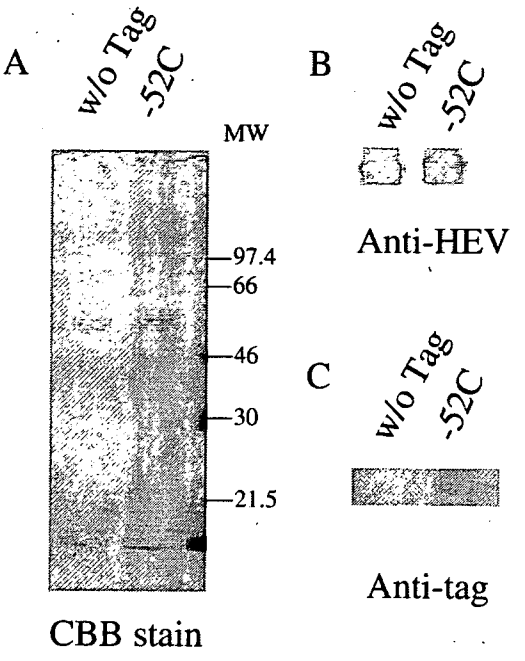
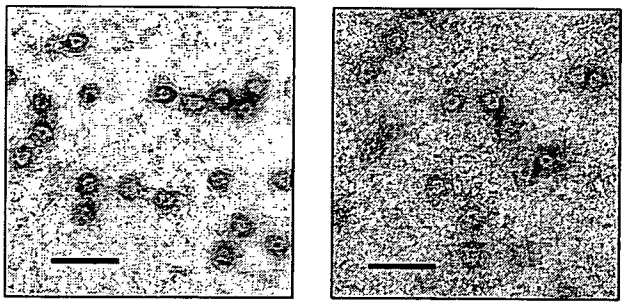


Fig. 3. Purification of the chimeric VLP. Purified VLP-52C was analyzed for its purity (A) and reactivity to anti-HEV (B) and anti-tag antibodies (C). A. Equal amounts (0.3 μg) of purified VLP-52C (–52C) and VLP without tag (w/o Tag) were separated on SDS-PAGE and stained by Coomasie brilliant blue staining. Positions of molecular weight markers are indicated on the right of the panel. B and C. Equal amounts (0.1 μg) of VLP-52C (–52C) and VLP without tag (w/o Tag) were analyzed by Western blotting using anti-HEV (B) and anti-tag (C) antibodies, respectively.

for preventing dORF2 from being incorporated into a VLP form. Rather, the amino acid sequences encoded by the HEV ORF2 genome prevented the formation of VLP.

We attempted to purify chimeric VLPs from the supernatant of Tn5 cells expressing chimeric dORF2 with a tag at either C-termini. The VLP-52C was slightly larger than the HEV-VLP without the tag (Fig. 3A). The purified VLP-52C retained the antigenicity of HEV as well as the intact tag epitope, as shown by the reactivity of specific antibodies (Figs. 3B and 3C).

Electron microscopic observation showed that VLP-52C was approximately 25 nm in diameter and indistinguishable from the



A. -52C B. w/o Tag

Fig. 4. Electron micrograph of VLP-52C. VLP-52C (A) and VLP without tag (B) were observed under electron microscopy after negative staining at a magnification of ×60,000. Inserted bar indicates 100 nm.

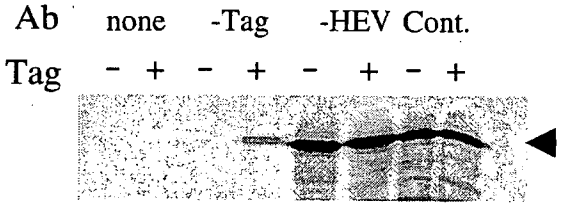


Fig. 5. Surface exposure of the tag epitope on VLP-52C. Surface exposure of the tag epitope on intact VLP-52C was examined by immunoprecipitation with the anti-tag antibody. Antibodies used are indicated at the top of panel. None, negative control without antibody; α-Tag, anti-tag antibody; α-HEV, anti-HEV antibody; cont., purified VLP-52C and VLP without tag were run as controls. The second row indicates either VLP with (+) or without the tag (−).

VLP without the tag (Fig. 4). Using two methods, we confirmed that the inserted epitope tag was exposed on the surface. The intact VLP-52C was immunoprecipitated with the anti-tag antibody, while the anti-HEV antibody immunoprecipitated both VLP-52C and the VLP without the tag (Fig. 5). Furthermore, the anti-tag antibody specifically reacted with the intact VLP-52C in an ELISA (data not shown). The results of immunoprecipitation and ELISA using intact chimeric VLP suggest that the tag epitope is exposed on the surface of the HEV-VLP.

Immune Responses to Chimeric HEV-VLPs through Oral Administration

Since many pathogenic viruses and bacteria establish their initial infections through the mucosal surface, vaccine strategies that can stimulate mucosal immunity have been widely studied (reviewed in Ogra *et al.*).[19] However, there are several difficulties in oral immunization with non-replicating molecules, such as low pH in the stomach, presence of proteolytic enzymes in the digestive tract, and presence of physical as well as biochemical barriers associated with the mucosal surface itself.[19] We previously reported that the HEV-VLP preserved original HEV construction and entered the epithelial cells of the small intestine by oral administration.[20] From these findings, mice were immunized with 50 μg of purified VLP-52C by the oral route four times at two-week intervals, with the mice having the ability to induce mucosal and systemic epitope-specific antibody responses. Specific IgG antibodies to the tag as well as to HEV were detected in intestinal fluids as early as two wpi (Fig. 6A). IgG levels in intestinal fluids continued to increase until the termination of the experiments. Specific IgA to both the tag and HEV also appeared in intestinal fluids from two wpi, paralleling the IgG levels (Fig. 6B). The IgA levels also continued to increase until the termination of experiments. As expected, the control mice immunized with VLP without the tag developed IgG and IgA only to HEV. In sera, levels of a specific IgG antibody to both the tag and HEV showed slightly higher OD values than those in non-immunized controls, but they never reached significant levels, as occurred in the intestinal fluids (Fig. 6C). The levels of specific IgA in sera were also low, although the OD values were also higher than the non-immunized controls (Fig. 6D). The control mice immunized with VLP without the tag showed similarly low OD values to HEV. The specific antibodies in the intestinal fluids were analyzed for their isotypes at 10 wpi. At this point, average OD values and SD for IgG and IgA in the intestinal fluids were 1.02±0.22 and 0.64±0.038, and 0.96±0.086 and 0.66±0.040, respectively, to HEV and the tag in three mice immunized with the chimeric VLP. In the control mice immunized with VLP without the tag, the average OD values and SD for IgG and IgA were 0.88±0.047 and 0.64±0.027, and 0.11±0.024 and 0.084±0.013, respectively, to HEV and the tag. All

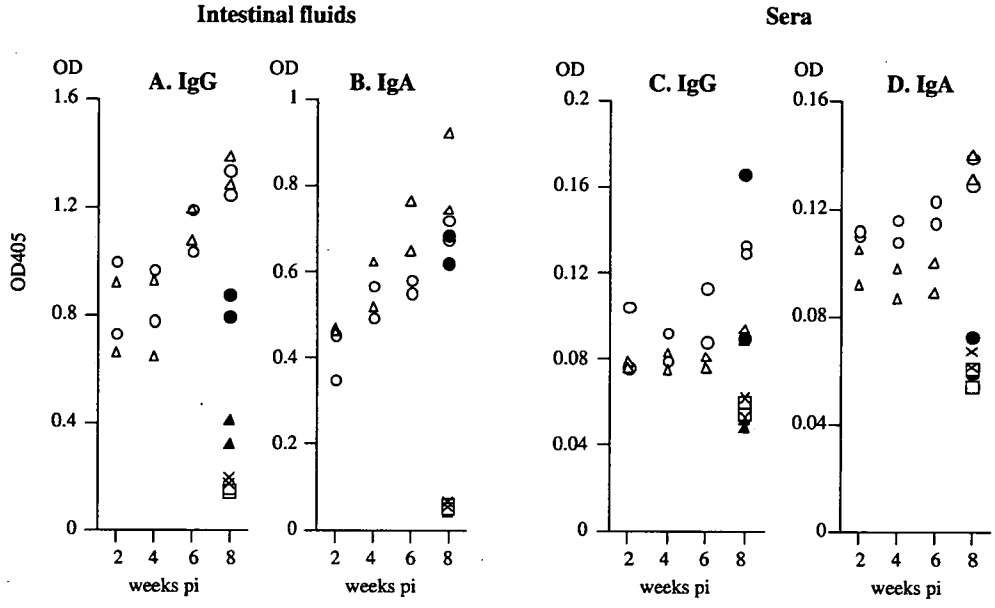


Fig. 6. IgG (A and C) and IgA (B and D) levels in intestinal fluids (A and B) and sera (C and D) of orally immunized mice. Circles and triangles indicate HEV-specific and the tag epitope-specific antibody levels, respectively, in individual mice. Two immunized mice were sacrificed at each time point (two, four, six and eight wpi). Specific antibody levels to HEV and the tag epitope of control mice immunized with VLP without the tag (closed circles and closed triangles, respectively) and background levels to HEV and the tag epitope of non-immunized mice (squares and crosses, respectively) are also shown. Antibody levels are indicated as OD405 in ELISA when sera and intestinal fluids were diluted at 1:100 and 1:2, respectively.

subclasses of IgGs to HEV — except IgG3, IgM, and IgA — were evident in all mice (Fig. 7B). Both to the tag and HEV, all mice failed to develop IgG3 above the detectable level (Figs. 7A and 7B). In the control mice immunized with VLPs without the tag, HEV-specific antibody reactions similar to the those with the chimeric VLPs were shown (Fig. 7B), while no detectable level of any isotype antibody specific to the tag was observed (Fig. 7A), as expected.

Induction of foreign epitope-specific antibody (Ab) responses by chimeric VLP administration is not easy compared with inducing cellular immune responses such as a cytotoxic T lymphocyte (CTL) response.[8,10,11,21,22] Moreover, our results showed Ab responses by oral

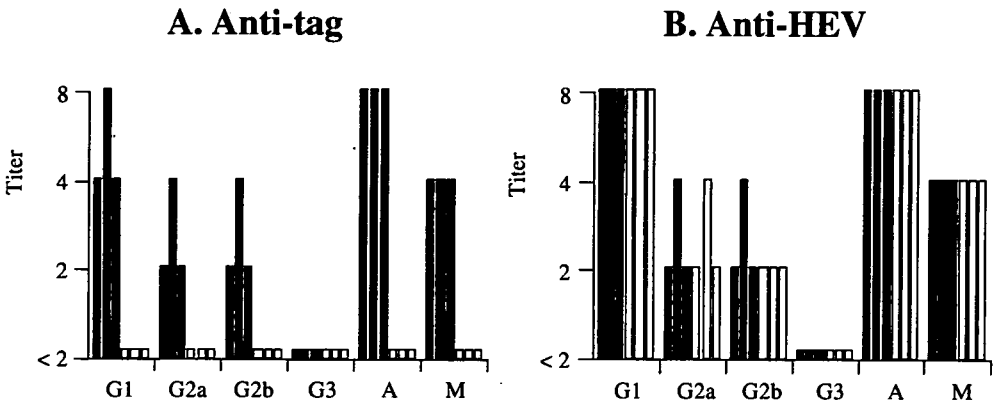


Fig. 7. Isotypes of antibodies specific to the tag epitope (A) and HEV (B) in intestinal fluids in orally immunized mice sacrificed at 10 wpi. Levels of IgA (A), IgM (M), and IgG subclasses (G1, G2a, G2b and G3) were examined by ELISA using isotype-specific secondary antibodies and are shown as end-point titers. Solid and open bars indicate antibody levels of each mouse immunized with the chimeric VLP and VLP without the tag epitope insertion, respectively.

administration to overcome the difficulties of a severe environment through the digestive tract. It is plausible that HEV-VLPs, which are derived from an orally transmissible virus, were incorporated into HEV-permissive epithelial cells in the small intestine because they retained structures and properties similar to those of HEV particles, producing an infection similar to that induced naturally.[17] It has been shown that the VLP structure should provide resistance to severe environments in the digestive tracts and enable specific binding to the mucosal surface if an appropriate VLP is chosen.[23] The delivery of a vaccine antigen (Ag) for induction of mucosal immune responses is usually achieved through the upper nasopharynx-associated lymphoid tissue (NALT), upper airway, salivary glands and tonsils.[24,25] Despite its obvious convenience, oral administration is rarely successful since it is quite difficult to protect vaccine Ag from the environment in the digestive tract.

The results of immunoprecipitation and ELISA using intact chimeric VLPs suggest that the tag epitope is exposed on the surface of the HEV-VLP. The successful induction of antibodies to the tag

also supports the hypothesis that the tag is exposed on the surface, since an internally localized B cell epitope in chimeric parvovirus-VLP failed to induce a specific antibody response.[11] Furthermore, this hypothesis is consistent with the results of three-dimensional analysis of the Norwalk virus-VLP particle, in which the C-terminal is exposed to the VLP surface.[26] Considering that B cell epitopes are generally hydrophilic and most likely exposed to the VLP surface, B cell epitope regions may not be directly involved in the protein-protein interactions to form a VLP. Our unsuccessful insertions into internal sites suggest that the integrity of internal regions must be maintained for proper protein folding and VLP formation. To find potential internal insertion sites, a precise three-dimensional structural map of the HEV-VLP may be necessary.

Oral vaccination has obvious advantages for a field trial in a large-scale public health vaccination program.[27] From a practical standpoint, oral administration is less stressful for vaccine recipients and does not require professional skill for administration. Moreover, delivery of vaccines via the intestinal tract is considered to be inherently safer than systemic injection. Encouraging results of phase I trials using Norwalk virus VLPs have recently been reported.[28] We also confirmed that chimeric HEV-VLPs carrying foreign CTL and B cell epitope at C-termini can elicit mucosal and systemic cellular immune responses as well as humoral immune responses by oral administration (submitted). It has become apparent that mucosal immune responses on different mucosal surfaces were achieved simultaneously, despite the initial stimulation of a single mucosal site.[29,30] Therefore, it is probable that oral administration of chimeric HEV-VLPs stimulates immune responses simultaneously on distant mucosal surfaces as well. This phenomenon significantly extends the potential use of chimeric HEV-VLPs as an oral vaccine vehicle.

A chimeric HEV-VLP has several advantages as an oral vaccine vector. First, large amounts can be easily obtained from standard cultivation protocols compared with amounts of other VLPs obtained. Second, the outcome of delivery of vaccine Ag in humans can be predicted using conventional laboratory animals, since HEV naturally infects various animals as well as humans through the same infectious

route and target cells.[6,31] Third, HEV-VLPs are stable at room temperature. Fourth, anti-HEV immune responses had no effect on boosting administration in the present study. Thus, HEV-VLPs are an attractive vaccine vector in developing countries because these VLPs can be preserved without the requirement of any particular equipment. These findings suggest that chimeric VLPs derived from orally transmissible viruses can be used as vaccine vectors to mucosal tissue by oral administration for the purpose of vaccination.

Acknowledgments

All experiments described in this chapter were carried out by Dr. Masahiro Niikura (Department of Microbiology and Molecular Genetics, Michigan State University) and Dr. Shiki Takamura (Department of Bioregulation, Mie University School of Medicine, Mie, Japan). This work was supported by Health Science Research Grants from the Ministry of Health, Labor and Welfare of Japan; the Ministry of Education, Culture, Sports, Science and Technology of Japan; and Regional Science Promotion Program.

References

1. Jameel S, Zafrullah M, Ozdener MH, Panda SK. (1996) Expression in animal cells and characterization of the hepatitis E virus structural proteins. *J Virol* **70**: 207–216.
2. Zafrullah M, Ozdener MH, Kumar R, *et al.* (1999) Mutational analysis of glycosylation, membrane translocation, and cell surface expression of the hepatitis E virus ORF2 protein. *J Virol* **73**: 4074–4082.
3. McAtee C, Zhang Y, Yarbrough PO, *et al.* (1996) Purification of a soluble hepatitis E open reading frame 2-derived protein with unique antigenic properties. *Protein Expr Purif* **8**: 262–270.
4. McAtee CP, Zhang Y, Yarbrough PO, *et al.* (1996) Purification and characterization of a recombinant hepatitis E protein vaccine candidate by liquid chromatography-mass spectrometry. *J Chromatogr B Biomed Appl* **685**: 91–104.
5. Robinson RA, Burgess WH, Emerson SU, *et al.* (1998) Structural characterization of recombinant hepatitis E virus ORF2 proteins in baculovirus-infected insect cells. *Protein Expr Purif* **12**: 75–84.

6. Li TC, Yamakawa Y, Suzuki K, *et al.* (1997) Expression and self-assembly of empty virus-like particles of hepatitis E virus. *J Virol* **71**: 7207–7213.
7. Xing L, Kato K, Li TC, *et al.* (1999) Recombinant hepatitis E capsid protein self-assembles into a dual-domain T = 1 particle presenting native virus epitopes. *Virology* **265**: 35–45.
8. Casal JI. (2001) Use of the baculovirus expression system for the generation of virus-like particles. *Biotechnol Genet Eng Rev* **18**: 73–87.
9. Niikura M, Takamura S, Kim G, *et al.* (2002) Chimeric recombinant hepatitis E virus-like particles as an oral vaccine vehicle presenting foreign epitopes. *Virology* **293**: 273–280.
10. Rueda P, Hurtado A, del Barrio M, *et al.* (1999) Minor displacement in the insertion site provokes major differences in the induction of antibody responses by chimeric parvovirus-like particles. *Virology* **263**: 89–99.
11. Rueda P, Marinez-Torrecuadrada JL, Sarraseca JC, *et al.* (2000) Engineering parvovirus-like particles for the induction of B-cell, CD4+ and CTL responses. *Vaccine* **18**: 325–332.
12. Yao Q, Zhang R, Guo L, *et al.* (2004) The cell-independent immune responses to chimeric hemagulutinin/simian human immunodeficiency virus-like particles vaccine. *J Immunol* **173**: 1951–1958.
13. Modelska A, Dietzschold B, Sleysh N, *et al.* (1998) Immunization against rabies with plant-derived antigen. *Proc Natl Acad Sci USA* **95**: 2481–2485.
14. Meng XJ, Purcell RH, Halbur PG, *et al.* (1997) A novel virus in swine is closely related to the human hepatitis E virus. *Proc Natl Acad Sci USA* **94**: 9860–9865.
15. Nishizawa T, Takahashi M, Mizuo H, *et al.* (2003) Characterization of Japanese swine and human hepatitis E virus isolates of genotype IV with 99% identity over the entire genome. *J Gen Virol* **84**: 1245–1251.
16. Tam AW, Smith MM, Guerra ME, *et al.* (1991) Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. *Virology* **185**: 120–131.
17. Li TC, Takeda N, Miyamura T. (2001) Oral administration of hepatitis E virus-like particles induces a systemic and mucosal immune response in mice. *Vaccine* **19**: 3476–3484.
18. Li TC, Suzaki Y, Ami Y, *et al.* (2004) Protection of cynomolgus monkeys against HEV infection by oral administration of recombinant hepatitis E virus-like particles. *Vaccine* **22**: 370–377.

19. Ogra PL, Faden H, Welliver RC. (2001) Vaccination strategies for mucosal immune responses. *Clin Microbiol Rev* **14**: 430–435.
20. Takamura S, Niikura M, Li TC, *et al.* (2004) DNA vaccine-encapsulated virus-like particles derived from an orally transmissible virus stimulates mucosal and systemic immune responses by oral administration. *Gene Ther* **11**: 628–635.
21. Morton G, Rueda P, Sarraseca JC, *et al.* CD8alpha-CD11b$^+$ dendritic cells present exogenous virus-like particles to CD8$^+$ T cells and subsequently express CD8 alpha and CD205 molecules.
22. Rueda P, Morton G, Sarraseca JC, *et al.* (2004) Influence of flanking sequence on presentation efficiency of a CD8$^+$ cytotoxic T-cell epitope delivered by parvovirus-like particles. *J Gen Virol* **85**: 563–572.
23. Ulrich R, Nassal M, Meisel H, Kruger DH. (1998) Core particles of hepatitis B virus as carrier for foreign epitopes. *Adv Virus Res* **50**: 141–182.
24. McCluskie MJ, Chu Y, Xia JL, *et al.* (1998) Direct gene transfer to the respiratory tract of mice with pure plasmid and lipid-formulated DNA. *Antisense Nucleic Acid Drug Dev* **8**: 401–414.
25. Morrow CD, Novak MJ, Ansardi DC, *et al.* (1999) Recombinant viruses as vectors for mucosal immunity. In J.-P. Kraehenbuhl & M.R. Neutra (eds), *Current Topics in Microbiology and Immunology* 236, pp. 255–273. Springer-Verlag, Berlin.
26. Prasad BV, Hardy ME, Dokland T, *et al.* (1999) X-ray crystallographic structure of the Norwalk virus capsid. *Science* **286**: 287–290.
27. Nagaraj K, Babu BV. (1997) Field trials of oral cholera vaccine in Vietnam. *Lancet* **349**: 1253–1254.
28. Ball JM, Graham DY, Opekun AR, *et al.* (1999) Recombinant Norwalk virus-like particles given orally to volunteers: phase I study. *Gastroenterology* **117**: 40–48.
29. Boyaka PN, Marinaro M, Vancott JL, *et al.* (1999) Strategies for mucosal vaccine development. *Am J Trop Med Hyg* **60**: Suppl., 35–45.
30. Mestecky J, McGhee JR. (1987) Immunoglobulin A (IgA): molecular and cellular interactions involved in IgA biosynthesis and immune response. *Adv Immunol* **40**: 153–245.
31. Nahde T, Muller K, Fahr A, *et al.* (2001) Combined transductional and transcriptional targeting of melanoma cells by artificial virus-like particles. *J Gene Med* **3**: 353–361.

JOURNAL OF INTERFERON & CYTOKINE RESEARCH 27:32–37 (2007)

DOI: 10.1089/jir.2007.0099

Identification of a Novel CXCL1-Like Chemokine Gene in Macaques and Its Inactivation in Hominids

HISAYUKI NOMIYAMA,[1] KAORI OTSUKA-ONO,[1] RETSU MIURA,[1] NAOKI OSADA,[2] KEIJI TERAO,[3] OSAMU YOSHIE,[4] and JUN KUSUDA[2]

ABSTRACT

Chemokines are a rapidly evolving cytokine gene family. Because of various genome rearrangements after divergence of primates and rodents, humans and mice have different sets of chemokine genes, with humans having members outnumbering those of mice. Here, we report the occurrence of lineage-specific chemokine gene generation or inactivation events within primates. By using human chemokine sequences as queries, we isolated a novel cynomolgus macaque CXC chemokine cDNA. The encoded chemokine, termed CXCL1L (from CXCL1-like) showed the highest similarity to human CXCL1. A highly homologous gene was also found in the rhesus macaque genome. By comparing the genome organization of the major CXC chemokine clusters among the primates, we found that one copy of the duplicated CXCL1 genes turned into a pseudogene in the hominids, whereas the gene in macaques has been maintained as a functionally active CXCL1L. In addition, cynomolgus macaque was found to contain an additional CXC chemokine highly homologous to CXCL3, termed CXCL3L (from CXCL3-like). These results demonstrate the birth-and-death process of a new gene in association with gene duplication within the primates.

INTRODUCTION

CYNOMOLGUS MACAQUE (*Macaca fascicularis*) and rhesus macaque (*Macaca mulatta*) are closely related old world monkeys commonly used in experimental and toxicologic studies for drug and vaccine development.[1–4] Although both macaques are considered phylogenetically very close to humans, possible genetic differences between macaques and humans that may cause interspecies differences in drug responses and toxicity should be taken into account when the data obtained from macaques are extrapolated to humans. To unveil the genetic differences in primates and also to help identify genes in the human genome, expressed sequence tag (EST) and genome sequencing projects of these macaques are underway.

Chemokines are a large family of cytokines that regulate inflammation, leukocyte trafficking, and immune cell development.[5–7] There are at least 46 chemokine members in humans. Based on the arrangement of the conserved cysteine residues, chemokines are classified into four subfamilies. Two main subfamilies are CXC and CC chemokines, which have the first two conserved cysteines separated by one amino acid or juxtaposed, respectively. Chemokines can also be divided into two functional subgroups. Inflammatory chemokines attract mainly monocytes and neutrophils and mediate innate immunity, whereas homeostatic chemokines are constitutively expressed in organs, such as lymphoid tissues, and are involved in relocation of lymphocytes and dendritic cells (DCs).

The inflammatory CXC and CC chemokines are known to form a large gene cluster.[7] Human CXC and CC inflammatory chemokine gene clusters reside on chromosomes 4 and 17, respectively, and the respective gene clusters in mice are located on chromosomes 5 and 11. Comparison of these gene cluster organizations shows that the chemokine gene content in each cluster is greatly different between human and mouse due to lineage-specific gene duplication or deletion events or both after the divergence of primates and rodents.[8,9] In contrast, the

[1]Department of Molecular Enzymology, Kumamoto University Graduate School of Medical Sciences, Kumamoto 860-8556, Japan.
[2]Division of Biomedical Research Resources, National Institute of Biomedical Innovation, Ibaraki, Osaka 567-0085, Japan.
[3]Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Ibaraki 305-0843, Japan.
[4]Department of Microbiology, Kinki University School of Medicine, Osaka-Sayama, Osaka 589-8511, Japan.
Sequence data from this paper have been submitted to the GenBank/EBI/DDBJ databases with accession nos. AB262775 (CXCL1), AB262776 (CXCL2), AB262777 (CXCL3), AB262778 (CXCL1L), and AB262779 (CXCL3L).

emergence of noncluster chemokines, most of which are homeostatic chemokines, apparently predated the divergence of primates and rodents.[8,9]

To determine if gene rearrangements within the chemokine clusters occurred even after the diversification of humans and nonhuman primates, we searched the EST and genome databases of nonhuman primates for novel chemokines.

MATERIALS AND METHODS

cDNA cloning

Chemokine cDNAs were cloned by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNAs were prepared from various tissues of cynomolgus macaque and reverse transcribed. The cDNAs were synthesized using PrimeSTAR HS DNA polymerase (Takara Bio, Kyoto, Japan) and cloned with Mighty Cloning kit (Takara Bio). The primer sequences were designed based on the rhesus macaque genome sequences and were CXCL1, 5′-CTCCAGCTCCTCGCACAG and 5′-AGCCACCAATGAGCTTCTTC; CXCL1L, 5′-AGTTCCCCTGCTCCTCTCAC and 5′-GCCAGTATTTCTGACCAACG; CXCL2, 5′-CCGAAACGCCTGCTGAG and 5′-CTTCAGGAACAGCCACCAAT; CXCL3 and CXCL3L, 5′-TCCCATCCTGCTGAG and 5′-CCGCAGGAAGTGTCAATGT. The cDNAs used in the cDNA cloning as templates are those of liver (CXCL1 and CXCL2), spleen (CXCL1L), and stomach (CXCL3 and CXCL3L). The PCR conditions were 30 cycles at 98°C for 10 sec, 60°C for 5 sec, and 72°C for 1 min.

Tissue expression analysis of CXCL1 and CXCL1L

The prepared cDNAs were amplified with Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). The primer sequences were CXCL1L, 5′-AGGGAATTCACCCCAAGAAC and 5′-GCAAACTCACCTGTTCAGCA; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-GCCAAGGTCATCCATGACAACTTTGG and 5′-GCCTGCTTCACCACCTTCTTGATGTC. The primers used for CXCL1 were as described.

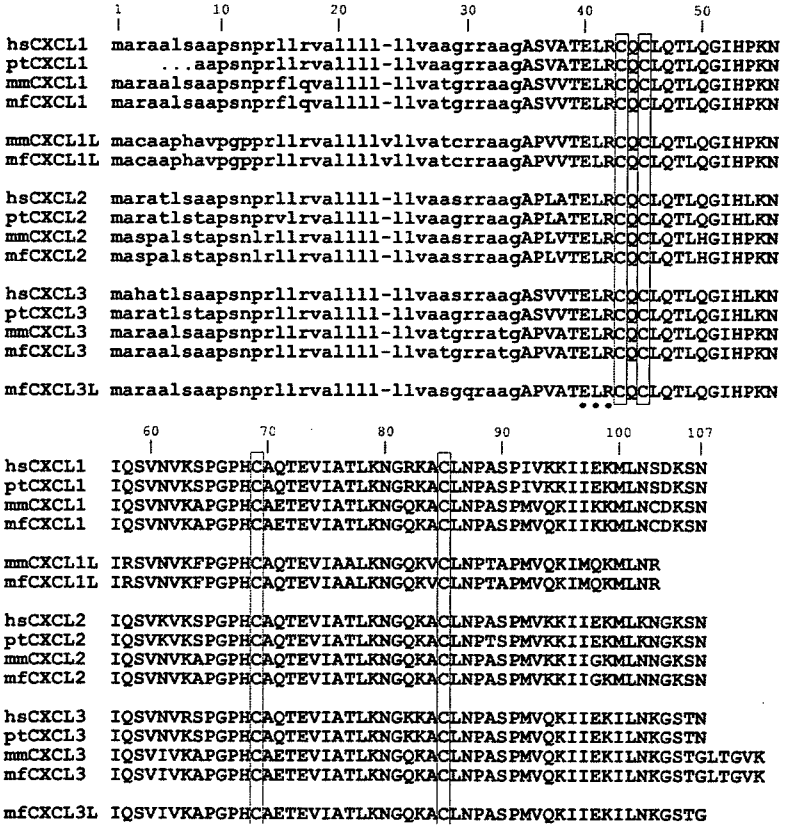


FIG. 1. Comparison of amino acid sequences of chemokines CXCL1, CXCL1L, CXCL2, CXCL3, and CXCL3L from human, chimpanzee, rhesus macaque, and cynomolgus macaque. Sequences other than those of cynomolgus macaque were taken from Ensembl (*www.ensembl.org/*) or UCSC Genome Browser (*genome.ucsc.edu/*). The amino-terminal sequence of the chimpanzee CXCL1 sequence is still unknown because of a gap in the genome sequence. Conserved four cysteine residues are boxed. ELR (Glu-Leu-Arg) motif is indicated by dots under the cynomolgus macaque CXCL3L sequence. Signal sequences are shown as lowercase letters. hs, human; pt, chimpanzee; mm, rhesus macaque; mf, cynomolgus macaque.

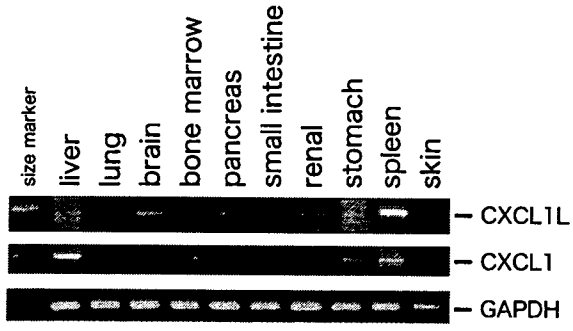


FIG. 2. RT-PCR analyses of CXCL1L and CXCL1 mRNAs in various cynomolgus macaque tissues. cDNAs were prepared from various tissues and amplified by PCR. GAPDH was used as an internal control.

The housekeeping gene GAPDH was used as an internal control. The PCR conditions were 35 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min. The product sizes were 170 bp (CXCL1L), 398 bp (CXCL1), and 313 bp (GAPDH).

Computational methods

Signal sequences were predicted by SignalP (*www.cbs.dtu.dk/services/SignalP/*). Dot-plot analysis was performed using PipMaker (*pipmaker.bx.psu.edu/pipmaker/*) with the all matches option. For phylogenetic analysis, the chemokine amino acid sequences were aligned with ClustalX (*bips.u-strasbg.fr/fr/Documentation/ClustalX/*). The Neighbor-Joining tree was constructed with PAUP* (*paup.csit.fsu.edu/*) using the protein-Poisson distances, and only >50% bootstrap values are shown at each node (1000 replications). CXCL1P (human and chimpanzee CXCL1 pseudogene, exons 1 plus 2 sequences) and CXCL7P1 (PPBPL1, human, chimpanzee and rhesus macaque CXCL7 pseudogene exon sequences) were also used in the tree construction. The codon frames were inferred from the CXCL1 and CXCL7 genes.

RESULTS AND DISCUSSION

When human chemokine sequences were used as queries, one novel chemokine sequence (clone QnpA-12174) was found in the cynomolgus macaque EST database (Japan National Institute of Biomedical Innovation JCRB Gene Bank, *genebank.nibio.go.jp/*). Because the cDNA was a chimeric and 5′-truncated clone, the rhesus macaque genome database[10] (UCSC Genome Browser Database, *genome.ucsc.edu/*) was searched with the sequence, and the corresponding gene was found to be located in the inflammatory CXC chemokine gene cluster on chromosome 5 (position 55,707,566–55,708,094, January 2006 assembly). Based on this gene sequence, a PCR primer

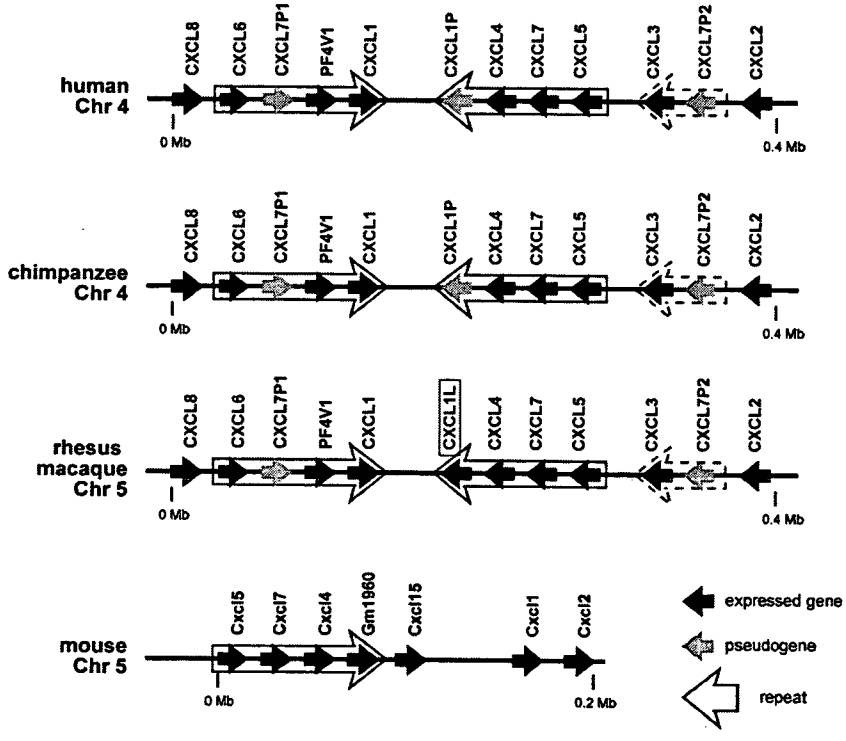


FIG. 3. Genome organization of CXC inflammatory chemokine gene clusters. Genome sequences taken from Ensembl or UCSC were analyzed and are shown schematically. Black and gray arrows denote functionally active gene and pseudogene, respectively, and arrowheads show the transcriptional orientation. Large arrows indicate duplicated regions.

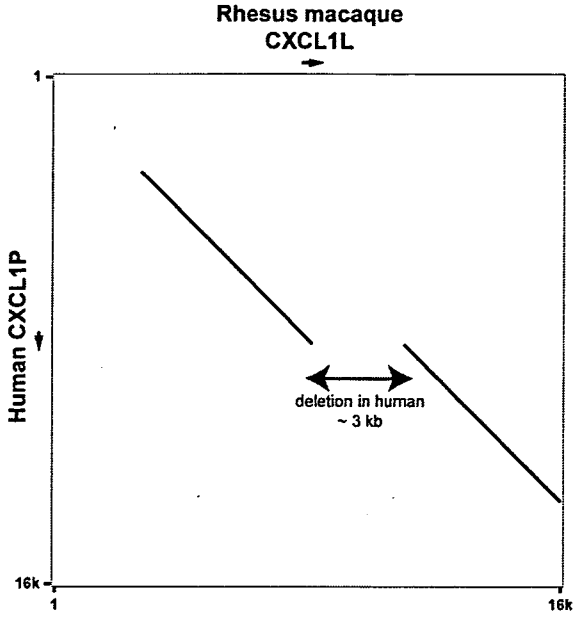


FIG. 4. Dot-plot analysis of the flanking sequences of the rhesus macaque CXCL1L gene and the human CXCL1 pseudogene (CXCL1P). The arrow with two heads shows a deletion of about 3 kb in length seen only in the human genome. The sizes of the genome sequences used were 16 kb for both species.

pair surrounding the coding sequence was prepared. A cDNA clone was isolated by PCR from a cDNA library prepared from cynomolgus macaque spleen. The cDNA encoded a polypeptide of 104 amino acid residues. As the encoded chemokine was highly similar to human CXCL1, CXCL2, and CXCL3, we also isolated cynomolgus macaque CXCL1, CXCL2, and CXCL3 cDNA clones from stomach or liver cDNA libraries for comparison (Fig. 1). The novel chemokine showed 80%, 76%, and 79% similarity to cynomolgus macaque CXCL1, CXCL2, and CXCL3, respectively, and, therefore, was called CXCL1L, from CXCL1-like. The corresponding rhesus macaque CXCL1L was identical to the cynomolgus macaque CXCL1L at the amino acid level but had one synonymous base substitution in the coding region. Interestingly, no orthologous gene for CXCL1L was found in other species, including hominids.

We then examined the expression of CXCL1L in various cynomolgus macaque tissues by semiquantitative RT-PCR. We also examined the expression of CXCL1 for comparison. CXCL1L mRNA was found to be expressed at high levels in spleen and to a lesser extent in brain, bone marrow, and pancreas (Fig. 2). In contrast, CXCL1 mRNA was expressed abundantly in liver, and the expression level in spleen was less than that in liver. Human CXCL1, CXCL2, and CXCL3 genes are likely to be expressed at an extremely low level in spleen according to the expression profiles (*www.ncbi.nlm.nih.gov/UniGene/*), and cynomolgus macaque CXCL1L and CXCL1 may have a unique function in the lymphoid organs.

When we prepared the cynomolgus macaque CXCL3 cDNA from stomach, a cDNA clone closely related to but slightly different from CXCL3 was also isolated. The cDNA encoded a chemokine of 107 amino acid residues that we named CXCL3L (from CXCL3-like) (Fig. 1). CXCL3L was just 5 amino acid residues shorter than CXCL3. Furthermore, although CXCL3L contained 13 base substitutions in the coding region, CXCL3L and CXCL3 differed by only 3 amino acid residues in the signal sequence. No counterpart of the CXCL3L gene was found in rhesus macaque or other primate species.

To obtain clues about the generation mechanism of the CXCL1L gene in macaques, we compared the maps of the inflammatory CXC chemokine gene clusters of human, chimpanzee, rhesus macaque, and mouse obtained from the websites (Ensembl and UCSC) (Fig. 3). The genome sequence of cynomolgus macaque is not available at present. In the primates, a large genome segment including four CXC chemokine genes was duplicated, forming an inverted repeat. Comparison of the maps shows that rhesus macaque CXCL1L gene locus corresponds to those of the CXCL1 pseudogene (CXCL1P) in the human and chimpanzee genomes. The pseudogene in both species contains exons 1 and 2 and the intron between them but lacks the downstream sequence.[11] To examine the genome organization at the nucleotide level, the sequences containing the rhesus macaque CXCL1L and human CXCL1P genes were compared by dot-plot analysis (Fig. 4). Figure 4 clearly shows that a deletion of approximately 3 kb in size is located 3′ downstream of the human CXCL1P gene. This result suggests that the human CXCL1P and rhesus macaque CXCL1L genes arose from a common ancestor gene and that a deletion event in the human genome made the gene inactive after the divergence of hominids and macaques.

Next, a phylogenetic tree was constructed to see the evolutionary relationship of the inflammatory CXC chemokines (Fig. 5). Protein coding sequences were used for the analysis, and the peptide sequences deduced from the human and chimpanzee CXCL1P sequences (exons 1 plus 2) were also included in the analysis for comparison. The tree shows that the macaque CXCL1L and human CXCL1P are indeed closely related, forming a unique branch, whereas the primate CXCL1, CXCL2, and CXCL3 form another relatively independent branch, suggesting the CXCL1L diverged from the common ancestor before CXCL1, CXCL2, and CXCL3 were generated.

Duplication of genes is recognized as the driving force of evolution, and multigene families evolve by a birth-and-death process of duplicated genes.[12] In the CXC chemokine gene cluster, CXCL1 and duplicated CXCL1L in the inverted repeat are both intact in macaques, whereas the latter gene became a pseudogene in hominids. Likewise, the CXCL7 gene in one copy of the repeat turned into a pseudogene CXCL7P1 in the primates (Fig. 3). In addition, another gene duplication appears to have occurred in cynomolgus macaque that generated the CXCL3L gene only in cynomolgus macaque. Given that inactivation of duplicated genes (CXCL1P and CXCL7P1) and emergence of a new gene (CXCL3L) are identified in different primate lineages, the birth-and-death process is still ongoing in the chemokine gene cluster of primates.

As the database search has not identified novel chemokine receptor genes in the macaque genome to date and macaque CXCL1L shows a high sequence similarity to human CXCL1, CXCL2, and CXCL3, the chemokine probably binds the chemokine receptor CXCR2 and has chemotactic activity for neutrophils. CXCL1L indeed contains the Glu-Leu-Arg (ELR)

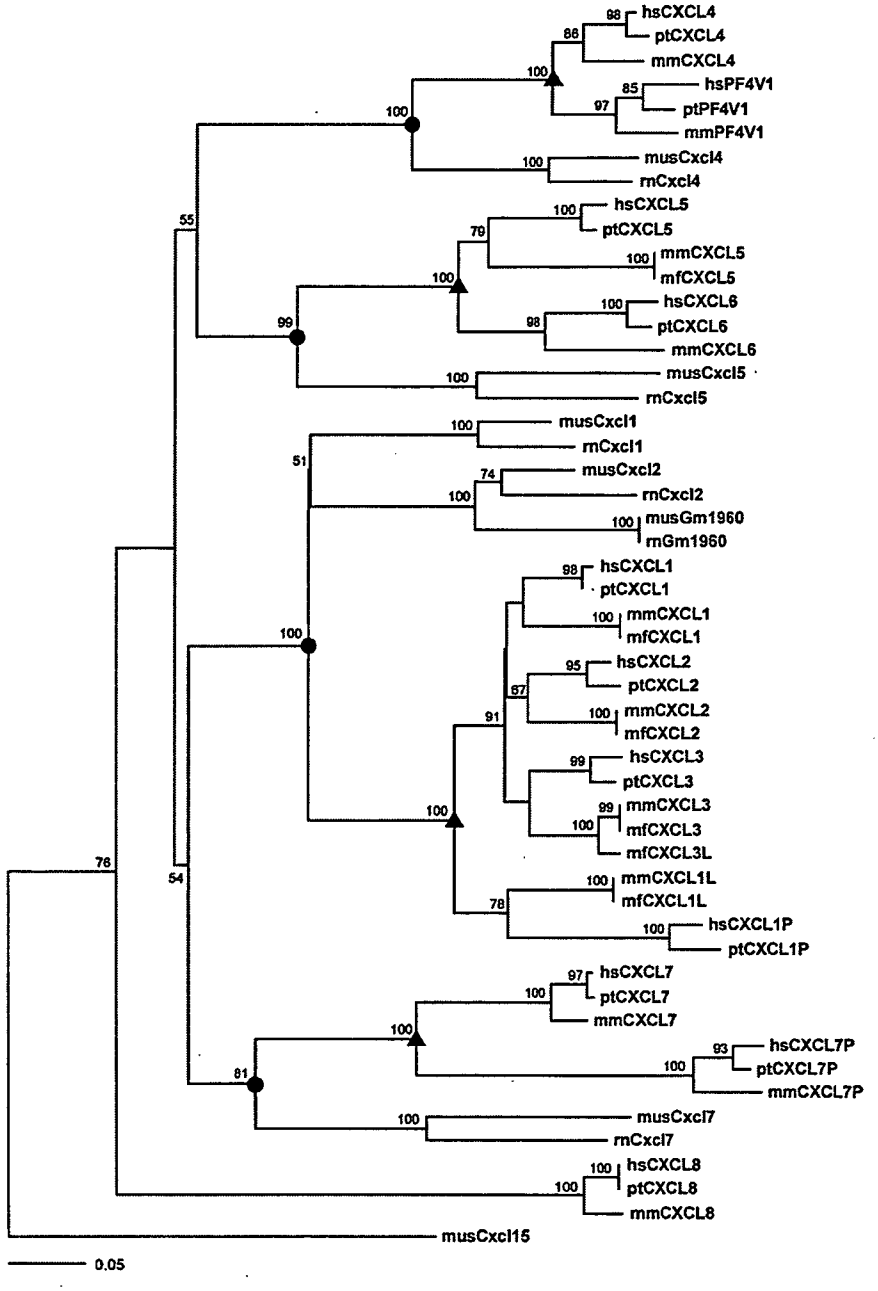


FIG. 5. Phylogenetic tree of CXC inflammatory chemokines. Circles at the nodes indicate divergence points between primates and rodents, and triangles show the points where segmental duplications have occurred in the primate lineage. Chemokine sequences were taken from Ensembl, UCSC, or Cytokine Family Database (*cytokine.medic.kumamoto-u.ac.jp/*). Mouse Cxcl15 was used as an outgroup. hs, human; pt, chimpanzee; mm, rhesus macaque; mf, cynomolgus macaque; mus, mouse; rn, rat.

motif preceding the first conserved cysteine residue that is essential for receptor binding, neutrophil activation, and angiogenic activity.[13,14] However, because gene duplication also allows one copy of the duplicated genes to acquire a new function,[12] we cannot exclude the possibility that CXCL1L might bind other receptors. Expression of CXCL1L at high levels in macaque spleen also suggests a new function in this lymphoid organ (Fig. 2). Recently, some chemokines were shown to be ligands for both chemokine receptors and those not categorized as chemokine receptors.[15,16] Thus, it will be interesting to see if CXCL1L has receptors and functions differently from CXCL1, CXCL2, and CXCL3.

ACKNOWLEDGMENTS

This work is supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and by MEXT. HAITEKU (2002–2006).

REFERENCES

1. Jacqz E, Billante C, Moysan F, Mathieu H. The non-human primate: a possible model for human genetically determined polymorphisms in oxidative drug metabolism. *Mol. Pharmacol.* 1988;34:215–217.
2. Thorgeirsson UP, Dalgard DW, Reeves J, Adamson RH. Tumor incidence in a chemical carcinogenesis study of nonhuman primates. *Regul. Toxicol. Pharmacol.* 1994;19:130–151.
3. Daddario-DiCaprio KM, Geisbert TW, Stroher U, Geisbert JB, Grolla A, Fritz EA, Fernando L, Kagan E, Jahrling PB, Hensley LE, Jones SM, Feldmann H. Post-exposure protection against Marburg haemorrhagic fever with recombinant vesicular stomatitis virus vectors in non-human primates: an efficacy assessment. *Lancet* 2006;367:1399–1404.
4. Misumi S, Nakayama D, Kusaba M, Iiboshi T, Mukai R, Tachibana K, Nakasone T, Umeda M, Shibata H, Endo M, Takamune N, Shoji S. Effects of immunization with CCR5-based cycloimmunogen on simian/HIVSF162P3 challenge. *J. Immunol.* 2006;176:463–471.
5. Moser B, Loetscher P. Lymphocyte traffic control by chemokines. *Nat. Immunol.* 2001;2:123–128.
6. Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. *Immunity* 2000;12:121–127.
7. Yoshie O, Imai T, Nomiyama H. Chemokines in immunity. *Adv. Immunol.* 2001;78:57–110.
8. Nomiyama H, Mera A, Ohneda O, Miura R, Suda T, Yoshie O. Organization of the chemokine genes in the human and mouse major clusters of CC and CXC chemokines: diversification between the two species. *Genes Immun.* 2001;2:110–113.
9. Nomiyama H, Egami K, Tanase S, Miura R, Hirakawa H, Kuhara S, Ogasawara J, Morishita S, Yoshie O, Kusuda J, Hashimoto K. Comparative DNA sequence analysis of mouse and human CC chemokine gene clusters. *J. Interferon Cytokine Res.* 2003;23:37–45.
10. Karolchik D, Baertsch R, Diekhans M, Furey TS, Hinrichs A, Lu YT, Roskin KM, Schwartz M, Sugnet CW, Thomas DJ, Weber RJ, Haussler D, Kent WJ. The UCSC Genome Browser Database. *Nucleic Acids Res.* 2003;31:51–54.
11. Shattuck-Brandt RL, Wood LD, Richmond A. Identification and characterization of an MGSA/GRO pseudogene. *DNA Seq.* 1997;7:379–386.
12. Nei M, Rooney AP. Concerted and birth-and-death evolution of multigene families. *Annu. Rev. Genet.* 2005;39:121–152.
13. Clark-Lewis I, Dewald B, Geiser T, Moser B, Baggiolini M. Platelet factor 4 binds to interleukin 8 receptors and activates neutrophils when its N terminus is modified with Glu-Leu-Arg. *Proc. Natl. Acad. Sci. USA* 1993;90:3574–3577.
14. Strieter RM, Burdick MD, Gomperts BN, Belperio JA, Keane MP. CXC chemokines in angiogenesis. *Cytokine Growth Factor Rev.* 2005;16:593–609.
15. Elagoz A, Henderson D, Babu PS, Salter S, Grahames C, Bowers L, Roy MO, Laplante P, Grazzini E, Ahmad S, Lembo PM. A truncated form of CKbeta8-1 is a potent agonist for human formyl peptide-receptor-like 1 receptor. *Br. J. Pharmacol.* 2004;141:37–46.
16. Nakayama T, Kato Y, Hieshima K, Nagakubo D, Kunori Y, Fujisawa T, Yoshie O. Liver-expressed chemokine/CC chemokine ligand 16 attracts eosinophils by interacting with histamine H4 receptor. *J. Immunol.* 2004;173:2078–2083.

Address reprint requests or correspondence to:
Dr. Hisayuki Nomiyama
Department of Molecular Enzymology
Kumamoto University Graduate School of Medical Sciences
Honjo 1-1-1
Kumamoto 860-8556
Japan

Tel: +81-96-373-5065
Fax: +81-96-373-5066
E-mail: nomiyama@gpo.kumamoto-u.ac.jp

Received 15 June 2006/Accepted 13 July 2006

Primates (2007) 48:140–146
DOI 10.1007/s10329-006-0008-z

SHORT COMMUNICATION

Development of a microsatellite marker set applicable to genome-wide screening of cynomolgus monkeys (*Macaca fascicularis*)

Toshihiko Kikuchi · Masayuki Hara · Keiji Terao

Received: 25 August 2005 / Accepted: 30 May 2006 / Published online: 22 November 2006

Abstract To develop a microsatellite marker set applicable to genome-wide screening of cynomolgus monkeys (*Macaca fascicularis*), 148 microsatellite markers were selected from the human genome database. The polymorphisms and inheritance of PCR products were determined by screening twenty unrelated monkeys and by analysis of three families, respectively. As a result, 106 primers (72%) gave PCR products of the size expected for humans and rhesus monkeys. Among these products, polymorphism and single-gene inheritance in cynomolgus monkeys was observed for 66 markers (62%). The average number of alleles at the 66 polymorphic loci was 5.86 (range 2–10), and average heterozygosity was 0.63 (range 0.10–0.88). This is the first report of microsatellite markers for cynomolgus monkeys. Chromosomal mapping of these markers is now in progress.

Keywords Microsatellite · Cynomolgus monkeys · Genome-wide screening

Introduction

Sets of microsatellite markers are available for genome-wide screening or kin selection for some non-human primate species (Morin et al. 1994a, b; Rogers et al. 2000; Hadfield et al. 2001; Rogers et al. 2006). Because many microsatellite markers on the human genome have already been identified, use of human microsatellite marker information is an efficient means of developing marker sets for other primate species (Domingo et al. 1997; Lukas et al. 2004). A first-generation genetic linkage map for the baboon has been prepared by using microsatellite marker information from the human genome (Rogers et al. 2000) and 280 microsatellite markers have been applied to linkage studies of osteoporosis in baboons (Havill et al. 2005). Two-hundred and forty-one polymorphic microsatellite markers have been identified in rhesus monkeys (Rogers et al. 2006). In contrast, few microsatellite markers have been reported for cynomolgus monkeys (*Macaca fascicularis*), even though cynomolgus monkeys have been widely used as a surrogate model in biomedical research for several familial diseases, for example endometriosis (Ami et al. 1993) and early onset macular degeneration (Suzuki et al. 2003). A microsatellite marker set for cynomolgus monkeys would assist colony management, conservation work, and paternity testing. We have therefore adopted a similar approach, using microsatellite marker sequence information available from the human genome database and from the rhesus monkey, to develop a set of polymorphic microsatellite markers for cynomolgus monkeys.

Materials and methods

Blood samples were obtained from 40 (19 male, 21 female) cynomolgus monkeys (*M. fascicularis*), from 5 to 24 years old, bred and reared at the Tsukuba Primate Research Center (TPRC), National Institute of Biomedical Innovation. They included 20 unrelated

T. Kikuchi · M. Hara · K. Terao (✉)
Tsukuba Primate Research Center,
National Institute of Biomedical Innovation,
1-1 Hachimandai, Tskukuba, Ibaraki 305-0843, Japan
e-mail: terao@nibio.go.jp

Table 1 Characteristics of 148 microsatellite markers in cynomolgus monkeys

Locus	Chr.	Origin	Accession number	Amplification	Polymorphism	Number of alleles	Heterozygosity	Size (bp)
D1S206	1	Hadfield et al.	Z16595	Y	P	4	0.61	222–230
D1S207	1	Hadfield et al.	Z16601	Y	P	7	0.82	126–158
D1S213	1	Hadfield et al.	Z16668	Y	P	9	0.88	99–121
D1S238	1	Hadfield et al.	Z16920	N				
D1S255	1	Hadfield et al.	Z17172	Y	P	5	0.75	83–99
D1S2797	1	Hadfield et al.	Z53878	Y	P	4	0.61	156–162
D1S2800	1	Hadfield et al.	Z53893	N				
D1S2868	1	Hadfield et al.	Z51655	Y	P	8	0.79	119–145
D1S468	1	Hadfield et al.	Z23994	Y	P	3	0.30	220–226
GCT10B12	1	CHLC	G09705	Y	P	3	0.22	146–152
D2S1397	2	CHLC	G08197	Y	M			226
D2S2333	2	Hadfield et al.	Z54050	N				
D2S2368	2	Hadfield et al.	Z51756	N				
D2S364	2	Hadfield et al.	Z24235	Y	P	7	0.80	216–248
D2S391	2	Hadfield et al.	Z24452	Y	P	5	0.74	145–163
D3S1279	3	Hadfield et al.	Z16741	Y	P	7	0.84	261–273
D3S1285	3	Hadfield et al.	Z16827	N				
D3S1292	3	Hadfield et al.	Z16871	Y	P	2	0.29	138–140
D3S1569	3	Hadfield et al.	Z23768	Y	P	6	0.77	112–138
D3S1614	3	Hadfield et al.	Z24572	Y	P	9	0.86	129–149
D3S1768	3	Andrade et al.	G08287	Y	P	8	0.73	194–226
D3S2400	3	CHLC	G08293	N				
D3S2401	3	CHLC	G08295	Y	P	4	0.60	204–232
D3S3810	3	CHLC	G10022	Y	M			156
GCT8C05	3	CHLC	G09574	Y	M			236
D4S1535	4	Hadfield et al.	Z23424	N				
D4S1575	4	Hadfield et al.	Z23905	Y	M			221
D4S1592	4	Hadfield et al.	Z24118	Y	M			172
D4S1626	4	Smith et al.	G08368	N				
D4S2374	4	Smith et al.	G08361	N				
D4S243	4	Smith et al.	M87736	Y	M			175
D4S2430	4	CHLC	G08403	Y	P	2	0.14	164–172
D4S2964	4	Hadfield et al.	Z53407	N				
D4S3090	4	CHLC	G09999	Y	P	3	0.32	106–112
D4S403	4	Hadfield et al.	Z16702	Y	P	3	0.27	183–193

Table 1 continued

Locus	Chr.	Origin	Accession number	Amplification	Polymorphism	Number of alleles	Heterozygosity	Size (bp)
D4S405	4	Hadfield et al.	Z16717	Y	M			325
D4S413	4	Hadfield et al.	Z16837	Y	M			195
D4S415	4	Hadfield et al.	Z16841	Y	P	5	0.31	311–315
D4S424	4	Hadfield et al.	Z17023	Y	P	5	0.62	202–218
D5S2313	5	CHLC	G09714	Y	M			126
D5S419	5	Hadfield et al.	Z16943	N				
D5S424	5	Hadfield et al.	Z16982	N				
C1_2_A	6	Foissac et al.	–	N				
C2_4_4	6	Foissac et al.	–	Y	P	9	0.82	206–224
C3_2_10	6	Foissac et al.	–	N				
C5_2_7	6	Foissac et al.	–	Y	M			313
D6S1014	6	CHLC	G08571	N				
D6S1015	6	CHLC	G08572	N				
D6S105	6	Martin et al.	X59425	Y	M			140
D6S1058	6	CHLC	G09538	Y	M			175
D6S1542	6	Foissac et al.	Z52075	Y	M			110
D6S1560	6	Foissac et al.	Z52491	N				
D6S1576	6	Foissac et al.	–	N				
D6S1610	6	Hadfield et al.	Z53131	Y	M			125
D6S1691	6	Andrade et al.	Z51673	Y	P	7	0.83	187–215
D6S1701	6	Foissac et al.	–	Y	M			162
D6S264	6	Hadfield et al.	Z16538	N				
D6S265	6	Martin et al.	–	Y	M			137
D6S273	6	Martin et al.	Z16657	N				
D6S2741	6	Martin et al.	–	Y	P	8	0.81	258–278
D6S276	6	Andrade et al.	–	Y	P	10	0.81	123–147
D6S2876	6	Martin et al.	–	Y	P	7	0.82	184–212
D6S2883	6	Martin et al.	–	Y	P	9	0.83	259–283
D6S289	6	Hadfield et al.	Z16884	Y	P	6	0.79	211–233
D6S291	6	Foissac et al.	Z16904	Y	P	4	0.27	168–174
D6S306	6	Foissac et al.	Z17120	Y	M			232
D6S388	6	Foissac et al.	L16310	Y	M			60
D6S434	6	Hadfield et al.	Z23378	Y	P	5	0.62	184–204
D6S439	6	Foissac et al.	Z23903	Y	M			264
DQCAR	6	Martin et al.	–	Y	M			180
DQCARII	6	Martin et al.	–	Y	M			176
G51152	6	Martin et al.	–	Y	M			155
HL/AF	6	Smith et al.	–	N				
MIB	6	Martin et al.	–	Y	M			321
MICA	6	Martin et al.	–	Y	P	4	0.62	196–204
MOGCA	6	Martin et al.	–	Y	P	5	0.65	117–137
Na/HLA F2	6	Smith et al.	–	N				
D7S1789	7	Smith et al.	G08582	Y	M			135
D7S1826	7	Smith et al.	G09628	Y	P	9	0.78	116–154
D7S1830	7	Smith et al.	G20169	N				
D7S507	7	Hadfield et al.	Z16885	Y	M			125

Table 1 continued

Locus	Chr.	Origin	Accession number	Amplification	Polymorphism	Number of alleles	Heterozygosity	Size (bp)
D7S640	7	Hadfield et al.	Z23671	Y	P	3	0.10	104–126
D7S794	7	Andrade et al.	G08607	N				
D8S1100	8	Smith et al.	G08663	Y	M			190
D8S1106	8	Smith et al.	G09378	Y	P	10	0.87	152–188
D8S1119	8	Smith et al.	G08654	Y	M			152
D8S1476	8	CHLC	G08719	N				
D8S272	8	Hadfield et al.	Z17250	Y	P	10	0.89	218–240
D8S505	8	Hadfield et al.	Z23478	Y	P	5	0.72	151–161
D9S1918	9	CHLC	G10043	N				
D9S290	9	Hadfield et al.	Z24641	Y	P	6	0.56	152
D10S1412	10	Andrade et al.	G08780	Y	P	5	0.60	155–170
D10S1432	10	Smith et al.	G08816	Y	P	7	0.76	140–176
D10S1652	10	Hadfield et al.	Z52339	Y	P	6	0.78	158–178
D10S1686	10	Hadfield et al.	Z51102	N				
D10S192	10	Hadfield et al.	Z16555	Y	P	7	0.81	179–211
D10S587	10	Hadfield et al.	Z24180	Y	M			295
D10S611	10	Smith et al.	G08794	Y	P	6	0.74	164–202
D11S1366	11	Smith et al.	G08865	Y	M			242
D11S1902	11	Smith et al.	L30089	Y	M			170
D11S1975	11	Smith et al.	G08849	Y	M			240
D11S2002	11	Smith et al.	G09598	Y	P	6	0.82	261–279
D11S902	11	Hadfield et al.	Z16521	Y	P	4	0.14	121–129
D11S908	11	Hadfield et al.	Z16617	N				
D11S925	11	Andrade et al.	Z17002	Y	P	7	0.81	225–239
D11S968	11	Hadfield et al.	Z17248	Y	P	6	0.56	138–148
D11S987	11	Hadfield et al.	Z21492	N				
D12S107	12	CHLC	–	N				
D12S1617	12	Hadfield et al.	Z52584	Y	P	7	0.87	250–262
D12S336	12	Hadfield et al.	Z23945	N				
D12S345	12	Hadfield et al.	Z24176	Y	M			290
D12S364	12	Hadfield et al.	Z24574	Y	P	7	0.84	140–162
D12S79	12	Hadfield et al.	Z16516	Y	M			150
D12S85	12	Hadfield et al.	Z16624	Y	P	4	0.32	104–120
D12S86	12	Hadfield et al.	Z16630	N				
GCT10G11	12	CHLC	G16002	Y	M			150
D13S317	13	Smith et al.	G09017	N				
D13S765	13	Andrade et al.	G09003	Y	P	7	0.77	180–214

Table 1 continued

Locus	Chr.	Origin	Accession number	Amplification	Polymorphism	Number of alleles	Heterozygosity	Size (bp)
D13S894	13	Smith et al.	G09022	Y	P	4	0.73	232–244
D15S1007	15	Hadfield et al.	Z53384	N				
D15S1012	15	Hadfield et al.	Z53420	Y	M			225
D15S1082	15	CHLC	G09930	Y	M			150
D15S644	15	Smith et al.	G07916	Y	P	9	0.85	218–244
GCT1C8	15	CHLC	G12438	Y	M			166
D16S403	16	Andrade et al.	Z16477	Y	P	5	0.79	160–174
D16S404	16	Hadfield et al.	Z16486	Y	P	5	0.27	190–200
D16S415	16	Hadfield et al.	Z16928	Y	P	9	0.71	192–230
D16S515	16	Hadfield et al.	Z24558	Y	P	5	0.72	238–260
D17S1290	17	Smith et al.	G07956	Y	P	5	0.64	216–244
D17S1852	17	Hadfield et al.	Z53709	Y	P	3	0.29	304–324
D17S804	17	Andrade et al.	Z17033	Y	P	5	0.65	160–176
D17S831	17	Hadfield et al.	Z50910	Y	P	9	0.84	187–207
D17S921	17	Hadfield et al.	Z23462	Y	P	4	0.34	167–181
D17S938	17	Hadfield et al.	Z23864	N				
D18S1102	18	Hadfield et al.	Z52586	N				
D18S536	18	Smith et al.	G08011	Y	P	7	0.72	146–180
D18S537	18	Smith et al.	G07990	N				
D18S72	18	Andrade et al.	Z17153	Y	P	4	0.29	216–222
D18S851	18	Smith et al.	G08002	Y	M			253
D18S861	18	Smith et al.	G07976	Y	M			160
D18S880	18	CHLC	G09488	Y	M			170
D19S210	19	Hadfield et al.	Z16612	N				
D19S571	19	Hadfield et al.	Z51499	N				
D20S117	20	Hadfield et al.	Z17123	N				
D20S608	20	CHLC	G09708	N				
D21S1256	21	Hadfield et al.	Z24038	N				
D22S274	22	Hadfield et al.	Z16730	Y	P	4	0.19	178–186
D22s280	22	Hadfield et al.	Z17028	Y	P	4	0.72	203–209

Polymorphism was determined with 20 unrelated monkeys

Chr. chromosome, Y amplification, N no amplification, P polymorphic, M monomorphic

individuals (9 male, 11 female) and ten males and ten females who were members of three families consisting of seven combinations of related parents and offspring.

Genomic DNA was extracted from 5 to 10 mL heparinized peripheral blood by use of the Wizard Genomic DNA purification kit (Promega, California, USA). One-hundred and forty-eight microsatellite markers were selected for this study, including 104 markers reported in rhesus monkeys (Smith et al. 2000; Hadfield et al. 2001; Andrade et al. 2004), 20 markers from the database of the CHLC (Cooperative Human Linkage Center: http://www.gai.nci.nih.gov/CHLC/),