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Genomic plasticity of the MHC class I *A* region in rhesus macaques: extensive haplotype diversity at the population level as revealed by microsatellites

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Abstract The *Mamu-A* genes of the rhesus macaque show different degrees of polymorphism, transcription level variation, and differential haplotype distribution. Per haplotype, usually one "major" transcribed gene is present, *A1* (*A7)*, in various combinations with "minor" genes, *A2* to *A6*. In silico analysis of the physical map of a heterozygous animal revealed the presence of similar *Mamu-A* regions consisting of four duplication units, but with dissimilar positions of the *A1* genes on both haplotypes, and in combination with different minor genes. Two microsatellites, D6S2854 and D6S2859, have been selected as potential tools to characterize this complex region. Subsequent analysis of a large breeding colony resulted in the description of highly discriminative patterns, displaying copy number variation in concert with microsatellite repeat length differences. Sequencing and segregation analyses revealed that these patterns are unique for each *Mamu-A* haplotype. In animals of Indian, Burmese, and Chinese origin, 19, 15, or 9 haplotypes, respectively, could be defined, illustrating the occurrence of differential block duplications and subsequent rearrangements by recombination. The haplotypes can be assigned to 12 unique combinations of genes (region configurations). Although most configurations harbor two transcribed *A* genes, one or three genes per haplotype are also present. Additionally, haplotypes lacking an *A1* gene or with an *A1* duplication appear to exist. The presence of different transcribed *A* genes/alleles in monkeys from various origins may have an impact on differential disease susceptibilities. The high-throughput microsatellite technique will be a valuable tool in animal selection for diverse biomedical research projects.

Keywords MHC · Microsatellites · Macaque · Physical map · Duplications · Disease susceptibility

Doxiadis and Groot contributed equally to the manuscript.

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Introduction

Gene products of the major histocompatibility complex (MHC), a multicopy gene family present in all jawed vertebrates, play a crucial role in adaptive immune responses. MHC class I molecules are involved in the binding and presentation of intracellularly generated peptides to $CD8^+$ T cells, whereas class II molecules present peptides from extracellular origin to $CD4^+$ T cells. The key feature of the *Mhc* class I and II genes is the high degree of allelic variation (polymorphism) and gene copy number variation (diversity) that is observed between as well as within species (Kelley et al. 2005). Because of its prominent role in disease susceptibility and/or resistance (Stephens 2005; Goulder and Watkins 2008; Barton and Worthington 2009; Kaur and Mehra 2009; Ramagopalan et al. 2009), transplantation biology (van Rood 1975; Doxiadis

et al. 2004), reproductive success (Ziegler et al. 2005) and even stress management (Gleimer and Parham 2003), the MHC system and its polymorphisms have been extensively studied in humans (HLA; Bodmer 1987; Little and Parham 1999) and nonhuman primates such as rhesus macaques, which are often used as preclinical model species (Bontrop 2001; Carrington and Bontrop 2002; Bontrop and Watkins 2005; t Hart et al. 2005; Vierboom et al. 2005).

Equivalents of the classical *HLA-A* and *HLA-B* genes are present in rhesus macaques as well, and are designated *Mamu-A* and *Mamu-B,* whereas an ortholog of *HLA-C* is missing in rhesus and other macaque species (Boyson et al. 1996; Vogel et al. 1999). The *Mhc* class I and II gene families have been subjected to several rounds of duplications (Kulski et al. 1997, 1999; Dawkins et al. 1999) and have evolved according to birth and death processes (Klein et al. 1993; Nei et al. 1997). Thus, new genes have been created by gene duplications or complex recombination processes, whereas others have been deleted or were inactivated and became pseudogenes. Different duplication models have been proposed, of which the segmental or tandem block duplication models appear to give the most plausible explanation for the contemporary class I gene organization (Kulski et al. 1997; Dawkins et al. 1999). A tandem duplication history of 28 duplicons has been suggested for the *Mamu-A* region, which is indeed three times larger than in humans, and each tandem appears to contain at least one class I-like sequence, specific *Alu* elements, and an endogenous retroviral HERV16 segment (Kulski et al. 2004). As a consequence, on each chromosome (haplotype), more than one copy of a *Mamu-A* gene always seems to be present. Furthermore, the number and content of the *A*-genes may be different per haplotype, a phenomenon classified as region configuration polymorphism (Doxiadis et al. 2000). This phenomenon is most prominently observed for the *B* region of rhesus macaques, in which up to eight *Mamu-B* alleles are transcribed per haplotype, of which one to three show a high, the others a low transcription level. Differential transcription levels, however, are also described for *Mamu-A* genes (Otting et al. 2005, 2007, 2008).

Most of the region configurations contain a polymorphic *Mamu-A1* gene characterized by its high transcription level in combination with one or two oligomorphic genes designated *Mamu-A2* up to *Mamu-A6*. In former times, the polymorphic *A* locus transcribed at high levels, and encoding the serotype in Indian origin rhesus macaques, was called *A1*. Subsequently, a second *A* transcript present on the same haplotype was named *A2,* a third *A3,* and so on. Furthermore, the division in loci was performed according to the clustering of alleles in phylogenetic analyses; the *A2* locus alleles, for example, have a 162 bp insertion in intron 2 in common. (Otting et al. 2005). Some haplotypes contain an additional highly transcribed locus, which was named *A7* according to its different phylogenetic relationship and the presence of another *A1* gene on the same chromosome (Otting et al. 2007, 2009). A schematic representation illustrating the configurations of transcribed *Mamu-A* genes on a haplotype and its nomenclature has been provided (Fig. 1). Configuration nos. 8 to 12 (Fig. 1) have been discovered in the course of the present study and will be discussed later. One should keep in mind that nothing is known about the exact order and distances of the *A* genes on the genome except for configurations 1 and 5, which have been confirmed by genomic sequencing data (Daza-Vamenta et al. 2004; Shiina et al. 2006).

In the past, products of the *Mamu-A1* gene of rhesus macaques have been characterized by serological typing procedures (Bontrop et al. 1995). This method, however, is restricted to monkeys of specific, mainly Indian, origin, since adequate typing sera are missing for monkeys of different origins or from other macaque species. Until now, laborious full-length complementary deoxyribonucleic acid (cDNA) cloning and sequencing has mostly been performed for accurate typing of the polygenic *Mamu-A* family (Otting et al. 2007, 2009; Pendley et al. 2008; Campbell et al. 2009) or segmental analysis by pyrosequencing (Wiseman et al. 2009). Therefore, high-throughput, robust, and cost-effective typing techniques are urgently needed.

Closely linked markers, such as microsatellites, have already been successfully used for characterization of the whole MHC region (Penedo et al. 2005; Wiseman et al. 2007; Wojcechowskyj et al. 2007), the *B* (Bonhomme et al. 2008; Doxiadis et al. 2009b), and the *DR* region in different macaque and other primate species (Doxiadis et al. 2007, 2009a, 2010; de Groot et al. 2008, 2009). In a pilot study, six microsatellites mapping close to the human *HLA-A* gene have been tested as candidates for the characterization of the *Mamu-A* region, and two of them, D6S2854 and D6S2859, have been chosen because of their robustness and length variability. Since the *Mamu-A* loci may be duplicated on a given haplotype, a duplication of adjacent microsatellites could be expected as well and was indeed observed in the pilot study. Therefore, an in silico search of the physical map of one heterozygous rhesus monkey (Daza-Vamenta et al. 2004; Shiina et al. 2006) has been performed to identify the copy number of the markers, their position, and association with *Mamu-A* genes and pseudogenes. As transposable elements (TEs) such as HERV16 seem to play a role in duplication processes (Kulski et al. 2004; Doxiadis et al. 2009b), repeat masking was applied to learn more about the presence and position of TEs in relation to the localization of the two microsatellites and the *Mamu-A* genes/pseudogenes. Finally, a large breeding colony of rhesus macaques, covering different geographic origins, has been analyzed using the two microsatellites. The results were confirmed by full-length sequencing of

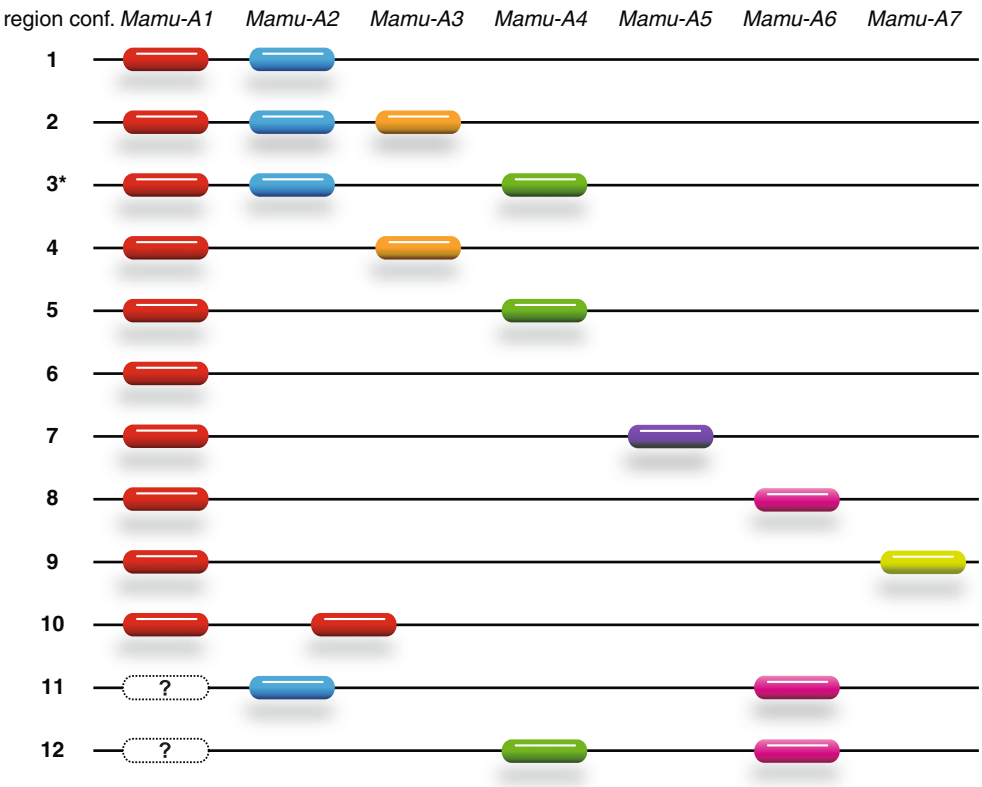

Fig. 1 Schematic representation of *Mamu-A* region configurations observed in Indian, Chinese, and Burmese rhesus macaques. Only transcribed *Mamu-A* loci are represented and color-coded. The order and distances of all loci, including those of region configuration 1 and 5 of the physical map, are schematically drawn. * Region configuration 3 has not been detected in this study

Mamu-A alleles to validate the practical efficiency of this high-throughput haplotyping method.

Materials and methods

Animals and DNA/RNA isolation

The BPRC houses a self-sustaining colony of approximately 1,000 rhesus macaques mainly of Indian but also of Burmese and Chinese origin. A large collection of DNA samples as well as B-cell lines is available. For this study, genomic DNA of 819 Indian, 101 Burmese, and 14 Chinese/Indian breed rhesus macaques was analyzed. The DNA was extracted from EDTA blood samples or from immortalized B-cell lines using a standard salting-out procedure. Ribonucleic acid (RNA) was isolated from PBMCs or B cells (Rneasy kit, Qiagen, Heiden, Germany).

Mamu-A D6S2854 and D6S2859 genotyping

The polymerase chain reaction (PCR) amplification of microsatellite D6S2854, a (TAAA)n repeat, was performed under the same conditions as previously described (Wiseman et al. 2007), with the exception that the 5′ primer (D6S2854-forward-VIC: TCATGAGCGTGGCACTGCAC) is VIC labeled and the 3′ primer (D6S2854-reverse: CCGTATGTTG-CAACCAGGAG) is unlabeled. D6S2859 was observed in humans as a $(TA)_n$ repeat (Gourraud et al. 2004). In rhesus macaques, it is observed as a mixed dinucleotide $(TA)_x(CA)_y$ repeat, and the primers have been newly designed according to the physical map of the rhesus macaque MHC (Daza-Vamenta et al. 2004) and named D6S2859-forward-FAM: CGTTCACCCTGGCATTCCAT and D6S2859-reverse: CCCCTGATCCAGAAGCCTTG. Both primer pairs were synthesized by Applied Biosystems (Foster City, CA, USA). Both PCR reactions were multiplexed in a 25 μl reaction volume containing one unit of *Taq* polymerase (Invitrogen, Paisley, Scotland) with 0.3 μM of the forward and reversed primer of D6S2859, 0.1 μM of the forward and reversed primer of D6S2854, 5 mM $MgCl_2$, 0.2 mM of each dNTP, 1×PCR buffer II (Invitrogen), and 100 ng DNA. The cycling parameters were a 5 min 94°C initial denaturation step, followed by five cycles of 1 min at 94°C, 45 s at 58°C, and 45 s at 72°C. The program was followed by 25 cycles of 45 s at 94°C, 30 s at 58°C, and 45 s at 72°C. A final extension step was performed at 72°C for 30 min. The amplified DNA was prepared for genotyping and analyzed on an ABI 3130*XL* genetic analyzer (Applied Biosystems). STR analysis was performed using the Genemapper software (Applied Biosystems).

cDNA cloning and sequencing

RNA was subjected to one-step reverse transcriptase (RT)-PCR as recommended by the supplier (Promega, Madison,

WI, USA). The primers (5′MAS) AATTCATGGCGCCCCGAACCCTCCTCCTGG, and (3′MAS) CTAGACCACACAAGGCGGCTGTCTCAC were used, which are specific for class I *A* transcripts in macaques. The final elongation step was extended to 10 min to generate a 3′dA overhang. The RT-PCR products were cloned in the pDrive cloning vector using the Qiagen PCR cloning kit according to the manufacturer's guidelines (Qiagen). After transformation in *Escherichia coli-XLblue*, 16 to 48 colonies were selected, and plasmid DNA was isolated using a standard mini-preparation procedure, and sequencing reactions were performed using the BigDye terminator cycle sequencing kit on an ABI 3130*XL* genetic analyzer (Applied Biosystems). The resulting sequences were analyzed by using the SeqMan program of the Lasergene software (DNASTAR, Madison, WI, USA) and/or MacVector™ version 10.6.0 (Oxford Molecular Group).

Nomenclature

Mamu-A alleles have been named according to established nomenclature proposals (Robinson et al. 2003; Ellis et al. 2006), which had been adapted to the recently published nomenclature for factors of the HLA system (Marsh et al. 2010). Briefly, *Mamu-A1–A7* reflect the locus names, followed by an asterisk and a 3-digit or 2-digit lineage name for the *A1* or *A2–A7* lineages, respectively (e.g., *Mamu-A1*001*). A lineage is defined as a family of alleles that share a common ancestor. The lineage name is followed by the allele name, separated by a colon (e.g., *Mamu-A1*001:01*). If a nucleotide substitution reflects a silent mutation, this will be defined by another two digits (e.g., *Mamu-A1*001:01:01*). The *Mamu-A* haplotypes are defined by a number indicating the region configuration (Fig. 1), followed by a dot, the number of the *A1* lineage, and, if needed, a letter indicating the allelic variation of one of its genes and/or variation of the microsatellite lengths: for instance, configuration 1.007a represents the haplotype of region configuration 1 with an *A1*007* lineage member and the allele *A1*007:01* (Table 1).

Results

Localization of microsatellites, *Mamu-A* genes, and TEs on the physical map

Two independent research groups published a physical map of the MHC of the same rhesus macaque. One team brought out a contig map covering the whole class I and II region of about 5.2 mb, whereas the other group mapped only the *Mamu-A* and *Mamu-B* region (Daza-Vamenta et al. 2004; Shiina et al. 2006). The rhesus macaque used for constructing the physical map is heterozygous (Daza-Vamenta et al. 2004) and the alleles of the *Mamu-B* (Doxiadis et al. 2009b) and *Mamu-DRB* genes (Doxiadis et al. 2008) had been defined. Both research groups analyzed only one haplotype for the *Mamu-A* region, and careful comparison suggests that they analyzed opposite haplotypes. Therefore, we tried to compare both *A* regions in silico by using the *Mamu*-SD locus which maps telomeric of the *A* region as a fixed anchor point (Shiina et al. 2006). On both haplotypes, four *A*-like genes/pseudogene can be observed centromeric from *Mamu-SD* (Fig. 2). Close to the *SD* locus, a pseudogene, named *Mamu*-A3 (nomenclature taken from Daza-Vamenta et al. 2004; Shiina et al. 2006), is located on both haplotypes followed by a transcribed *Mamu-A1*001:01* gene on the first haplotype (Shiina et al. 2006) and *A4*14:03* on the second haplotype, respectively (Daza-Vamenta et al. 2004). Furthermore, both haplotypes harbor another *Mamu-70* pseudogene and a second, transcribed *A* gene, which corresponds to *Mamu-A2*05:04* on the first and to *A1*004:01:01* on the second haplotype, respectively (Fig. 2). Thus, the physical map confirmed two of the five *Mamu-A* region configurations (Fig. 1, region configurations 1 and 5) that were defined earlier by segregation and sequencing analyses in Indian rhesus macaques.

One copy of the D6S2854 marker is located in close proximity to each *A*-like gene (Fig. 2, blue), whereas marker D6S2859 (Fig. 2, red) is present only once on these haplotypes, namely, between the *Mamu-70* pseudogene and the centromeric *A* gene. Analysis of this 253-kb-long segment using the RepBase database (http://www.girinst.org/censor/index.php) revealed that D6S2854 is part of an *AluJo(r)* element, whereas D6S2859 is situated within a long HERVP71A retroviral structure. Furthermore, one long retroviral HERV16 segment maps next to the *A3* pseudogene, whereas the other three *A* genes/pseudogenes of both haplotypes are accompanied by two HERV16 segments, one telomeric and one centromeric to the respective *A* locus (Fig. 2 and Table S1A, B). Additionally, the pseudogene A3 of both haplotypes and *A1*001:01* or *A4*14:03*, respectively, as well as the *A1*004:01* gene on haplotype 2 are joined by a long interspersed element, L1, that is situated next to the marker D6S2854. Thus, this part of the region harboring the classical *Mamu-A* genes seems to be comparable on both haplotypes (Fig. 2 and Table S1A, B), with a duplication unit characterized by one or two HERV16 elements, one *Mamu-A* (like) gene/pseudogene, an *AluJo(r)* element, and an additional L1 or HERVP71A segment. The most centromeric *A* gene of the first haplotype, *Mamu-A2*05:02*, however, misses the L1 element in contrast to its counterpart, *A1*004:01* on the second haplotype. Interestingly, the *Mhc* region centromeric to this point on the map is totally different in both

Table 1 *Mamu-A* haplotypes defined by STR genotyping and cDNA analysis

Haplotype	Freq (%)	*Mamu-*			D6S2854	D6S2859
(A) Mamu-A haplotypes of Indian-origin rhesus macaques (N= 819)						
1.001	11,9	***A1*001:01/02***	***A2*05:04***		**177,181**	**201**
1.006a	4,2	*A1*006:02*	*A2*05:01*		173,181,196	171,185
1.006b	0,7	*A1*006:02*	*(A2*05)*		173,181,196	171,183
1.007a	3,9	*A1*007:01/02/03*	*A2*05:07*		181,192	176,186
1.007b	0,6	*A1*007:02*	*A2*05:07*		181,185,192	176,186
1.012a	1,8	*A1*012:01*	*A2*05:19/11*		null	165,195
1.012b	3,7	*A1*012:01*	*A2*05:11*		null	167,195
1.019a	1,7	*A1*019:01*	*A2*05:05*		173,214	189,201
1.021	3,1	*A1*021:01/02*	*A2*24*		173,181,185,192,196	159,211
1.025	0,3	*A1*025:01*	*A2*05:09*		185,192	187
1.027	3,3	*A1*027:01*	*A2*05*		181,192	187
2.008	20,0	*A1*008:01:01*	*A2*05*	*A3*13:03*	173,181,196	171,203
4.002	15,2	*A1*002:01*	*A3*13:02*		192	166 (low)
4.016	2,1	*A1*016:02*	*A3*13:06*		181,192	166
5.004a	22,2	***A1*004:01:01***	***A4*14:03***		**181,185,196**	**171**
5.023	2,1	*A1*023:01*	*A4*14*		180,221	166
5.026a	1,6	*A1*026:01*	*(A4*14)*		181,196	175
6.003	1,4	*A1*003:02*			177,192,196	173
n.d.	0,2	n.d.			192,221	185
(B) Mamu-A haplotypes of Burmese-origin rhesus macaques (N= 101)						
1.007c	0,9	*A1*007:05*	*(A2*05)*		192	173
5.004a	11,1	***A1*004:01:01***	***A4*14:03***		**181,185,196**	**171**
6.008	15,7	*A1*008:01:02*	?		173,181,196	171,203
6.032	4,2	*A1*032:02:01*	?		181,184,192,196	167
6.041	3,2	*A1*041:01*	?		181,192	176
6.050	2,8	*A1*050:01*	?		173,181,192	171,236
6.051	12,5	*A1*051:01*	?		173,181	167,201
6.056	1,4	*A1*056:03*	?		188	175
6.110	5,6	*A1*110:01*	?		177,192	145
7.049a	1,9	*A1*049:02*	*(A5*30)*		190,196,216	171,189
7.049b	5,1	*A1*049:04*	*A5*3002*		190,196,216	171,189
8.007	0,9	*A1*007:01*	*A6*01:02*		181,185,192	176,186
9.059	2,8	*A1*059:01*	*A7*02:01*		177,181,212	185
10.043	7,4	*A1*043:01*	*A1*065:01*		173,185,192,196	169,176,201
11.0	24,5	?	*A2*05:26*	*A6*01:01*	192	199
(C) Mamu-A haplotypes of Chinese-origin rhesus macaques (N= 14) are shown						
1.007a	3,9	*A1*007:03*	*A2*05:07*		181,192	176,186
1.007d	19,6	*A1*007:04*	*(A2*05)*		192	173,186
1.007e	5,9	*A1*007:05*	*A2*05:02*		181,192	176,186
1.019b	19,6	*A1*019:03*	*(A2*05)*		173,193,204	171,189
5.004b	7,8	*A1*004:01:02*	*(A4*14)*		181,185,216	173
5.004c	7,8	*A1*004:01:01*	*(A4*14)*		181,185,216	185
5.026b	7,8	*A1*026:02*	*(A4*14)*		181,185,196	173
7.049a	3,9	*A1*049:02*	*(A5*30)*		190,196,216	171,189
12.0	23,5	?	*A4*14:04*	*A6*01:04*	181,185,196	186,196

The two haplotypes, 1.001 and 5.004a, which are identical to the physical map, are shown in **bold.** A lineage name in parentheses () indicates that an allele of this lineage is supposed to be present but has not yet been detected. A "?" indicates that the absence of the respective locus member cannot be guaranteed. A lineage without an allele name indicates that a member of this lineage has been detected but not enough clones have been sequenced for allele designation

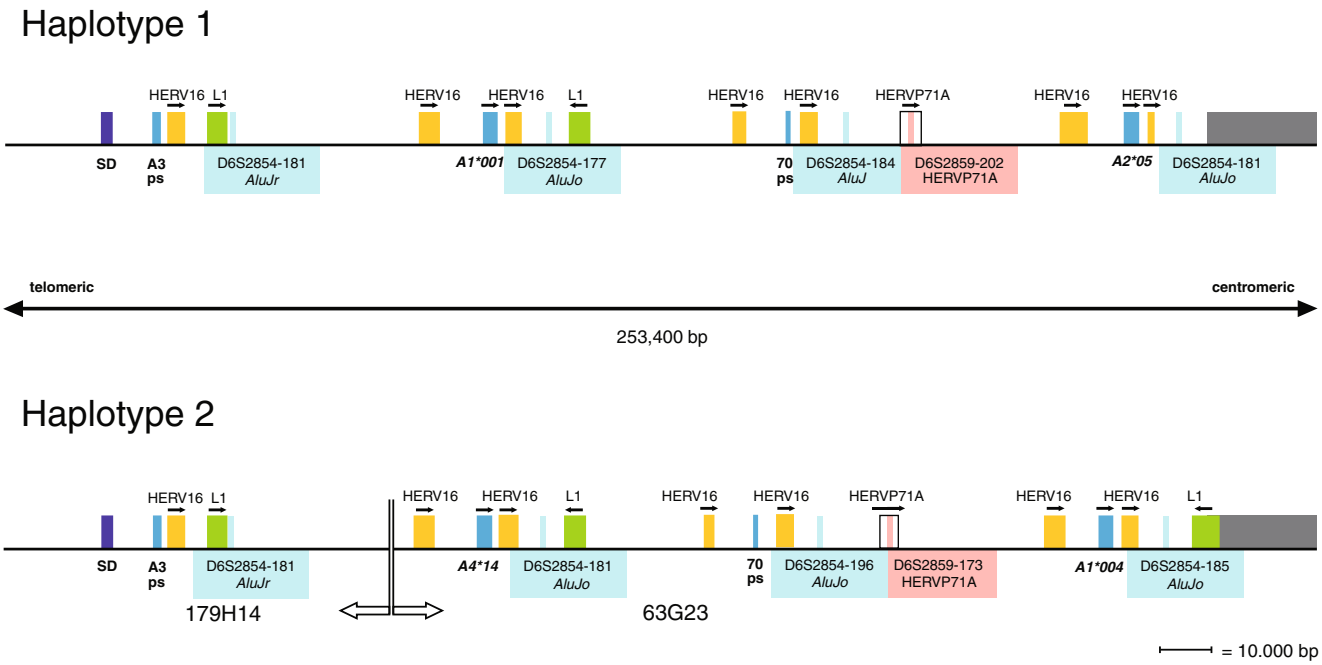

Fig. 2 Detailed physical map of the *Mamu-A* region of a heterozygous rhesus macaque. Location of *Mamu-A* genes/pseudogenes, microsatellite D6S2854 and D6S2959, L1, and HERV16/HERVP71A loci are indicated in scale. Haplotype 1 is based on the pysical map of Shiina and coworkers and haplotype 2 on data of Daza-Vamenta and colleagues (Daza-Vamenta et al. 2004; Shiina et al. 2006)

haplotypes with respect to the content of repeat elements (Fig. 2, grey shadowed).

Definition of *Mamu-A* haplotypes

The physical map (Fig. 2) illustrates that the microsatellite D6S2854 is part of an *Alu* element next to an *A* gene or *A*-like pseudogene, and D6S2859 is also localized within the part of the region that encodes for classical *A* gene products. Additionally, preliminary tests had shown that both markers are polymorphic in length and that the typing results were highly reproducible. Thus, both microsatellites appeared to be suitable as haplotyping reagents, and 934 monkeys of the rhesus macaque breeding colony were tested using the two markers. The animals were mainly of Indian origin, but breeding groups involving animals originating from Burma and mixed breeds of Chinese/Indian origin were also included. Genotyping with both markers resulted in patterns of various lengths (see below) that could be distributed on haplotypes by segregation analyses, since all rhesus macaques are part of well-defined breeding groups covering at least five generations. To link unambiguously the microsatellite patterns to *Mamu-A* haplotypes, at least one animal representing a unique haplotype was additionally characterized for its *Mamu-A* transcripts by full-length cDNA sequencing. This approach resulted in the definition of 19, 15, and 9 *Mamu-A* haplotypes for the Indian, Burmese, and Chinese origin rhesus macaques, respectively (Table 1). The haplotypes have been classified into 12 region configurations (Fig. 1) and subdivided based on the presence of different *A1* lineages, according to the nomenclature described earlier. For most haplotypes, alleles of the *Mamu-A1* locus have been defined *plus* transcripts of at least one other minor locus. As an exception to the rule that each haplotype contains one *A1* locus encoding the major *A* transcript, there are, on the one hand, two *Mamu-A* haplotypes, one detected in Burmese and one in Chinese origin monkeys, without an *A1* gene transcript (Table 1B, no. 11.0 and Table 1C, no. 12.0). On the other hand, one Burmese haplotype displays a duplication of the *A1* locus (Fig. 1, region configuration 10; Table 1B, no. 10.043). Additionally, another Burmese haplotype is characterized by the presence of two "majors," an *A1* and an *A7* (Fig. 1 region configuration 9; Table 1B, no. 9.059). The *Mamu-A* haplotypes no. 1.001 and 5.004a (Table 1A, B, bold) correspond to those of the physical map, which are characterized by an *A1*001:01:01* allele together with an *A2*05:04* (Fig. 2, haplotype 1) and *A1*004:01:01* together with an *A4*14:03* (Fig. 2, haplotype 2), respectively. Additionally, the microsatellite length patterns of marker D6S2854 and the length of D6S2859 of both haplotypes (Table 1A, no. 1.001 and 5.004a) correspond to those calculated from the physical map (Fig. 2). Only one marker,

D6S2854-184, present in haplotype 1 (Fig. 2) cannot by detected by microsatellite analysis, most probably due to three nucleotide substitutions observed in the 3′ primer.

Microsatellite typing of the present, extended rhesus macaque panel showed a high degree of length variation of the amplicons for both markers, varying from 173 to 221 bp for D6S2854 and from 145 to 236 bp for D6S2859. Furthermore, the copy number of microsatellites present per haplotype can vary as well, namely, from zero to five for D6S2854 and from one to three for D6S2859 (Table 1). Nearly all *Mamu-A* haplotypes analyzed so far are characterized by unique microsatellite patterns. If two haplotypes share the same D6S2854 pattern, the lengths of D6S2859 amplicons are mostly different (e.g., Table 1A, no. 1.006a/b) and vice versa (e.g., Table 1A, no. 1.007a/b). Exceptions are rarely observed and concern haplotypes that can be differentiated by related *A1* alleles if they share the same STR patterns (e.g., Table 1B no. 7.049a/b). Far more haplotypes that are characterized by the same *A* alleles can be subdivided by different microsatellite patterns (e.g., Table 1A no. 1.012a/b). Furthermore, one haplotype that could not be determined by *Mamu-A* cDNA sequencing because RNA of the respective monkey was not available could be defined by microsatellite typing as well (Table 1A). Thus, haplotyping for *Mamu-A* can be easily performed by this simple and robust technique using only two microsatellites.

Comparison of *Mamu-A* region configurations and haplotypes of different populations

In this study, most of the monkeys originated from India, and 19 *Mamu-A* haplotypes were distinguished (Table 1). These haplotypes belong to region configurations 1–6, most of which are characterized by the presence of one *A1* gene in concert with one, sometimes two, and even no "minor" *A* genes. The latter configuration was described previously in cynomolgus macaques but was observed for the first time in Indian origin rhesus monkeys. In addition, five of the six configurations reported in this study have also been reported for the cynomolgus monkey (Otting et al. 2007). However, five region configurations, nos. 7–11, defined in Burmese origin monkeys, are unknown for Indian macaques (Fig. 1, Table 1 A, B). Although far fewer animals of Burmese (N=101) than of Indian origin (N=819) had been analyzed, the number of haplotypes (N=15) is nearly as high.

The number of haplotypes in comparison to animals tested is most remarkable in mixed breeds of Chinese/Indian origin: in only 14 animals tested, nine *Mamu-A* haplotypes could be defined by segregation analysis to be of Chinese origin. Within these nine haplotypes, another novel region configuration could be described in which a *bonafide A1* gene appears to be missing. In Burmese animals (Table 1 B, no. 11.0), one other region configuration was observed that seems to lack an *A1* transcript. In these two haplotypes, however, the otherwise minor *A* gene provides the major transcript. Therefore, the number of region configurations/haplotypes will certainly increase if more rhesus macaques of Chinese origin are to be analyzed, and the number of Chinese haplotypes may then exceed those of Indian monkeys.

Most of the *Mamu-A* haplotypes are characterized by a specific *A1* lineage member. There is, however, at least one *A1* lineage, namely, *A1*007*, that is linked to different minor *A* genes, thus belonging to at least two region configurations (e.g., Table 1A no. 1.007a, b and Table 1B, no. 8.007). Additionally, this lineage is oligomorphic and present in all three macaque populations as well as in the cynomolgus monkey (Otting et al. 2009). Moreover, a few haplotypes (Table 1, no. 1007a, 5.004a, and 7.049a) are shared between monkeys of different populations, and, interestingly, haplotype 5.004a is the most frequent one in the Indian origin macaques. In most cases, however, these haplotypes are not identical but show allelic variation (indicated by letters a–e).

Discussion

The physical map of two haplotypes harboring the *Mamu-A* region has been compared. The content of this 253-kb-long segment within the alpha block, which is composed of four duplicons containing an *A* gene/*A*-like pseudogene and specific TEs, appears to be nearly identical in both haplotypes (Fig. 2, Table S1 A/B). This is in contrast to other parts of the *Mhc* of this heterozygous monkey, especially of the *Mamu-B* but also the *Mamu-DR* region (Daza-Vamenta et al. 2004; Bonhomme et al. 2008; Doxiadis et al. 2009b). Furthermore, the most centromeric part of the *Mamu-A* region is not identical in both haplotypes with regard to genes/pseudogenes and enclosed TEs. The haplotype variation starts from the most proximal L1 segment next to *A1*004* on haplotype 2 (Fig. 2, grey shadowed). Since L1 elements are autonomous transposons, known to be responsible for genetic instability by causing insertions and deletions in mammalian genomes, this nearly intact L1 element may have been the reason for the chromosomal rearrangements observed in the past (Goodier and Kazazian 2008; Belancio et al. 2009). Although the *Mamu-A* region is nearly identical in the two haplotypes studied, the two major *A1* genes are not on the same position of the physical map and are accompanied by different minor genes. The observation indicates that recombination-like events have taken place within the macaque alpha block after the Old World monkey-

Hominoidea split about 25 million years ago (mya) (Kulski et al. 2004). This suggestion is supported by cDNA analysis as well as by microsatellite typing, which show the linkage of a certain *A1* lineage (e.g., *A1*007*) with different minor *A* genes, and the existence of a haplotype with a duplication of *A1* or of others that lack the *A1* gene. In addition, there are haplotypes that harbor more or fewer than two transcribed *Mamu-A* genes. Furthermore, it is possible that others with three *A* genes may have remained undetected because of their low transcription levels. Another indication for the flexibility of this region is given by the microsatellite patterns that show up to five copy numbers for marker D6S2854 (Table 1). However, a lower copy number than expected may be caused by primer inconsistencies and/or the presence of different copies with the same amplicon length on one haplotype as has been shown for D6S2854-181 on the physical map (Fig. 2). Nevertheless, the copy number and length variation of both microsatellites, D6S2854 and D6S2859, appear to be highly specific for a given *Mamu-A* haplotype.

Six of the 12 *Mamu-A* region configurations have also been observed in cynomolgus monkeys and therefore seem to be old entities originating before the divergence of rhesus and cynomolgus macaques ~1.3 mya (Stevison and Kohn 2009), although a relatively recent gene flow between species is unlikely but cannot be excluded (Street et al. 2007). While region configurations may be shared between monkeys of different origin and even different species, many of the *Mamu-A1* lineages, and nearly all of the allelic variations appear to be population-specific. This finding may be due to the evolutionary history of the monkeys. Macaques originated in Africa about 5 mya and expanded eastward. It is believed that rhesus macaques have their origin west of their present range, where the earliest macaque fossils are found, and then dispersed to China through an Indian wet zone. One would expect such a scenario to have led to a reduced genetic heterogeneity in Chinese origin rhesus monkeys as compared to Indian origin animals due to founder effects. This is, however, precisely the opposite of what published data show, namely, an overall higher genetic variability of Chinese- in comparison to Indian origin monkeys, proven by single nucleotide polymorphisms (SNPs) and mtDNA analysis (Smith and McDonough 2005; Ferguson et al. 2007), a greater diversity of *Mhc* class I sequences (Karl et al. 2008; Solomon et al. 2010) and *Mamu-B* haplotypes (Wiseman et al. 2009), and contrary to what can be concluded from our results presented in this study. One possible explanation may be that the lower level of genetic heterogeneity observed in Indian origin rhesus macaques reflects a severe and ancient genetic bottleneck caused by desiccation of the wet zone, which has led to the extinction of Indian origin monkeys (Smith and McDonough 2005). Furthermore, all data comparing the gene content of Chinese versus Indian origin rhesus macaques lead to the conclusion that the genetic background of Chinese and Indian origin monkeys is remarkably divergent (Smith and McDonough 2005; Ferguson et al. 2007; Otting et al. 2007, 2008; Karl et al. 2008; Wiseman et al. 2009). Additionally, a recent publication reports that about one half of the *Mhc* class I sequences, which the authors have defined in Burmese origin rhesus macaques, are novel, probably representing Burma origin specific class I alleles (Naruse et al. 2010). This observation is supported by our results. Thus, the genetic repertoire of rhesus macaques of different origins appears to be highly diverse. These findings may have major implications for immunologically related, preclinical studies in macaques, in which MHC class I molecules appear to play a role. Not only in humans but also in chimpanzees and macaques, there is, for example, a strong association of *Mhc* class I *A* and *B* alleles with a particular outcome of HIV or SIV infections (Mothe et al. 2002; O'Connor et al. 2003; Loffredo et al. 2007, 2009; Goulder and Watkins 2008; de Groot et al. 2010). Most of the studies are performed in rhesus macaques of Indian origin, and several class I molecules such as Mamu-A1*001, Mamu-B*017, and Mamu-B*008 are associated with a successful control of SIV infections (reviewed in Goulder and Watkins 2008), whereas in Burmese origin macaques, *Mamu-A1*065:01* appears to be responsible for SIV control (Goulder and Watkins 2008; Tsukamoto et al. 2008), and *Mamu-A1*001* has not been detected in these monkeys. Interestingly, the *Mamu-A1*065:01* allele is part of the only haplotype (Table 1B, no. 10.043) that harbors two *A1* alleles, and the same haplotype has also been observed by Naruse and coworkers in Burmese monkeys. Since the *Mamu-A1* alleles with a high transcription level are thought to encode immunologically active molecules that present peptides to $CD8^+$ T cells, it would be advantageous to be able to present a higher number of peptides as a "double lock strategy" after viral infections.

Recent data have shown the differential subcellular localization of some class I molecules. Strong cell surface but low intracellular expression has been detected for certain *Mamu-A1*-, as well as Mamu-A2-, Mamu-A3-, and Mamu-B-encoded molecules, whereas others demonstrated low surface but high intracellular expression (Rosner et al. 2010). These data indicate that not only Mamu-A1 molecules have a peptide-presenting function, and this would explain the frequent existence of haplotypes without *A1* alleles, as detected in Chinese and Burmese origin macaques. Furthermore, the different MHC class I repertoires of monkeys of various origins may be one of the possible reasons that animals are more or less susceptible to diseases such as SIV/SHIV (Ling et al. 2002). Thus, our data underline the necessity to type carefully for the MHC

of all animals used in preclinical, biomedical research. The high-throughput and robust STR typing method presented here can be a useful tool in achieving this aim.

Concluding remarks

The physical map of the MHC region of a heterozygous animal shows different positions of the *A1* genes on both haplotypes in combination with different minor *A* genes. This observation suggests that recombination-like events reshaped the order of the genes within the alpha block during evolution. In addition, the existence of a total of 40 different *Mamu-A* haplotypes that are characterized by microsatellite copy number variation illustrates the occurrence of differential block duplications and subsequent rearrangement by recombination. The presence of different, transcribed *Mamu-A* genes per haplotype in monkeys of various origins may have an impact on the differential disease susceptibilities of these animals, as has been documented for the susceptibility to SIV/SHIV. Therefore, an analysis of the genetic background of monkeys prior to biomedical studies is highly recommended. The high-throughput microsatellite typing technique will be a valuable tool in animal selection for this type of study; it will also be useful in other research areas as well as in colony management, or with regard to species conservation issues.

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