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Sequence analysis of the MHC class II DPB1 gene in chimpanzees (*Pan troglodytes*)

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Summary

The diversity of the MHC class II region in non-human primates is a focus of biomedical research because this region plays a crucial role in the recognition of antigens in the immune system. In particular, the chimpanzee [*Pan troglodytes* (*Patr*)], which belongs to the superfamily *Hominoidea*, has been used as a human model for the study of diseases such as human hepatitis C virus (HCV), human hepatitis B virus (HBV) and human immunodeficiency virus (HIV) infections, to which only humans and chimpanzees are susceptible. In the present study, polymorphisms of the MHC-DPB1 gene (*Patr*-DPB1) in a chimpanzee colony in Japan were examined using a stepwise polymerase chain reaction (PCR) technique. In order to design a suitable primer pair which would amplify exon 2 of the *Patr*-DPB1 gene, a fragment of approximately 8 kb from exon 1 to exon 3 was amplified from chimpanzee genomic DNA. After designing a 500-bp primer pair at the 3′ region of intron 1 and the 5′ region of intron 2, analysis of DPB1 exon 2 alleles of each chimpanzee was carried out. Twenty-two chimpanzees were used in our study, and we identified seven alleles by sequence analysis on the *Patr*-DPB1 gene, including one new allele. The obtained nucleotide sequence patterns suggest that *Patr*-DPB1 alleles emerge by genetic variations such as the exchange of sequence motifs and the accumulation of point mutations.

Introduction

Major histocompatibility complex (MHC) class II molecules are highly polymorphic cell surface glycoproteins that mediate both humoral and cell-mediated immune responses. They are heterodimers comprised of two subunits which consist of an α chain and a β chain (Korman *et al.*, 1985; Slierendregt *et al.*, 1993), and the extracellular part of the α chain includes α1 and α2 domains; similarly, the β chain has β1 and β2 domains (Jones, 1997). The primate MHC class II region consists of DR, DQ and DP subregions (Slierendregt *et al.*, 1993; de Groot & Bontrop, 1999). In both humans and chimpanzees (*Pan troglodytes*), two pairs of genes are located in the DP subregion: DPA1/DPB1 and DPA2/DPB2 (Bontrop *et al.*, 1999). Although the DPA2/DPB2 genes are not functional, the DPA1 and DPB1 genes are both functional and polymorphic, leading to variation in the DP protein (Slierendregt *et al.*, 1995). Polymorphisms of MHC-DPA1/DPB1 genes are confined largely to the second exon, which encodes the functional domain that forms the peptide-binding region of the molecules (Marsh & Bodmer, 1989; Bugawan *et al.*, 1991; Klein *et al.*, 1993).

Evolutionary stability and the *trans*-species mode of inheritance have been found in many primate MHC DQ and DRB lineages (Gyllensten *et al.*, 1991; Bontrop *et al.*, 1999). Additionally, *Pan troglodytes* (*Patr*)-DPA1 alleles are closely related to some human equivalents (Otting & Bontrop, 1995; de Groot & Bontrop, 1999). However, maintenance of the DPB1 allelic variation might be controlled by a different mechanism from that of other MHC class II genes, and this mechanism may have been generated during evolutionary history. First, the primate MHC-DPB1 gene shows a relatively species-specific polymorphism, which is thought to occur by segmental recombination and point mutation within the same species. Secondly, in a survey of substitution patterns in hominoid species, the number of synonymous changes at the antigen recognition site (ARS) in hominoid DPB1 alleles was found to be only about half that for hominoid DRB1 and DQB1 alleles (Gyllensten *et al.*, 1990, 1996; Satta *et al.*, 1994), suggesting that the allelic diversification at the DPB1 loci has evolved faster than that of other MHC class loci. Lastly, in contrast to all other class II loci, the DPB1 gene appears to have been subjected to strong positive selection only in the human lineage (Hughes & Nei, 1989; Bergstrom & Gyllensten, 1995).

Because of the species-specific character of the MHC-DPB1 gene, we attempted to develop a DNA-based typing method for *Patr*-DRB1 alleles for use with the polymerase chain reaction (PCR) technique. This typing technique, in combination with direct sequencing analyses, can be effectively used to detect unique *Patr*-DPB1 alleles.

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Materials and methods

DNA extraction

The 22 chimpanzees used in this study were housed at the Kumamoto Primate Park, Sanwa Kagaku Kenkyusho, in Japan. Table 1 lists the name, registration number, age and sex of each animal. All chimpanzees except Kirara, Kurara and Miyuki were captured in Africa and transported to Japan. The pedigree of this chimpanzee colony is available at http://www.nbr-chimp.org/chim/zoo/sanwa/sanwa-m_all.html. Miyuki and Tetsu are the parents of Kirara and Kurara. There were no other genetic relationships among the chimpanzees used in this study.

Genomic DNA was isolated from a peripheral blood sample using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Design of a primer set for *Patr*-DPB1 exon 2

In order to create a specific primer pair which would amplify exon 2 in each *Patr*-DPB1 allele, 8-kb PCR, covering the area from exon 1 to exon 3 on the DPB1 gene, was performed using the genomic DNA of Sanzou and Iyo (Fig. 1a and b). The primer pair (DPB-E1+ and DPB-E3–)

Table 1. Distribution of *Patr*-DPB1 alleles in individual chimpanzees

Name	Number	Age (years)	Sex	*Patr*-DPB1 alleles
Genki	53	33	Male	*13, *17
Ichigo	115	24	Female	*16, *25
Iyo	C54	27	Female	*13, *22
Izou	24	26	Male	*16, *22
Kirara[a]	277	7	Female	*13
Koiko	C51	27	Female	*11, *17
Konatu	105	27	Female	*17, *29
Kurara[a]	278	7	Female	*13
Maruku	109	24	Male	*11, *13
Miro	C30	31	Female	*13, *16
Miyuki[a]	204	19	Female	*13
Nozomi	112	26	Female	*11, *16
Remu	106	22	Female	*13
Roman	C69	30	Female	*13
Sango	C35	28	Female	*16, *22
Sanzou	C33	30	Male	*11, *13
Siomi	85	24	Female	*16
Sumiko	86	24	Female	*11, *13
Susumu	82	26	Male	*16, *17
Takabo	104	27	Male	*11, *13
Tamae	108	25	Female	*16, *17
Tetsu[a]	44	28	Male	*13, *16

[a] Miyuki and Tetue are the parents of Kirara and Kurara (identical twins).

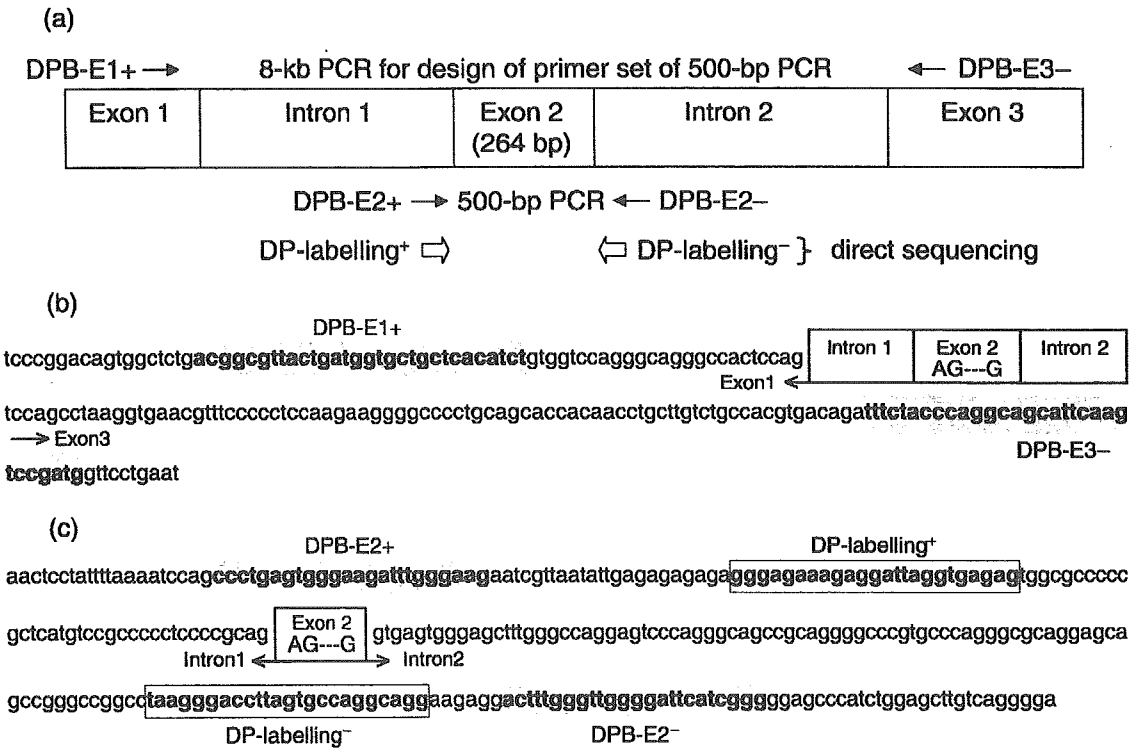

Figure 1. Designing specific primers. (a) Schematic diagram of the primer pairs used in the present study. (b) A primer pair for 8-kb PCR. DPB-E1+ and DPB-E3– (grey rectangle) are located in exon 1 and exon 3, respectively. The intron 1, exon 2 and intron 2 sequences are omitted. (c) Primer pairs for the exon 2 fragments. Grey rectangular boxes are DPB-E2+ and DPB-E2–. White boxes are DP labelling + and DP labelling – primers for direct sequences of exon 2.

was designed using a chimpanzee DP cDNA clone as previously described (Hatta *et al.*, 2002).

The reaction mixture contained 0.25 units of LA Taq polymerase (Takara, Kyoto, Japan), 2.5 mM dNTP, 2.5 mM $MgCl_2$, 4 μM of each primer, and 2 μl DNA template. Amplification was first performed with 14 cycles of denaturation at 94 °C for 20 s, and annealing and extension at 68 °C for 20 min. This was followed by 17 cycles of denaturation at 94 °C for 20 s, and annealing and extension at 68 °C for 20 min in stepwise elongation with an incubation time of 15 s/cycle. After electrophoresis in a 0.8% agarose gel (Wako, Osaka, Japan), the purified 8-kb target PCR product was ligated into a PCR-XL-TOPO vector (Invitrogen, Carlsbad, CA) and transformed into a DH5α™ competent cell (Toyobo, Osaka, Japan). The obtained clones were checked by PCR against the primer pair (sense: 5′-GCTACGCGTTTAATGGGACACAG-3′; antisense: 5′-CTCGGCGCTGCAGGGTCACGG-3′) that was used to amplify the 258-bp product of chimpanzee DPB1 exon 2. Each of four plasmid clones from Sanzou and Iyo was isolated using the NucleoSpin® Plasmid QuickPure kit (Macherey-Nagel, Düren, Germany).

The plasmid samples were then labelled with a Big Dye™ terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and analysed with an ABI PRISM™ 377 DNA Sequencer (Applied Biosystems). Based on the obtained sequences, a PCR primer pair for each chimpanzee was designed (DPB-e2+ and DPB-e2–; Fig. 1a and c) in order to amplify a 500-bp product containing the entire exon 2 (264-bp) sequences.

PCR and cloning

PCR was applied to the DNA of the 22 chimpanzees as follows. Amplification was performed using AmpliTaq Gold (Applied Biosystems) with the DPB-E2+ and DPB-E2– primers. The target products were then cloned into a pCRII-TOPO vector (Invitrogen). After transformation of the vector, 10 clones were selected for each chimpanzee.

Direct sequencing

Direct sequencing was carried out to confirm the sequence of the clones of *Patr*-DPB1 exon 2 amplified by PCR. The 500-bp products were gel-purified with a Wizard® SV gel and PCR Clean-Up System (Promega, Madison, WI) and were sequenced directly using direct sequencing primers (DP labelling + and DP labelling –; Fig. 1a and c).

MHC *Patr*-DPB1 nomenclature

The official designation for the *Patr*-DPB1 allele detected in this study was obtained from R. E. Bontrop and Natasja G. de Groot (Biomedical Primate Research Centre-TNO, Rijswijk, the Netherlands). This new allele has been registered in the DNA Data Bank of Japan (DDBJ).

Results and Discussion

Distribution of the *Patr*-DPB1 allele in each chimpanzee

Table 1 shows the patterns of *Patr*-DPB1 alleles in each chimpanzee. The *Patr*-DPB1*13 allele was found at very high frequency, being present in 13 of 22 chimpanzees. Of the five chimpanzees homozygous for DPB1*13, Kirara and Kurara are identical twins whose parents are Miyuki (*13 homozygote) and Tetsu (*13 and *16 heterozygote). A previous survey of the Biomedical Primate Research Center (BPRC) chimpanzee population showed that the *Patr*-DPB1*13, *16 and *17 alleles were the most frequently observed (Otting *et al.*, 1998). The *Patr*-DPB1*13 allele may occur frequently in the gene pool of wild chimpanzees.

Patr-DPB1 polymorphism

To date, 28 alleles of the *Patr*-DPB1 gene have been reported in the chimpanzee (Otting *et al.*, 1998; Bontrop *et al.*, 1999). In the present study, the 22 chimpanzees had seven different alleles, including one novel *Patr*-DPB1 allele. The nucleotide sequences of *Patr*-DPB1 exon 2 and the deduced amino acid sequences are depicted in Figs 2 and 3, respectively. The hypervariable region (HV) of the MHC-DPB1 exon 2 nucleotide sequence in human and non-human primate species is restricted to six major variability regions (Slierendregt *et al.*, 1995). The variations of the new allele were found in these regions, supporting the hypothesis that this new allele is not the result of random PCR error. The *Patr*-DPB1*29 allele was similar to the *Patr*-DPB1*04 allele, but was found to differ from the *Patr*-DPB1*04 allele in three non-synonymous substitutions: at codon 55 (GCT: Ala → GAT: Asp) in HV3, codon 69 (GAG: Glu → AAG: Lys) in HV4, and codon 76 in HV5 (ATG: Met → ATC: Ile) (small grey rectangular box in Fig. 2). Furthermore, the non-synonymous substitution was present at codon 24, which is located in the region immediately adjacent to the HV1-6 region (TTC: Phe → TAC: Tyr) (large grey rectangular box in Fig. 2). These variations may be considered to be point mutations.

Typing techniques for *Patr*-DPB1 alleles

Cloning and sequencing methods using PCR techniques are the traditional approach for typing MHC alleles. In MHC-DPB1 alleles which include point mutations, it is important to design a suitable primer pair for amplification of the target gene. For example, the owl monkey *Aotus nancymaae* has a unique sequence motif PLI/NRK at the C-terminus of *Aona*-DPB1 exon 2, and an attempt to amplify gene segments with a primer pair suitable for HLA-DPB1 exon 2 sequences failed (Diaz *et al.*, 2002). Also, the PCR technique using primer pairs derived from humans is limited because of the polymorphic patterns of *Patr*-DPB1 alleles. In our preliminary examination using a primer pair reported in a previously published paper (Bugawan *et al.*, 1991), three or four

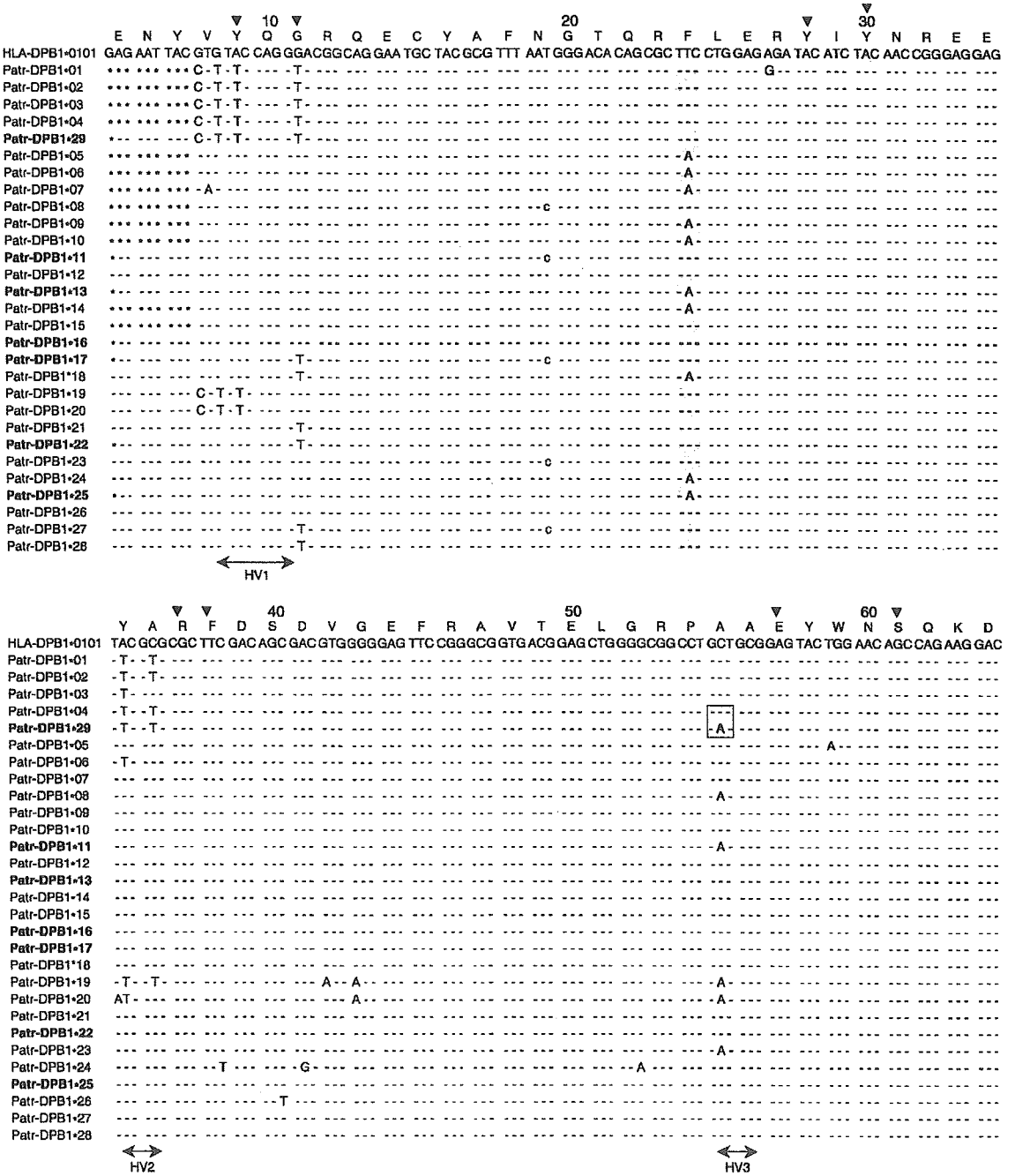

Figure 2. Alignment of the nucleotide sequences of *Patr*-DPB1 exon 2 alleles. The six previously known alleles and one new allele detected in this study are indicated by bold type (*Patr*-DPB1*29; DDBJ accession number AB183471). The HLA-DPB1*0101 allele is shown at the top of the figure. Dashes (–) indicate identity with this allele. Synonymous substitutions appear in lowercase, while non-synonymous substitutions are in uppercase. The inferred amino acid sequence of the HLA-DPB1*0101 allele is shown with the conventional one-letter code. Codons 9, 11, 28, 30, 37, 38, 57, 61, 67, 70, 71, 74, 78, 82 and 86 are antigen recognition sites (▼).

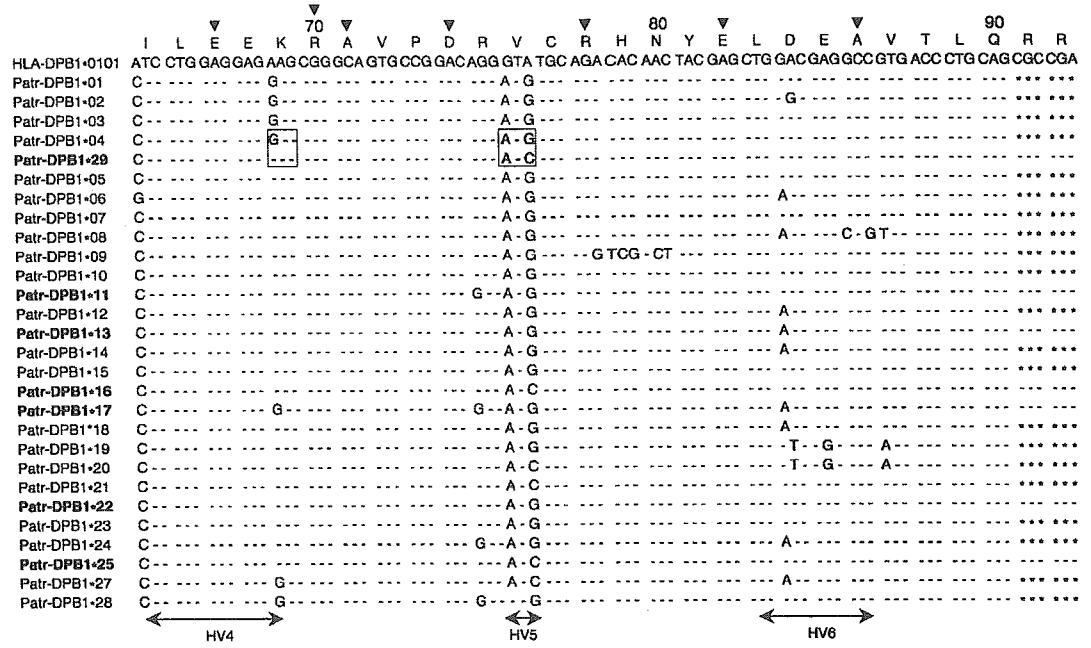

Figure 2. *Continued*

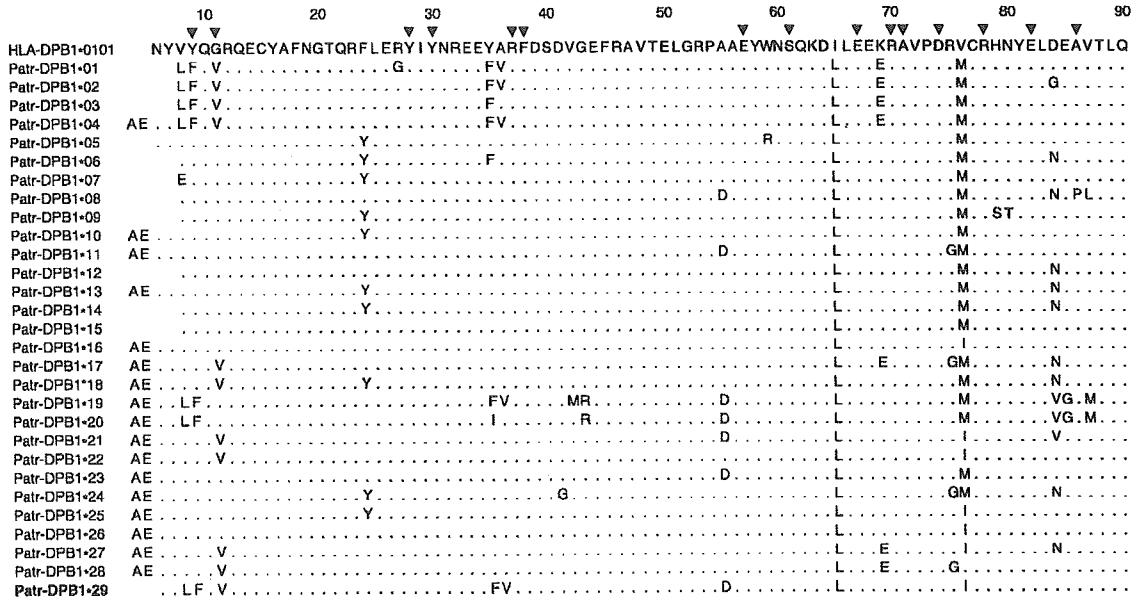

Figure 3. Alignment of the deduced amino acid sequences of 28 known *Patr*-DPB1 exon 2 alleles and one new allele detected in this study. The new allele is indicated by bold type. The HLA-DPB1*0101 allele is shown at the top of the figure. Dots (.) indicate identity with the HLA-DPB1*0101 allele. The symbol ▼ indicates antigen recognition sites.

independent alleles among selected clones were detected in some chimpanzees.

By designing a specific primer pair for the chimpanzee, we successfully analysed the exon 2 nucleotide sequences of *Patr*-DPB1 loci using cloning and direct sequencing. The application of direct sequencing was particularly useful to exclude incorrect TA cloning sequencing resulting from PCR error. The methods used in the present study should facilitate the identification of *Patr*-DPB1 alleles, and contribute towards maintenance of *Patr*-DPB1 allelic

diversity in chimpanzee groups in Japan through colony management.

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Identification and analysis of MHC class II DRB1 (Patr-DRB1) alleles in chimpanzees

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allelic polymorphism; microsatellite repeat length variability; Patr-DRB1 allele; PCR technique

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Abstract

The *MHC-DRB1* gene is known to display the most extensive allelic polymorphisms among MHC class II genes. We attempted the selective identification of chimpanzee (*Pan troglodytes*) DRB1 (Patr-DRB1) alleles using the polymerase chain reaction (PCR) technique in three steps: first, we performed Patr-DRB1*02 lineage-specific 8-kb PCR for *02 lineage detection in each chimpanzee; second, we performed 620-bp PCR for amplification of full-length exon 2; and finally, we carried out an insert check using the pattern of microsatellite repeat length variability. In the genomic DNA of 23 chimpanzees, nine Patr-DRB1 alleles containing two new alleles were detected. Our approach provides a relatively effective method of identifying Patr-DRB1 alleles in individual chimpanzees and should also contribute to our understanding of the features of MHC molecules in non-human primates.

Introduction

The classical major histocompatibility complex (MHC) class II molecules are heterodimeric surface glycoproteins composed of polymorphic α and β chains (1–3). MHC class II molecules play a key role in orientation in the immune response due to their restricted expression on professional antigen-presenting cells (APCs) (4, 5). Additionally, differential peptide binding of distinct MHC class II molecules can lead to different T-helper cell activation, and MHC class II molecules are therefore associated with susceptibility or resistance to infectious diseases (6, 7).

Chimpanzees have a significantly favorable response to human immunodeficiency virus (HIV)-1 and human hepatitis B virus (HBV) (8, 9). Furthermore, the chimpanzee model of human hepatitis C virus (HCV) infection is suitable for determining the identity of epitopes targeted by $CD4^+$ cells.

High levels of genetic homology between chimpanzees (*Pan troglodytes*) and humans are a major reason why the chimpanzee represents an important experimental animal for comparative bioscience (10). In both humans and chimpanzees, the MHC class II genes are divided into DP, DQ, and DR subregions. The DR subregion appears to display the most extensive polymorphism in MHC class II genes in which the β chain is encoded by any of a series of polymorphic DRB loci, while the α chain is encoded by the virtually monomorphic DRA locus (11, 12). Chimpanzees have three functional loci (DRB1, DRB3, and DRB5) among several DRB loci (DRB1, DRB3, DRB4, DRB5, DRB6, DRB7, DRW8, and DRW9). The functional DRB loci are characterized by an extensive amount of allelic variation that is confined primarily to the second exon. Patr-DRB2, Patr-DRB8, and Patr-DRB9, denoted as HLA-DRB2, HLA-DRB8, and HLA-DRB9, respectively, are thought to be present, but the alleles have not yet been determined (13).

The analysis of the *DRB1* gene among the DR regions has generally been considered to be the most important

parameter for studying phylogeny and disease association (14–18). In particular, the *Patr-DRB1* gene has been shown to be the most polymorphic of the functional *DRB* genes (2). Therefore, a rapid and precise typing method for *DRB1* genes is necessary for defining polymorphisms and performing sequence analysis of a large number of individuals for group management as well as for genetic analysis of disease susceptibility. In the present study, we developed a useful method for the determination of Patr-DRB1 alleles and examined Patr-DRB1 allelic diversity in a chimpanzee group in Japan.

Materials and methods

DNA samples

The 23 chimpanzees used in this study were housed at the Kumamoto Primate Park, Sanwa Kagaku Kenkyusho, in Japan. The name, registration number, age, and sex of each animal are listed in Table 1. All but six of the chimpanzees (Miyuki, Kirara, Kurara, Kotetu, Paru, and Zugo) were captured in Africa and transported to Japan. The genetic relationships among the chimpanzees are summarized in Figure 1. To the best of our knowledge, there were no other genetic relationships among the chimpanzees used in this study. The pedigree of this chimpanzee colony is provided at http://www.nbr-chimp.org/chim/zoo/sanwa/sanwa-m_all.html.

Table 1 Patterns of Patr-DRB1 alleles in individual chimpanzee

Name	No.	Age	Sex	Patr-DRB1
Ichigo	115	25	♀	*0302, *0307
Iyo	C54	28	♀	*0204, *0307
Izou	24	27	♂	*0201, *0311
Kirara	277	9	♀	*0305, *0307
Koiko	C51	28	♀	*0307
Konatu	105	28	♀	*0312
Kotetu	244	15	♂	*0201, *0312
Kurara	278	9	♀	*0305, *0307
Maruku	109	25	♂	*0204, *0302
Miyuki	204	20	♀	*0204, *0305
Nozomi	112	27	♀	*0302
Paru	228	16	♀	*0307
Remu	106	24	♀	*0201, *0204
Roman	C69	31	♀	*0201, *0307
Sango	C35	29	♀	*0204, *0307
Sanzou	C33	31	♂	*0201, *0307
Siomi	85	25	♀	*1001, *0302
Sumiko	86	25	♀	*0309
Susumu	82	27	♂	*0307
Takabo	104	28	♂	*0201, *0311
Tamae	108	26	♀	*0204, *0302
Tetu	45	29	♂	*0204, *0307
Zugo	256	13	♂	*0307

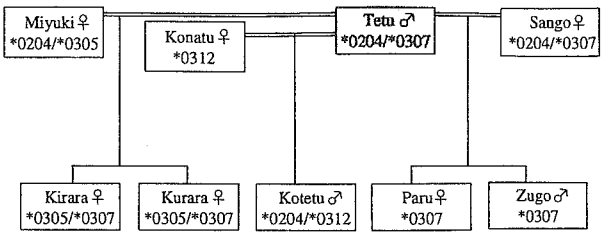

Figure 1 Pedigree patterns of the three chimpanzee families. The horizontal and vertical bars mean the partner (double line) and thier progenies (single line), respectively. Tetu (gray box) is an alpha male.

Genomic DNA was isolated from a peripheral blood sample using a QIAamp DNA Blood Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany).

Design of the primer set for Patr-DRB1 exon 2

In order to determine a specific primer pair which amplifies exon 2 in each Patr-DRB1 allele, 8-kb polymerase chain reaction (PCR) covering the area from exon 1 to exon 3 on the *DRB1* gene was performed using the genomic DNA of Sanzou and Iyo (Figure 2A,B). The primer pair (DRB-E1+ and DRB-E3–) was designed from a Patr-DR cDNA clone as previously described (19). The reaction mixture contained 0.25 U of LA Taq polymerase (Takara, Kyoto, Japan), 2.5 mM dNTP, 2.5 mM $MgCl_2$, 4 μM of each primer, and 2 μl of DNA template. Amplification was first performed for 14 cycles of denaturation at 94°C for 20 s with annealing and extension at 68°C for 20 min. This process was followed by 17 cycles of denaturation at 94°C for 20 s with annealing and extension at 68°C for 20 min in a stepwise elongation with an incubation time of 15 s/cycle. Only one band of 8 kb was detected during electrophoresis with an 0.8% agarose gel (Wako, Osaka, Japan). The purified 8-kb target product was ligated into a pCR-XL-TOPO vector (Invitrogen, Carlsbad, CA) and then transformed into a DH5™ competent cell (Toyobo, Osaka, Japan). The obtained clones were checked by PCR with the primer pair (sense: 5′-GCTTCGACAGCGACGTGGGGGAGT-3′; antisense: 5′-ACCCGCTCCGTCCCATTGAAGAAA-3′) that was used to amplify the 250-bp product of the chimpanzee DRB1 exon 2. Each of three plasmid clones from Sanzou and Iyo was isolated with a Nucleospin® Plasmid QuickPure kit (Macherey-Nagel, Düren, Germany).

The plasmid samples were labeled with a Big Dye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and analyzed with an ABI PRISM™ 377 DNA Sequencer (Applied Biosystems). Based on the sequence, the PCR primer pair for each chimpanzee was designed (DRB-Int1+ and

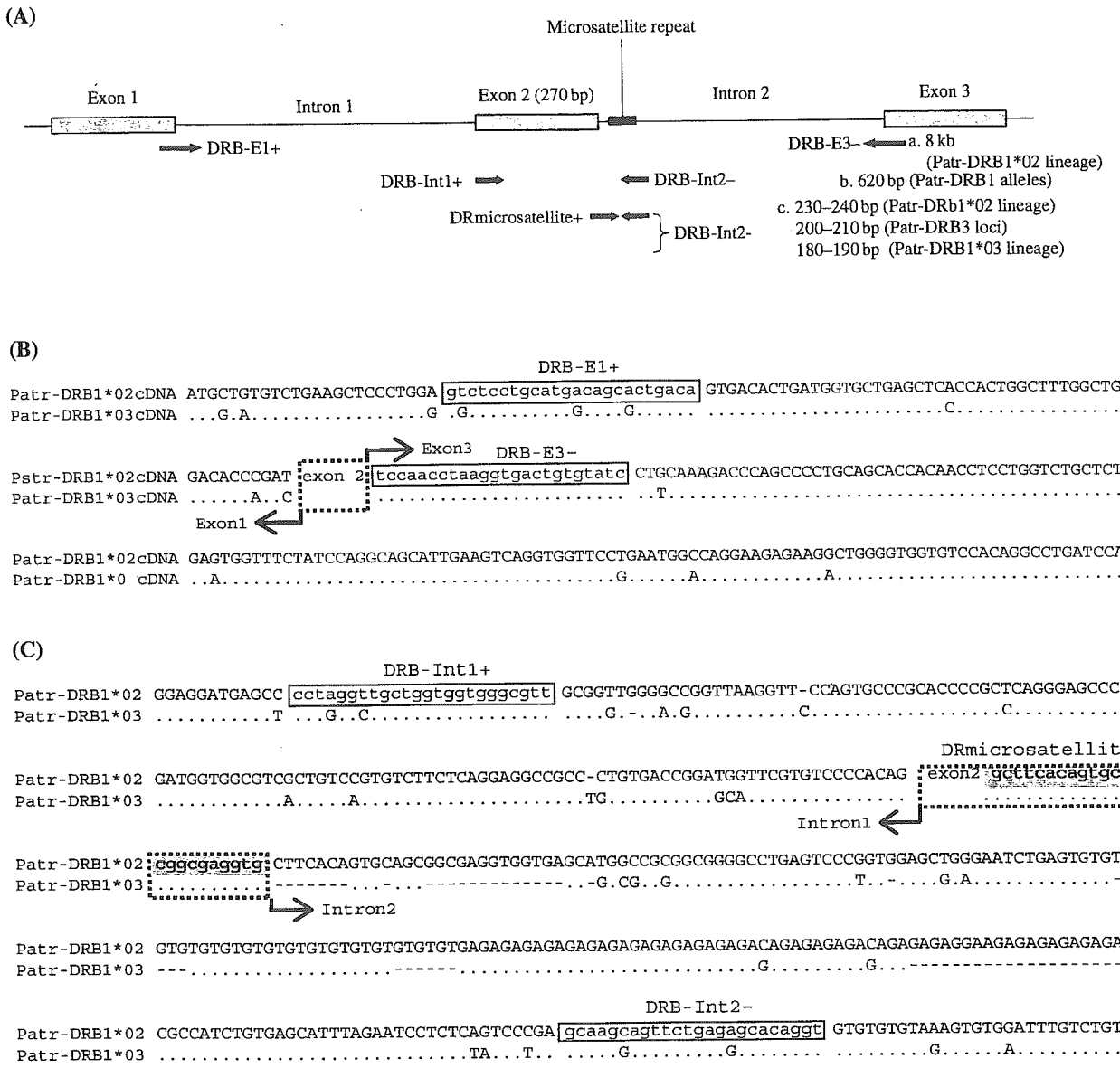

Figure 2 (A) Schematic diagram of the primer pairs used in this study. (a) 8-kb PCR for the detection of Patr-DRB1*02 lineage. (b) 620-bp PCR for the exon 2 amplification of Patr-DRB1 alleles. (c) An insert check for colony selection of Patr-DRB1 alleles. (B) A primer pair for 8-kb PCR. DRB-E1+ and DRB-E3– (a white rectangle) are located in exons 1 and 3, respectively. The upper and lower nucleotide sequences are the Patr-DRB1*02 lineage cDNA and Patr-DRB1*03 lineage cDNA (accession number AY663401), respectively. Dots (.) indicate identity with the Patr-DRB1*02 lineage. Exon 2 sequences are omitted. (C) Primer pairs for the exon 2 fragments and insert checking. The upper and lower nucleotide sequences are the Patr-DRB1*02 lineage and Patr-DRB1*03 lineage, respectively. Dots (.) indicate identity with the Patr-DRB1*02 lineage. Dashes (–) indicate the deleted sequence. The primer pairs for exon 2 appear inside the white rectangular boxes (DRB-Int1+ and DRB-Int2–). The primer pair for the insert check in clones appears in the gray and white boxes (DRmicrosatellite+ and DRB-Int2–). PCR, polymerase chain reaction.

DRB-Int2–; Figure 2A,C) to amplify a 620-bp product containing the entire exon 2 (270 bp) sequences. In addition, amplification of Patr-DRB1 lineages other than the Patr-DRB1*02 lineage was confirmed in one other chimpanzee (Siomi).

PCR, cloning, and the selection of DRB1 clones

PCR was applied to the DNA of the 23 chimpanzees as follows. Amplification was performed using AmpliTaq Gold (Applied Biosystems) with DRB-Int1+ and DRB-

Int2– primers, and the 620-bp products were cloned into a pCRII-TOPO vector (Invitrogen). Due to the high genetic homology of *Patr-DRB1*03* and the *DRB3* gene, the target products were detected in both the *DRB1* and *DRB3* genes (sequence data of detected Patr-DRB3 not shown). In order to select only DRB1 alleles, a specific insert check was carried out on the 12 selected clones. A new sense primer (DRmicrosatellite+) was designed from the microsatellite repeat region of intron 2, and PCR was carried out (Figure 2A,C). Identification of amplified products was performed on the 8% acrylamide gel. Based on the results of the electrophoresis, we again selected 6–8 clones of DRB1 alleles and sequenced them.

MHC Patr-DRB1 nomenclature

The official designation for the Patr-DRB1 allele detected in the present study was obtained from IMGT-NHP database (20). New alleles have been registered in the DNA Data Bank of Japan (DDBJ).

Results

Specific separation of DRB1 alleles in individual chimpanzees and microsatellite repeat patterns of each allele

In the DRB1 loci of the HLA, the lengths from exon 1 to exon 3 containing intron 1 on the HLA-DRB1*15 and HLA-DRB1*16 lineages (denoted as the Patr-DRB1*02 lineage in chimpanzees) and the HLA-DRB1*03 lineage (denoted as the Patr-DRB1*03 lineage in chimpanzees) (21) were approximately 8 and 11 kb, respectively. Similarly, the lengths from exon 1 to exon 3 of the Patr-DRB1*03 lineage was approximately 11 kb (accession number, AY663401).

In the present study, we were able to identify the existence of a Patr-DRB1*02 lineage by the amplification of 8 kb using the DRB-E1+ and DRB-E3– primer pair [Figure 2A, (a)]. Nine (Kirara, Kurara, Ichigo, Nozomi, Koiko, Susumu, Sumiko, Paru, and Zyugo) of the 23 chimpanzees did not have the target band of 8 kb. In fact, the Patr-DRB1*02 lineage was not detected in these chimpanzees in their sequence analysis (Table 1). We next amplified the second exon of DRB1 alleles with a 620-bp primer pair [Figure 2A, (b)] and cloned it in vector. We then attempted to carry out an insert check with the new primer pair [DRmicrosatellite+ and DRB-Int2–; Figure 2A, (c)]. Sequence analysis indicated that the lengths of the allele-specific PCR product were the following: the Patr-DRB1*02 lineage, Patr-DRB3 locus, and Patr-DRB1*03 lineage were 230–240, 200–210, and 180–190 bp, respectively (Figure 3). As a consequence, the detection quality of the DRB1 alleles was maximized

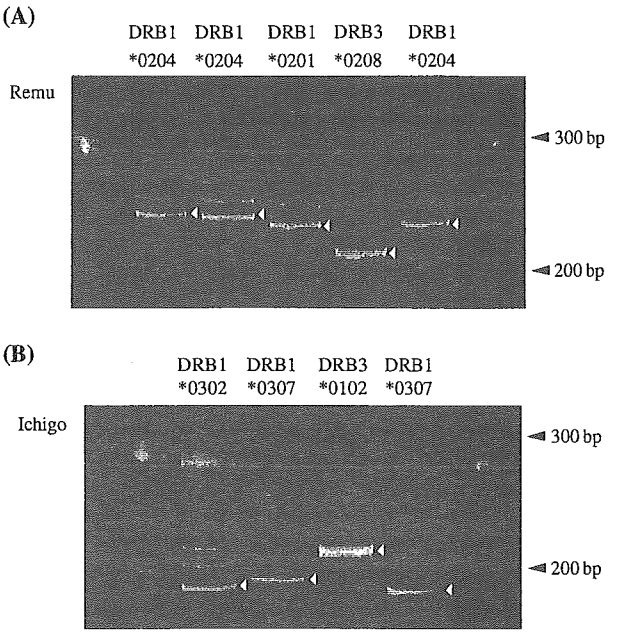

Figure 3 An acrylamide gel for each of the clones derived from Remu and Ichigo. PCR products were amplified by a DRmicrosatellite+ and DRB1-Int2– primer. The target band for each clone is represented by a white triangle. (A) Remu shows DRB1*0201, DRB1*0204, and DRB3*0208. (B) Ichigo shows DRB1*0302, DRB1*0307, and DRB3*0102. PCR, polymerase chain reaction.

by the present PCR technique and we were able to effectively detect the DRB1 alleles in each chimpanzee.

Moreover, we analyzed allelic diversity using a complex microsatellite located in intron 2, close to the polymorphic second exon. The detected polymorphism in the intron 2 microsatellite displays a complex pattern, with both sequence and length variability between alleles (Table 2).

Patterns of Patr-DRB1 alleles in individual chimpanzees

The patterns of the Patr-DPB1 alleles in each chimpanzee are summarized in Table 1. Nine alleles, including two new alleles (Patr-DRB1*0311 and Patr-DRB1*0312), were detected in this study and could be divided into three lineages: the Patr-DRB1*02 lineage, which included the Patr-DRB1*0201 and Patr-DRB1*0204 alleles; the Patr-DRB1*03 lineage, which included the Patr-DRB1*0302, Patr-DRB1*0305, Patr-DRB1*0307, Patr-DRB1*0309, Patr-DRB1*0311, and Patr-DRB1*0312 alleles; and the Patr-DRB1*10 lineage, which included only the Patr-DRB1*1001 allele. In this study, the Patr-DRB1*07 lineage was not detected.

The majority of chimpanzees (16/23) showed heterozygotes that combined with the DRB1*02 (1/16), DRB1*02/03 (11/16), DRB1*03/03 (3/16), and DRB1*1001 allele/03 lineages (1/16). The Patr-DRB1*0307 allele was found in

Table 2 Microsatellite variation of alleles detected in this study

Allele	5′ repeat	Middle repeat	3′ repeat	Dinucleotide^{-} repeat number of a complex microsatellite
Patr-DRB1*0201	$(GT)_{19-20}$	$(GA)_{11-12}CA(GA)_4CA(GA)_3GGAA$	$(GA)_6$	47–49
Patr-DRB1*0204	$(GT)_{16}$	$(GA)_{14}CA(GA)_4CA(GA)_3GGAA$	$(GA)_6$	47
Patr-DRB1*0302	$(GT)_{21}$	$(GA)_6AA$	$(GA)_6$	34
Patr-DRB1*0305	$(GT)_{16}$	$(GA)_4AA$	$(GA)_7$	28
Patr-DRB1*0307	$(GT)_{11}$	. . .	$(GA)_{21}$	32
Patr-DRB1*0309	$(GT)_{10}$	$(GA)_{18}CA$	$(GA)_4$	33
Patr-DRB1*0311	$(GT)_{20}$	$(GA)_5AA$	$(GA)_6$	32
Patr-DRB1*0312	$(GT)_9$	$(GA)_{16}CA$	$(GA)_4$	30
Patr-DRB1*1001	$(GT)_9$	$(GA)_{11}CA(GA)_4AA$	$(GA)_4$	30
Patr-DRB3*0208	$(GT)_6$	$(GA)_{14}(GA)_9GGAA(GA)_4CA$	$(GA)_3$	39
Patr-DRB3*0102	$(GT)_2$	$(GT)_7AT(GT)_6(GA)AA(GA)_6GGAA(GA)_4CA$	$(GA)_3$	38

13 chimpanzees. The identical twin chimpanzees, Kirara and Kurara, were found to share Patr-DRB1*0307 (Figure 1).

Sequence analysis of the Patr-DRB1 allele

The nucleotide sequences of Patr-DRB1 exon 2 and the deduced amino acid sequences are depicted in Figures 4 and 5, respectively. None of the alleles, including the two new alleles, displayed features characteristic of a pseudogene.

The common sequence motifs were concentrated in the upstream region in the exon 2 segment (codon 9–45) within the Patr-DRB1 lineages. All of the Patr-DRB1*03 lineages containing the two new alleles were found to show the EYSTS amino acid motif at positions 9–13 (the gray rectangular box in Figure 5). In addition, the Patr-DRB1*02 lineage was found to share an identical EEFMRFDSDV amino acid motif at positions 35–44 and the LQPKG amino acid motif at positions 9–13 (except the Patr-DRB1*0205 allele; solid-lined boxes in Figure 5). The FLDRYFY motif at positions 26–32 was found in two Patr-DRB1*02 alleles (Patr-DRB1*0202 and Patr-DRB1*0205) and six Patr-DRB1*03 alleles (Patr-DRB1*0306, Patr-DRB1*0308, Patr-DRB1*0309, Patr-DRB1*0310, Patr-DRB1*0311, and Patr-DRB1*0312; dotted-lined box in Figure 5).

On the other hand, there appeared to be broad variation in the downstream region of exon 2 (codon 60–90). The variations of the two new alleles (Patr-DRB1*0311 and Patr-DRB1*0312) were found in this region. Patr-DRB1*0311 was similar to Patr-DRB1*0310, but a different non-synonymous substitution was found at codon 84 (Gly→Arg; the gray box in Figure 4). Additionally, Patr-DRB1*0312 was similar to Patr-DRB1*0309, but two non-synonymous substitutions were found at codon 57 (Asp→Ser) and codon 61 (Trp→Tyr; solid-lined box in Figure 4). Furthermore, several alleles were encoded to valine, alanine, and leucine at position 86; Patr-DRB1*0305, Patr-DRB1*0306, and Patr-DRB1*0310 were changed to valine, Patr-DRB1*0307, Patr-DRB1*0309, and Patr-DRB1*0312 to alanine, and Patr-DRB1*0701 to leucine.

Discussion

Studies of the MHC class II DR molecule have been progressing with the intent of developing therapeutic and preventive vaccines (22). Specifically, the influence of $CD4^+$ T cells against individual epitopes in HCV has been studied in humans and chimpanzees (10, 23). Most individual epitopes fit the DR supertype motif and show high affinities with the DRB1 molecule, DRB1*1001, DRB1*0701, and DRB1*0402. It is believed that DRB1 alleles may play an important role in vaccine development.

Useful identification of MHC DRB alleles in the rhesus macaque (*Macaca mulatta*) and common marmoset (*Callithrix jacchus*) has been achieved through denaturing gradient gel electrophoresis (DGGE) and PCR-single-strand conformation polymorphism (PCR-SSCP) (24–26). In the chimpanzee, the traditional approach for typing DRB alleles involves cloning and sequencing methods using PCR (1, 2, 10, 21). However, DNA sequencing specific to each *DRB* gene from individual chimpanzees has been difficult, because there is high genetic homology within functional DRB loci. The traditional approach for cloning and sequencing exon 2 on *DRB* genes for a given colony has been reported to be disadvantageous, in which a minimum of 30 different molecular clones must be selected (10). Furthermore, in our preliminary experiments using the primer set derived in a previous study (1), we were unable to detect DRB1 alleles among 25–30 different clones in the tested chimpanzees (data not shown). Therefore, in the present study, we hypothesized that a PCR technique using three separate steps could facilitate the identification and characterization of unique Patr-DRB1 alleles and provide a rapid and unambiguous detection method for Patr-DRB1 alleles.

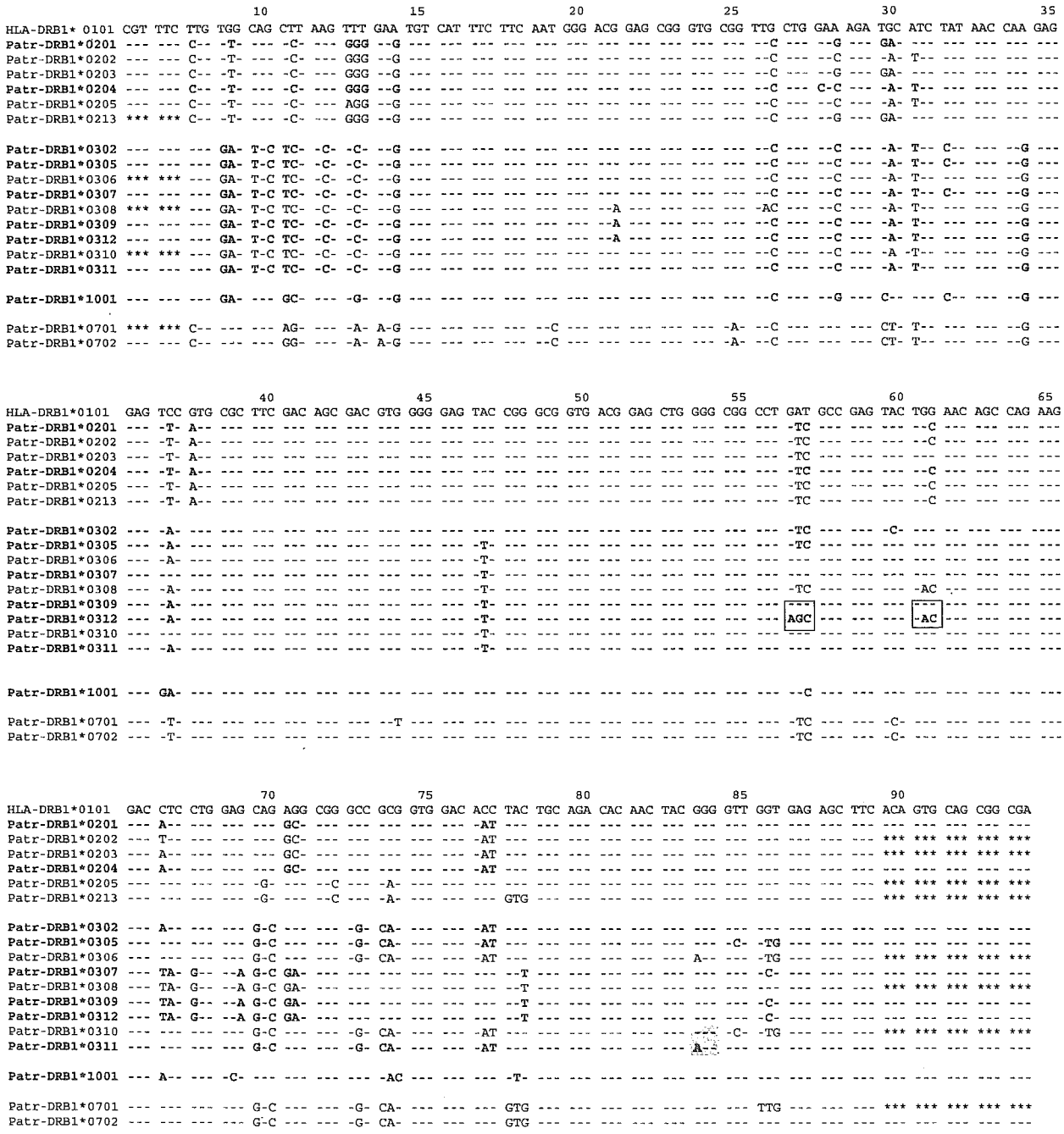

Figure 4 Alignment of the nucleotide sequences of Patr-DRB1 exon 2 alleles. The seven known alleles and two new alleles detected in this study are highlighted by bold print (Patr-DRB1*0311, DDBJ accession number AB201752; Patr-DRB1*0312, DDBJ accession number AB201753). An HLA-DRB1*0101 allele is given at the top. Dashes (–) indicate identity with the consensus sequence and the asterisk (*), a lack of availability of sequence information.

We were able to analyze the full length of exon 2 on Patr-DRB1 loci using a 620-bp primer pair. The sequence data of exon 2 of Patr-DRB1 alleles suggest that the β-pleated sheet and the α-helical portion have evolved distinctly from one another. In the β-pleated sheet portions, similar amino acid motifs within the Patr-DRB1*02

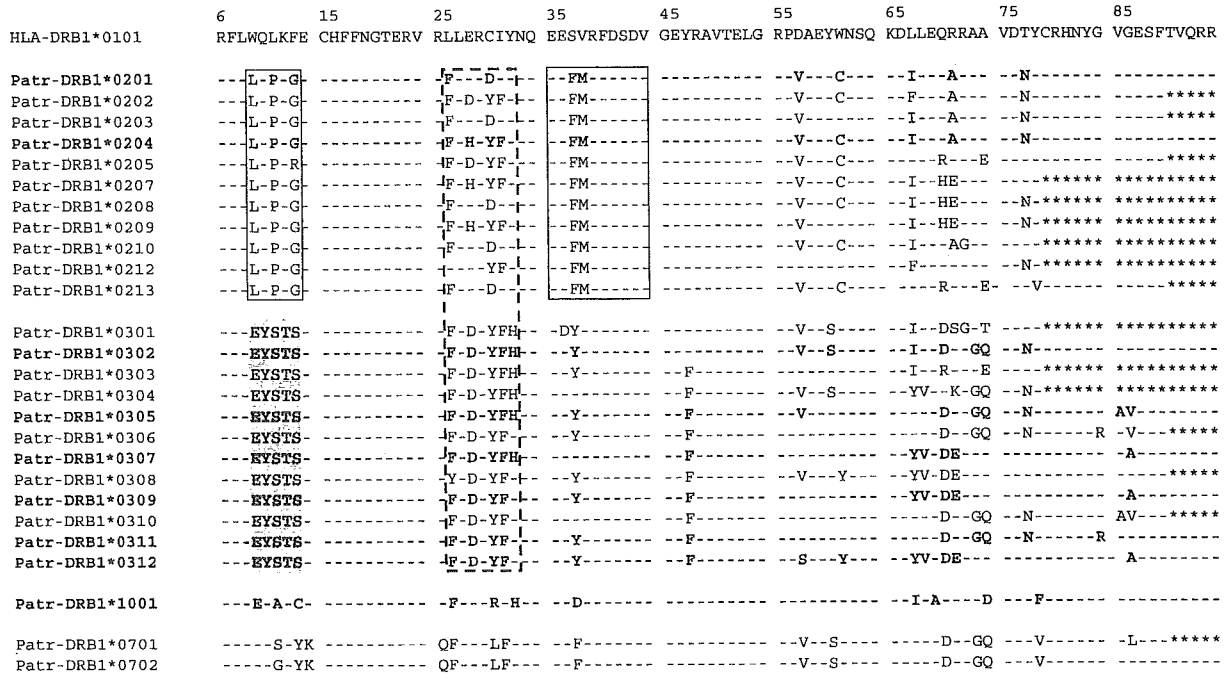

Figure 5 Alignment of the deduced amino acid sequences of Patr-DRB1 exon 2 alleles. The detected alleles in this study are highlighted by bold print. An HLA-DRB1*0101 allele is given at the top. Dashes (–) indicate identity with the consensus sequence and an asterisk (*), a lack of availability of sequence information.

and Patr-DRB1*03 lineages have converged at positions 26–32. Interestingly, the FLDRYFY motif shares some alleles of the HLA-DRB1*04 lineage. In the present study, this motif of some alleles of the Patr-DRB1*02 and Patr-DRB1*03 lineages was found to be conserved in some of the β-pleated sheet portions of the HLA-DRB1*04 lineages. These variations were thought to have been introduced by point mutations in combination with recombination within the β-pleated sheet portion in humans and chimpanzees. In addition, similar variations have been found in the β-pleated sheet portion in other primate species (26–29). Specifically, the Patr-DRB1*02 lineage shows the LQPKG amino acid motif at positions 9–13, except in the Patr-DRB1*0205 allele, and the EEFMRFDSDV amino acid motif at positions 35–44. These amino acid motifs, which appear to be of a more recent origin than the EYSTS motif of the MHC-DRB1*03 lineages, are shared in all primates and are likely to have been conserved after speciation of the Patr-DRB1*02 lineage and found only in the homnoids.

On the other hand, new alleles appear to be generated primarily by changes in the α-helical loop portion. These variations are consistent with those identified in previous studies (21, 30, 31), which suggest that the allelic variation at MHC-DRB1 has been generated primarily through exchanges in the α-helical loop portion of exon 2. Additionally, amino acid variations of Patr-DRB1 alleles at position 86 have been described in other non-human primate species (28, 32). Because the side chain of position 86 is directly related to the antigen-binding site, position 86 is known to influence DR dimer stability and the length of the peptide for the antigen-biding site (33). Therefore, these findings suggest that the differential peptide-binding function in non-human primates may be achieved by employing other types of amino acids.

In the present study, we used a 620-bp PCR technique to analyze not only the full length of exon 2 but also the sequence of a complex microsatellite located downstream of the end of exon 2. The microsatellite structure of HLA-DRB1 alleles is different among individual allelic lineages (34). However, until now, intron sequence variability of the *Patr-DRB* gene presenting among alleles within a lineage has provided only limited information. In the data provided in Table 2, the general structure of the microsatellite was strongly associated with individual lineages. Interestingly, microsatellite repeat structures such as the (GT)x(GA)y structure in Patr-DRB1*0307 differ from those in other Patr-DRB1*03 lineages. However, the microsatellite repeats of all Patr-DRB1*03 lineages have comparable lengths.

Because 620-bp PCR detected *Patr-DRB3* genes as well as *Patr-DRB1* genes, we decided to carry out a

microsatellite-PCR technique using the variable repeat length of a complex microsatellite located in intron 2 of the Patr-DRB1 and Patr-DRB3 loci. Based on the variation of microsatellite repeat patterns between Patr-DRB1 alleles and Patr-DRB3 alleles, we successfully selected Patr-DRB1 alleles.

Identification and analysis of Patr-DRB1 alleles using this method should be useful in facilitating colony management of chimpanzee groups in Japan and are expected to contribute to studies on disease association and susceptibility associated with MHC class II molecules. In addition, the data of the microsatellite sequences of the chimpanzees analyzed in the present study will provide a basis for examining the evolution of microsatellite sequences and for tracing the origin of individual Patr-DRB1 alleles.

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臨床研究と展望　実験動物由来感染症

Bウイルス感染症

本藤　良　斉藤　彩　植田富貴子

Infectious disease of simian herpes B virus

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Abstract

The epidemiology and the method of diagnosis were elucidated for simian herpes B virus (SHBV) infection. It is important that usefulness was demonstrated for the methods of DNA diagnosis having high sensitivity and specificity for SHBV and HSV-1, 2 types, and of detectable serological diagnosis for each specific antibody.

The methods allowed the final diagnosis of the human infection due to the reactivation of latent SHBV and the HSV infection from human to monkey. These results would be able to become important and fundamental knowledge for the sero-epidemiological analysis of the infectious stile.

Key words: α-herpesvirinae, simian herpes B virus, DNA diagnosis, PCR-microplate hybridization, sero-epidemiology

はじめに

Bウイルス感染症は，旧世界ザル由来の人獣共通感染症の一つである．1932年に米国の研究者Brebnerが外見上正常なアカゲザルに咬まれ，急性進行性髄膜脳炎で死亡したのが初発報告例である．1933年にその脳からウイルスが分離され，その性状が明らかとなった[1]．

Bウイルスは，正式名称cercopithecine herpesvirus 1(オナガザルヘルペスウイルス1)，一般にsimian herpes B virus(SHBV)と呼ばれ，ヘルペスウイルス科のαヘルペスウイルス亜科に分類されている．分子量は約130kd，核酸は二本鎖DNA，約160kbでG-C含有量が約75％である．

1. SHBV感染症の疫学

a. 自然宿主と感染様式

自然感染は，旧世界ザルのマカク属に分類されるサル間で主に感染環を形成している．そのなかにはニホンザルも含まれる．初感染の後，多くは不顕性感染の経過をとり，ヘルペスウイルスの特性から後根神経節に潜伏感染を引き起こし，ストレスや免疫抑制などの要因により再活性化を繰り返す．口腔領域などに水疱や潰瘍などの回帰性ヘルペス像を呈し，病巣組織にCowdry A型の核内封入体を形成する(図1)．この過程で唾液，結膜(涙液)，陰部粘膜(尿)から

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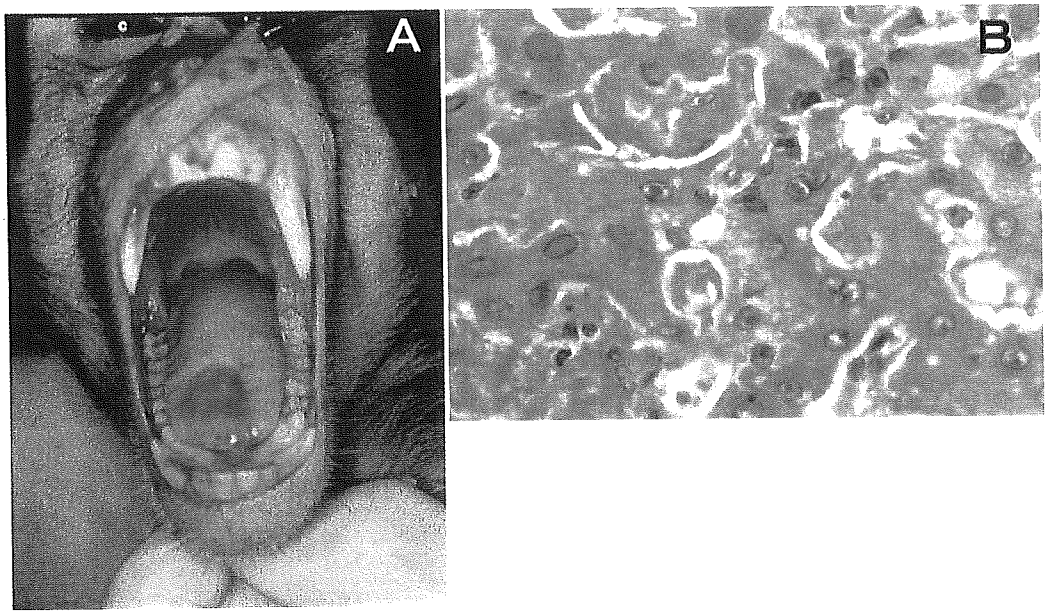


図1 SHBV感染カニクイザルの病像
A: 口腔領域および舌にみられる潰瘍.
B: 肝臓組織病巣にみられたCowdry A型核内封入体(HE).
(岐阜大学・柳井徳磨博士, Dr. Simon M提供)

ウイルスが分泌されて感染源となり, 咬傷や引っ掻き傷などの接触感染により伝播するものと考えられている. 感染ザルでは軽症で, ヒトおよび新世界ザルが感染すると脳脊髄炎症状を呈し, 致命率はヒトで約50%である.

b. 感染症の発生状況

現在までに, 幸いにして我が国でのSHBV感染と発症に関する報告は皆無であるが, 外国例では少なくとも40数例の症例が報告されている. 多くはサルに関連した研究者や動物飼育管理者による実験室内感染であるが, 潜伏ウイルスの再活性化による再発症例や第二次感染が起こり得ることも明らかにされている. また, ウイルス感染のモデル実験などにサル類の使用数が増加していることに加えて, 最近ではペットを含めた動物園などの展示ザルからのヒトへの感染が懸念されている[2-5].

c. マカク属サルのSHBV感染状況

我が国において, 輸入マカク属サルのSHBV抗体の保有調査が行われている. 輸入野生カニクイザルでの調査では, ①1979-87年: 2,018頭中の51.2%, ②1994-95年: 1,200頭中の41.9%がSHBV抗体陽性であった(国立感染症研究所・筑波霊長類センター). また, 国立大学・実験用飼育ザル類での調査(1997年)では, ③マカク属のサル類947頭中の40%が抗体陽性で, そのうちニホンザルでは34%が抗体陽性であった[6].

SHBV抗体陽性ザルは, 後根神経節に潜伏の状態で生涯ウイルスを保有することで重要な知見である.

2. SHBV感染症の分子疫学

著者ら[7,8]は, SHBV感染・抗体陽性の輸入カニクイザル, A群(10頭)およびB群(20頭)の左右三叉神経節からPCR(polymerase chain reaction)法による潜伏SHBVゲノムの検出を試みた. その結果を**表1**に示した. SHBV抗体陽性ザルのうち, 三叉神経節に潜伏感染を起こしているものが約35-50%であることが判明した. これらの潜伏ウイルスがストレスや免疫抑制などの要因により再活性化を繰り返すことにより, 唾液からウイルスが分泌されて, 主要感染源となり得るものと考えられる.

表1 カニクイザル三叉神経節におけるSHBVの潜伏感染

No.	個体番号	性別	体重(kg)	SHBV・検出 PCR	SHBV・検出 hybridization	No.	個体番号	性別	体重(kg)	SHBV・検出 PCR	SHBV・検出 hybridization
1	96CO819	M	3.65	右：(−) 左：(−)	(−) (−)	11	96CO576	F	3.15	右：(−) 左：(−)	(−) (−)
2	96CO527	M	2.90	右：(+) 左：(+)	(+) (+)	12	96CO220	F	2.90	右：(−) 左：(−)	(+) (−)
3	97CO123	M	3.05	右：(−) 左：(−)	(+) (−)	13	96CO304	F	2.60	右：(−) 左：(−)	(−) (−)
4	96CO389	M	2.45	右：(+) 左：(−)	(+) (−)	14	96CO384	F	2.60	右：(−) 左：(−)	(+) (+)
5	97CO633	M	2.50	右：(−) 左：(−)	(−) (−)	15	96CO306	F	2.35	右：(−) 左：(−)	(−) (−)
6	96CO129	M	3.30	右：(−) 左：(−)	(−) (−)	16	96CO296	F	2.65	右：(+) 左：(+)	(+) (+)
7	96CO627	M	3.20	右：(−) 左：(−)	(−) (−)	17	C965546	F	3.00	右：(−) 左：(−)	(−) (−)
8	96CO743	M	2.85	右：(−) 左：(−)	(−) (−)	18	C967547	F	2.55	右：(−) 左：(−)	(−) (−)
9	96CO115	M	3.10	右：(−) 左：(+)	(+) (+)	19	C965285	M	3.05	右：(−) 左：(−)	(−) (−)
10	96CO558	F	3.00	右：(−) 左：(−)	(−) (−)	20	C965411	M	3.25	右：(−) 左：(−)	(−) (−)

※輸入カニクイザルB群(20頭).

3. SHBV感染症の臨床症状

SHBVが分泌されているサルから咬傷などにより感染した場合，局所での第一次増殖後，末梢神経を伝達して中枢神経組織に到達し，上行性脊髄炎や脳脊髄炎を起こし，経時的な臨床症状を呈する．①早期症状：接触部の激痛やそう痒感，外傷部周囲の水疱や潰瘍，リンパ節腫大．②中期症状：発熱，接触部の感覚異常や麻痺，結膜炎など．③晩期症状：頭痛と項部硬直，悪心と嘔吐，脳幹部症状(眩暈，交差性知覚障害，脳神経麻痺)，意識障害，脳炎などである[9]．

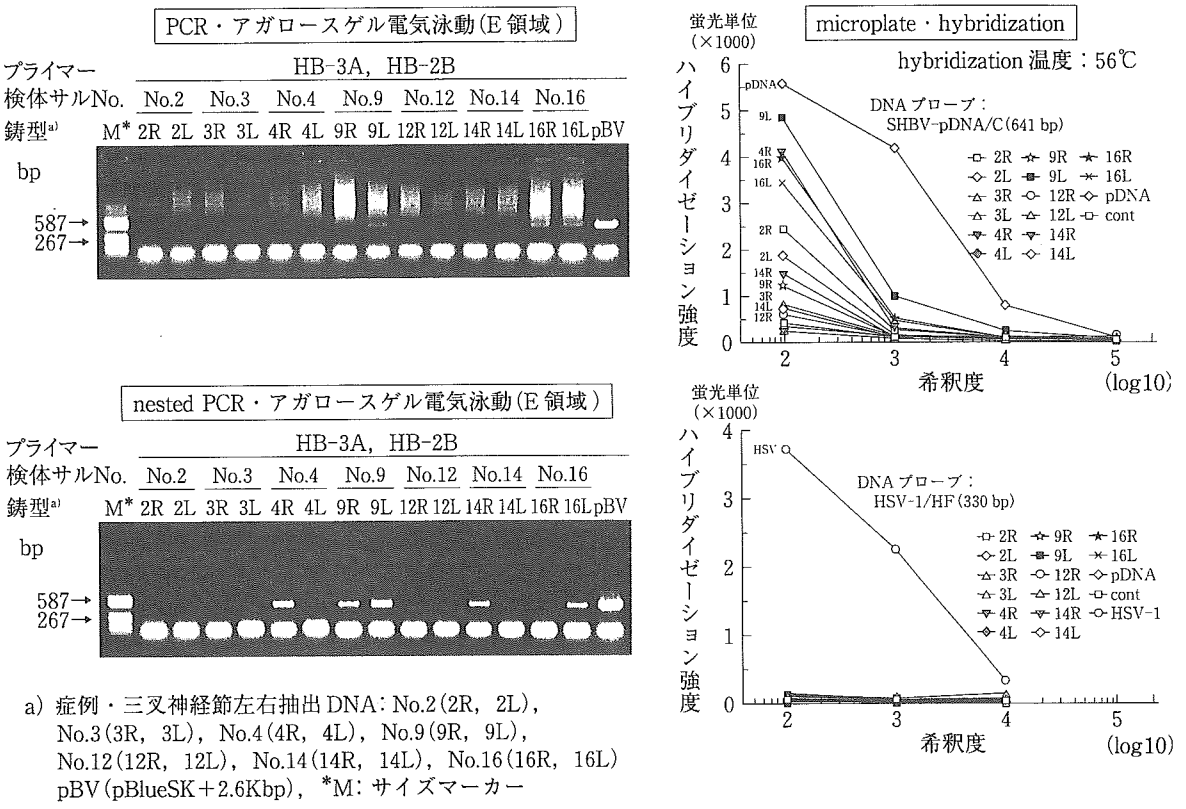
4. SHBV感染症の診断法

a. 医療機関での診断

サルによる創傷の後，創傷部皮膚に水疱の発疹や神経症状を呈した場合にはSHBVの感染を疑う．確定診断は，皮膚病変部，脊髄液および血清を検体として，特異ウイルスゲノムの検出や抗体の検出で行う．医師の依頼にかぎり，国立感染症研究所・筑波霊長類センターで実施している．なお，サルのSHBV抗体検査は予防衛生協会(筑波霊長類センター内)で実施している．また，SHBV感染の患者を診断した場合は，最寄りの保健所に7日以内に届出義務がある(感染症法・第12条)．

b. 実験室での診断

SHBV感染症の確定診断については，非常に困難が伴うところである．ウイルス分離では，バイオハザードレベルでP3からP4施設が必要であり，現実的には不可能と考えてよい．また，抗体の検出では近縁ヘルペスウイルス，特に同じ亜科に属する単純ヘルペスウイルス1，2型(herpes simplex virus: HSV−1，2)との相同性



a) 症例・三叉神経節左右抽出 DNA：No.2(2R, 2L), No.3(3R, 3L), No.4(4R, 4L), No.9(9R, 9L), No.12(12R, 12L), No.14(14R, 14L), No.16(16R, 16L) pBV(pBlueSK+2.6Kbp), *M：サイズマーカー

→ PCR の条件：1) denature：94℃ 2 分　3) extension：72℃ 4 分
2) annealing：65℃ 3 分　4) DMSO：2.5 %

図 2　カニクイザル三叉神経節からの潜伏 SHBV ゲノムの検出
PCR-microplate hybridization 法.

が高く極めて近縁で，本ウイルス感染症との類別診断の難しいところである.

1) 遺伝子診断法

ウイルス分離に代わる方法として，PCR 法による特異ウイルスゲノムの検出が一般的である. 著者ら[7,8]は，近縁ヘルペスウイルスと相同性の低い，SHBV ゲノム上の Us 領域内(ORF, Us5: gJ, Us6: gD)をターゲット領域とし，PCR 法と microplate-hybridization 法[10,11]を併用した，特異性と感度の高いゲノムの検出と同定およびゲノムコピー数の定量法を用いて，微量検体からの SHBV ゲノムの検出を実施している(**図 2**).

2) 血清学的診断法

ELISA やウエスタンブロット法および免疫蛍光間接法で実施されている. ヒト血清では，HSV-1，2 型との共通抗原性が高く，その交叉性からヒト感染症例での類別診断は困難である. 著者ら[12]は，SHBV および HSV-1，2 型のゲノム構造糖蛋白(gD, gG)[13,14]を抗原とした蛍光 ELISA 法による，高感度で特異性の高い血清学的診断法の開発を試み，その実用性を明らかにした.

本法は，至適反応条件および至適抗原濃度の条件下において，各抗血清の希釈度に対比した，蛍光単位の標準反応曲線を求め，それに対比した血清の最終希釈度をもって，抗体価を判定する手法である.

本手法により，SHBV の gD 抗原では，HSV-1，2 型感染血清との交叉反応も低く，SHBV 特異抗体の検出が可能である(**表 2**). また，HSV-1，2 型の各 gG 抗原では，SHBV 感染血清との交叉反応もなく，HSV-1，2 型の各特異抗体の検出と HSV-1，2 型の重複感染例も特異的に識別が可能である(**表 3**).