Mafa-A-s/Mafa-A-a, Mafa-A13-s/Mafa-A1013-a
1102	*A11*	33	Mafa-A-s/Mafa-A-a

Table 3 Summary of typing alleles with sequencing and multiplex PCR-SSP analysis on cynomolgus monkeys (*ND* not done)

Animal	Sequencing	Multiplex PCR-SSP
9234	*A11*	ND
8225	*A07, A14*	*A07, A08, A14*
5117	*A11, A14*	*A04, A11, A14*
4045	*A11, A14*	*A04, A11, A14*
5076	*A04, A11, A14*	*A04, A11, A14*
2010	*A03, A07*	*A03, A07*
3005	*A03, A14*	*A03,* A14
1102	*A11*	*A04, A07, A11*

Table 3. Since the presence of the *Mafa-A*08* allele was only demonstrated by the multiplex PCR-SSP, the PCR amplicon was subjected to partial nucleotide sequence analysis. The result revealed that the DNA fragment identified as *Mafa-A*08* allele by multiplex PCR-SSP contained, in addition to that of an *Mafa-A* allele per se, the sequence derived from an allele that had not been found before. The results of similar analyses with large numbers of animals were consistent with the view that one to four *Mafa-A* alleles were present in an animal (data not shown). These results strongly suggested that the *A* locus in cynomolgus monkeys had been duplicated.

Discussion

The polymorphic nature of MHC genes is particularly important in antigen recognition because it provides a way of presenting huge numbers of antigens by comparably limited numbers of MHC molecules. Numerous reports on the association of MHC alleles with susceptibility to HIV-1 infection in humans have been published; the prevalence of *HLA-B*14*, *-B*27*, and *-B*57* was shown to be significantly high among slow progressors, while a possible association of *HLA-B*35* and *-B*44* with shorter survival time was also demonstrated in HIV-1 infected patients (Gierowska et al.1999; Hendel et al. 1999; Migueles et al. 2000; Nelson et al. 1997). In SIV-infected rhesus monkeys, *Mamu-A*01* and *-A*1303* have been shown to be associated with not only longer survival time but also lower set-point viral load (Muhl et al.2002). However, as far as we know, there is no literature describing the associations of MHC polymorphism with progression of the disease in SIV-infected cynomolgus monkeys. Moreover, even the nucleotide sequence analyses have not been performed with regard to the class I MHC genes in cynomolgus monkeys. We therefore attempted to determine the nucleotide sequences of the MHC *A* locus genes. *Mafa-A* was amplified using *A*-locus-specific primer pairs. The results showed that at least 14 independent alleles of the class I *A* locus were found in cynomolgus monkeys.

We also have succeeded in developing a multiplex PCR-SSP method which enables us to readily detect several *Mafa-A* alleles simultaneously. When cDNA derived from cynomolgus monkey 8225 was analyzed by this method, however, the presence of a *Mafa-A* allele not detected by the usual RT-PCR was demonstrated. It seemed likely, therefore, that the primer pair designed to amplify *Mafa-A*08* was not specific enough to discriminate the *Mafa-A*08* allele from one with a very similar nucleotide sequence. Similarly, the primer pair designed to detect *Mafa-A*04* amplified a DNA fragment from not only the authentic *Mafa-A*04* allele but also an allele quite similar to *Mafa-A*04*. It seemed, therefore, premature to use only PCR-SSP for detection of either *Mafa-A*04* or *Mafa-A*08*; however, the method could be applied for detection of other alleles. Since we could not rule out completely the possibility that the primers designed to detect other alleles would cross-react with alleles yet to be found, further accumulation of the nucleotide sequences of *Mafa-A* alleles is required.

It was reported that rhesus MHC class I haplotype consisted of at least one *Mamu-A* and at least two *Mamu-B*

(Boyson et al. 1996b). No evidence for the presence of a *HLA-C* homologue was observed, suggesting that the evolution of the *C* locus in gorillas, chimpanzees, and humans is a fairly recent occurrence (Adams et al. 1999, 2000; Boyson et al. 1996b; Cooper et al. 1998; de Groot et al. 2000, Lawlor et al. 1990, 1991). This study showed that an individual cynomolgus monkey had two or three *Mafa-A* alleles, suggesting that the *A* locus of cynomolgus monkeys might be duplicated.

The amino acids that composed the Mafa-A pocket were compared with those for the *Mamu-A* pocket. From a crystallographic analysis, a peptide of 8–11 amino acids was demonstrated to bind into the groove of the MHC molecule, which was made up of α_1 and α_2 domains. In the case of the most HLA molecules, the second amino acid of the peptide is thought to play a crucial role in binding to the HLA molecule in conjunction with the C-terminal amino acid of the peptide. In the case of *Mamu-A*01*, however, the most important anchor residue was

Fig. 5 Amino acid residues of pockets B, C, D, and F probably determining the peptide-binding specificity of cynomolgus monkey MHC class I molecule. Amino acids identical with *Mamu-A*01* are indicated by *dashes*

MHC allele	B pocket residue No.										
	7	9	24	25	34	45	63	66	67	70	99
Mamu-A*01	Y	Y	A	V	V	M	E	N	M	E	V
Mamu-A*1303,NA4	-	-	-	-	-	-	-	-	-	A	-
Mafa-A*01	-	-	-	-	-	-	N	I	-	-	Y
Mafa-A*02	-	-	-	-	-	-	N	I	-	-	Y
Mafa-A*03	-	-	-	-	-	-	S	I	-	-	Y
Mafa-A*04	-	-	-	-	-	-	N	Y	-	-	L
Mafa-A*05	-	-	-	-	-	-	-	-	-	-	Y
Mafa-A*06	-	-	-	-	-	-	-	-	-	A	-
Mafa-A*07	-	H	-	-	-	-	-	K	-	-	Y
Mafa-A*08	-	H	T	-	-	K	N	I	S	N	Y
Mafa-A*09	-	-	-	-	-	E	-	I	Y	-	Y
Mafa-A*10	-	-	S	-	-	-	-	K	-	-	Y
Mafa-A*11	-	H	T	-	-	K	N	I	S	N	F
Mafa-A*12	-	-	-	-	-	E	-	I	Y	A	Y
Mafa-A*13	-	-	-	-	-	-	-	I	S	N	Y
Mafa-A*14	-	-	-	-	-	-	-	-	-	A	Y

MHC allele	C pocket residue No.								
	9	22	70	73	74	97	99	114	116
Mamu-A*01	Y	F	E	N	A	R	V	E	Y
Mamu-A*1303,NA4	-	-	A	-	-	-	-	-	-
Mafa-A*01	-	-	-	M	-	T	Y	-	F
Mafa-A*02	-	-	-	-	-	M	Y	D	F
Mafa-A*03	-	-	-	-	-	I	Y	S	F
Mafa-A*04	-	-	-	-	-	-	L	D	H
Mafa-A*05	-	-	-	-	-	K	Y	H	S
Mafa-A*06	-	-	A	-	-	-	-	-	-
Mafa-A*07	H	-	-	M	-	V	Y	S	-
Mafa-A*08	H	-	N	-	Y	K	Y	-	F
Mafa-A*09	-	-	-	-	Y	K	Y	-	S
Mafa-A*10	-	-	-	T	Y	T	Y	-	-
Mafa-A*11	H	-	N	T	Y	-	F	S	-
Mafa-A*12	-	-	A	T	Y	-	Y	S	-
Mafa-A*13	-	-	N	T	Y	-	Y	S	H
Mafa-A*14	-	-	A	T	Y	T	Y	D	S

MHC allele	D pocket residue No.					
	99	114	155	156	159	160
Mamu-A*01	V	E	S	M	Y	L
Mamu-A*1303,NA4	-	-	R	-	-	-
Mafa-A*01	Y	-	Q	-	-	-
Mafa-A*02	Y	D	Q	H	-	-
Mafa-A*03	Y	S	Q	W	-	-
Mafa-A*04	L	D	-	-	-	-
Mafa-A*05	Y	H	-	F	-	-
Mafa-A*06	-	-	Q	I	-	-
Mafa-A*07	Y	S	Q	H	-	-
Mafa-A*08	Y	-	Q	H	-	-
Mafa-A*09	Y	-	Q	-	-	-
Mafa-A*10	Y	-	Q	W	-	-
Mafa-A*11	F	S	-	F	-	V
Mafa-A*12	Y	S	-	F	-	V
Mafa-A*13	Y	S	Q	I	-	-
Mafa-A*14	Y	D	Q	Q	-	-

MHC allele	F pocket residue No.			
	77	80	81	116
Mamu-A*01	N	T	L	Y
Mamu-A*1303,NA4	A	N	-	-
Mafa-A*01	D	N	-	F
Mafa-A*02	-	N	-	F
Mafa-A*03	G	N	-	F
Mafa-A*04	-	N	-	H
Mafa-A*05	-	N	-	S
Mafa-A*06	-	N	-	-
Mafa-A*07	A	N	-	-
Mafa-A*08	-	N	-	F
Mafa-A*09	S	N	-	S
Mafa-A*10	-	-	-	-
Mafa-A*11	-	-	A	-
Mafa-A*12	S	N	-	H

identified at the third position of the antigen peptide (Allen et al. 1998; Dzuris et al. 2000; Sidney et al. 2000). On the other hand, anchor motif analysis of *Mamu-B*03* showed that the second as well as the C-terminal amino acids of the peptide was indispensable for binding to the MHC molecule (Dzuris et al. 2000). The second amino acid of the antigenic peptide interacts with the B pocket composed of the amino acids shown in Fig. 5. A close look at the amino acid sequences suggested that a peptide presented by *Mafa-A*06* might have a similar motif to the peptide presented by *Mamu-A*1303*, as amino acids involved in the formation of the B pocket were identical in both MHC molecules. Since it was shown that *Mamu-A*01* and -*A*1303* were associated with longer survival and lower set-point viral load (Nelson et al. 1997) in SIV-infected rhesus monkeys, it would be of interest to look at whether the presence of the *Mafa-A*06* allele in a cynomolgus monkey would affect the regulation of viral replication of SIV.

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