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Received July 16, 2001
Accepted September 5, 2001

Corresponding Editor: James L. Hamrick

A Radiation Hybrid Mapping Panel for the Rhesus Macaque

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The genomes of nonhuman primates have recently become highly visible candidates for full genome analysis, as they provide powerful models of human disease and a better understanding of the evolution of the human genome. We describe the creation of a 5000 rad radiation hybrid (RH) mapping panel for the rhesus macaque. Duplicate genotypes of 84 microsatellite and coding gene sequence tagged sites from six macaque chromosomes produced an estimated whole genome retention frequency of 0.33. To test the mapping ability of the panel, we constructed RH maps for macaque chromosomes 7 and 9 and compared them to orthologous locus orders in existing human and baboon maps derived from different methodologies. Concordant marker order between all three species maps suggests that the current panel represents a powerful mapping resource for generating high-density comparative maps of the rhesus macaque and other species genomes.

Radiation hybrid (RH) mapping is becoming a powerful tool for whole genome characterization in model organisms. RH maps provide a useful resource for high-resolution comparative mapping, comparative candidate positional cloning, and ultimately a guide to whole genome sequence assembly (Band et al. 2000; Kwitek et al. 2001; Murphy et al. 2000; O'Brien et al. 2001; van de Sluis et al. 2000). There is increasing interest in charting the genomes of nonhuman primates for both their close evolutionary kinship with mankind and the potential that nonhuman primates, especially the rhesus macaque, possess for effectively and accurately modeling human biological phenomena (Dawes 2001; McConkey and Varki 2000; VandeBerg et al. 2000). Here we describe the construction and initial characterization of a rhesus macaque RH panel as a resource for comparative evolutionary inference with other genomes and effective utilization of this species as an animal model. We demonstrate its potential resolving power by producing comparative chromosome maps for two macaque chromosomes and comparison of these to human and baboon maps of homologous chromosomes. These comparative mapping data are useful for elucidating the evolutionary history of human chromosome 14 and 15.

Materials and Methods

The 5000 rad RH panel was constructed by fusing approximately 2×10^7 irradiated cells from a male rhesus macaque fibroblast donor cell line with an equivalent number of cells from the A23 thymidine kinase (TK)-deficient hamster cell line. The origin of the donor fibroblasts was a male rhesus macaque (Mm123–87) housed at the New England Regional Primate Center. The radiation dosage was 5000 rads, similar to what has been used for other domestic species (Band et al. 2000; Murphy et al. 2000). Fusions were plated onto alpha-MEM+20% fetal bovine serum, penicillin, streptomycin, oubain, and HAT supplement, and grown at 37°C. Appropriate controls for the TK selection and irradiation procedures showed no growth 10 days after fusion. Seven to 14 days after fusion, colonies were isolated and grown separately in the wells of 24-well microtiter plates. Ninety-three clones were selected and expanded based on retention of macaque-specific DNA, determined using previously optimized markers (Rogers et al. 2000) and an Inter-Alu PCR assay. DNA harvests yielded an average of 1.9 mg of DNA per hybrid.

Genotyping was performed with 25 ng of hybrid DNA from the 93 cell lines as well as 3 control samples (macaque genomic DNA, hamster [A23] genomic DNA, and a water blank) in a 10 μl reaction volume using Perkin-Elmer dual 384-well 9700 thermal cyclers under the following conditions: 10 min denaturation at 95°C, fol-

lowed by 35 cycles of 15 s at 95°C, 15 s at 50–60°C, and 30–45 s at 72°C, with a final 5-min extension at 72°C. PCR mixtures contained polymerase chain reaction (PCR) buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), $MgCl_2$ (1.5–3.0 mM), dNTPs (200 μM each), 40 nmol forward and reverse primers, and 0.5 units TaqGold DNA polymerase (ABI). Amplification products were resolved on 2% agarose gels, stained with Vista green, digitally recorded, and manually scored. RH genotypes were scored as either positive, negative, or ambiguous (clones which were discordant between duplicate typings). RH maps were constructed using RH2PT and RHMAXLIK, both from RHMAP version 3.0 (Boehnke et al. 1996).

Results and Discussion

PCR-based duplicate genotyping of 84 loci distributed on 6 different macaque chromosomes (Table 1) revealed an average genome-wide retention frequency of 0.33 (range 0.15–0.62), suggesting the panel retains sufficient donor genome to construct moderate-resolution maps with full genome coverage. We noticed an increased retention frequency of markers adjacent to centromeres, as has been observed in RH panels from other species (e.g., Murphy et al. 2000; Stewart et al. 1997). Type I coding markers homologous to human chromosome 14 and 15 loci were initially amplified using touchdown PCR with conserved mammalian primers (Table 2) designed from multiple mammalian sequence alignments. PCR products were end sequenced for verification by BLAST search, from which macaque-specific primers were designed (GenBank accession nos. AF455790–AF455802). Human microsatellite loci, previously optimized for linkage mapping in the baboon genome (Rogers et al. 2000), were optimized for use in the RH panel using a touchdown PCR strategy. All markers were typed on the panel in duplicate, allowing us to assay two loci per 384-well plate.

Table 1. Marker retention frequencies (RFs) for 84 loci on six macaque chromosomes

Chromosome	No. of loci	Average RF	RF range
Mma1	22	0.27	0.15–0.62
Mma3	12	0.29	0.17–0.47
Mma4	6	0.31	0.21–0.44
Mma7	25	0.40	0.32–0.52
Mma9	12	0.33	0.17–0.52
Mma15	7	0.29	0.22–0.40
Total	84	0.33	0.15–0.62

Table 2. Conserved mammalian primer pairs for generating coding STSs

Locus	Human chromosome	Forward conserved primer	Reverse conserved primer
APEX	14	CTCTTCAAAGATATCATCAACATG	CACAGTGCTAGGTATAGGGT
CMA1	14	GACACATGGCAGAAGCTTGA	GATTTTGTCTTCCTGGGATTG
HNF3A	14	CAGGCTTGTGGCAATACTCTT	TTTAAAGTCCTTAACTGCAAATGA
PYGL	14	CTCTTCAAAGATATCATCAACATG	TGACATAGGCTTCGTAGTCTGC
ESR2	14	TTCTATAGTCCTGCTGTGATGA	CCAAGGACTCTTTTGAGGTTC
PGF	14	TGCTGGTACCTGCCCTCTAT	AGAGAATCTGGCTTGGCAGT
TSHR	14	GAATCCTTGAGTCTTTGATGTGTA	GGACTGTGATGTAGATCTTCAC
CKB	14	GCTCATTGACGACCACTTCC	AGGTGAGGGTTCCACATGAA
TJP1	15	AACTTTGACCTCTGCAGCAA	AACAGAAATCGTGCTGATGTG
THBS1	15	ACCTCATTTGTTGTGTGACTG	CAATTCTTCAATTCAGTGTGCAA
TCF12	15	TGGAATACAAGAGCAATGGTCA	GCTACAGTGAAAAATAAGCCAAA
FAH	15	TGGTCCTTATGAACGACTGG	GAGCATCCATGGGCACCAC
IGF1R	15	ACATCCGCAACGACTATCAG	CCAGCAGCGGTAGTTGTACTC

To test the mapping accuracy of the RH panel we analyzed RH scores from two chromosomes with a sufficient coverage of markers, Mma7 and Mma9. Two-point linkage analyses using RH2PT identified a single linkage group with an LOD of 8.0 for Mma7 and a single linkage group for Mma9 with an LOD of 6.0. The maximum likelihood order (assuming both equal retention and centromeric models) for the Mma7 loci was identical to the baboon linkage map and showed only a single discordant locus position with the human sequence-based map (Figure 1). It is noteworthy that this single discrepancy is between closely linked loci (*D14S72* and *APEX*) for which the RH ordering confidence is less than 100:1, and probably represents a mapping artifact that is likely to be resolved by increasing marker density. Similarly concordant locus orders were obtained for the maximum likelihood lo-

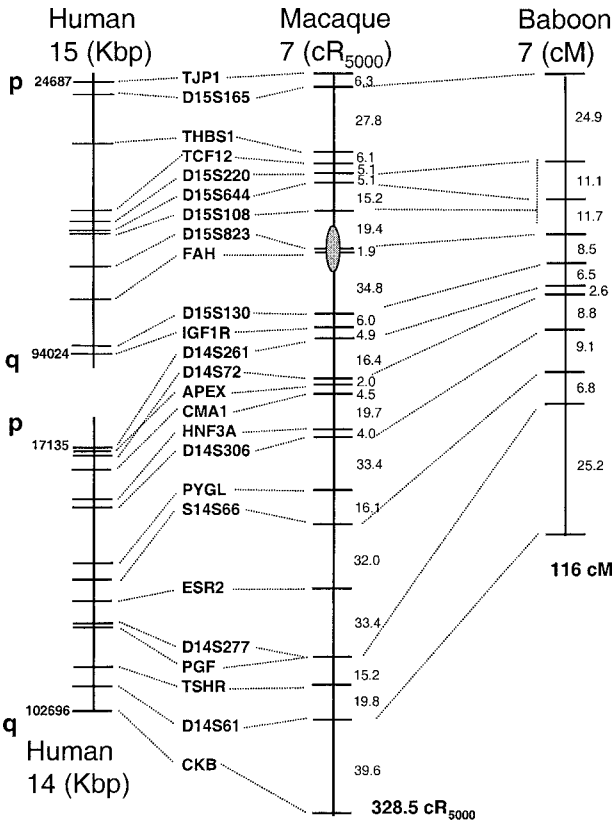

Figure 1. Comparative maps of *Macaca mulatta* chromosome 7 (distance intervals in centirays$_{5000}$), human sequence-based physical maps of chromosomes 14 and 15 (coordinates in kilobase-pairs), and the linkage map for baboon chromosome 7 (distance intervals in centiMorgans). Human microsatellite loci are denoted by the prefix DS and coding genes are given by their standard abbreviations. The human mapping coordinates are from the NCBI build of the human draft sequence data (http://www.ncbi.nlm.nih.gov). The baboon linkage data are from Rogers et al. (2000). The shaded oval denotes the inferred centromere position on macaque chromosome 7 based on elevated retention frequency.

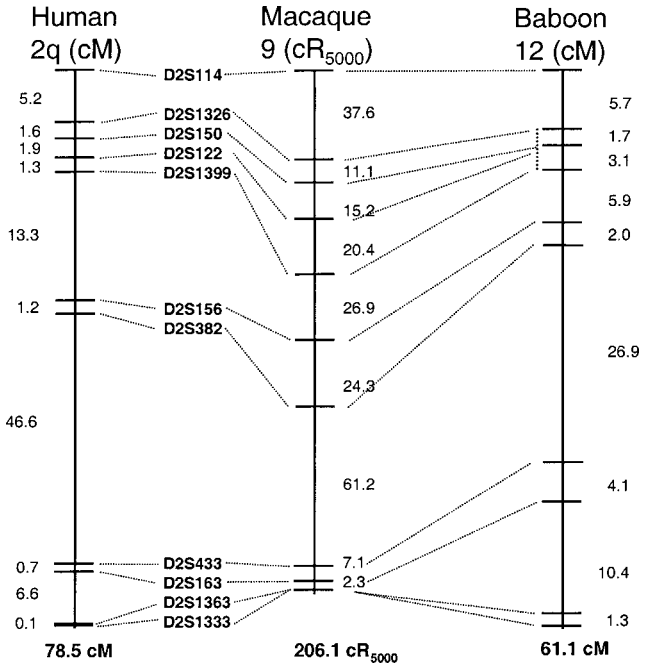

Figure 2. Comparative maps of *Macaca mulatta* chromosome 9 (distance intervals in centirays$_{5000}$), human chromosome 2q, and baboon chromosome 12. Human and baboon data are from Rogers et al. (2000). Details are as described in Figure 1.

cus order for 11 loci mapped to Mma9, in comparison to the human and baboon linkage maps (Figure 2). Comparison of these two preliminary macaque RH chromosome maps with the baboon linkage map predicts a 3 cR$_{5000}$/cM ratio in the macaque, assuming recombination rates do not differ significantly between macaque and baboon, both of which are Cercopithecine monkeys and have identical G-banded karyotypes (Moore et al. 1999).

Comparative RH maps can also reveal the evolutionary history of mammalian chromosomes (Murphy et al. 1999, 2001). Macaque chromosome 9 (Figure 1) represents a conserved synteny of human chromosomes 14 and 15, which likely reflects the ancestral condition for placental mammals (Chowdhary et al. 1998; Murphy et al. 2001; Wienberg et al. 2000). The interstitial centromere position within the human 15 homologous region is also inferred to be the ancestral condition for primates, and possibly placental mammals, given a similar centromere position in outgroup species such as cat and dolphin (Murphy et al. 2001; O'Brien et al. 1999). The conserved gene order between human, macaque, and baboon provides no evidence of an inversion moving the centromere to a position at the boundary of the chromosome 14 and 15 homologous segments, which then could have undergone a fission to produce the two chromosomes observed in humans and other apes. Instead these data imply a more complex evolutionary scenario, involving the loss of the interstitial centromere in the ancestral 14–15 chromosomes, followed by an evolutionary break at the 14–15 junction, and evolution of two new centromeres at the ends of both chromosomes.

Our initial characterization of a 5000 rad macaque RH panel suggests a mapping resolution similar to those panels described for other model organisms which have been utilized for construction of genome spanning maps (http://compgen.rutgers.edu/rhmap/). The high average retention frequency and concordance of locus orders with published baboon and human maps demonstrate the ability of the panel to construct accurate chromosome maps, while providing the means to dissect chromosome evolution within primates, and eventually across mammalian orders. The establishment of this mapping resource sets the stage for the generation of a high-density macaque RH map incorporating microsatellites and expressed sequence tags, to facilitate whole genome scans and provide an anchored comparative framework for whole genome sequencing.

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Received November 16, 2001
Accepted November 19, 2001

Corresponding Editor: Oliver A. Ryder

Partial Cytochrome *b* Sequences for Six Hymenoptera of the Eastern United States

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Mitochondrial DNA (mtDNA) haplotypes have been commonly used to determine honeybee subspecies relationships. To see if these markers would also be useful for comparisons of other Hymenoptera, we collected workers of six local species: *Vespa crabro,* the European hornet; *Bombus impatiens,* a bumblebee; *Vespula germanica,* the German yellow jacket; *Polistes fuscatus,* a paper wasp; *Halictus ligatus,* an alkali bee; and an unspecified *Megachile,* a leafcutting bee. MtDNA was isolated and digested with six endonucleases (*Ava*I, *Bgl*II, *Eco*RI, *Hin*dIII, *Hin*fI, *Xba*I). The digested DNA was electrophoresed and visualized on agarose gels with comparison to a standard fragment marker and similarly treated honeybee mtDNA. The fragments obtained were also purified and sequenced. Phylogenetic relationships between six wasp and bee species, *Apis mellifera,* and several other similar aculeate Hymenoptera were determined. Newly defined DNA sequences were posted to GenBank (AF281169–AF281174).

Mitochondrial DNA (mtDNA) is a short, circular molecule containing a double strand of nonnuclear DNA that has been widely used as a tool for identification and comparison in honeybees (*Apis mellifera* L.) (Schmitz and Moritz 1990; Smith and Brown 1988). Known markers have been used for distinguishing between Africanized and European honeybees (Hall and Muralidharan 1989; Hall and Smith 1991; Sheppard et al. 1991, 1994; Smith et al. 1989) and to study population genetics, biogeography, and phylogeny of the genus *Apis* (Meixner et al. 1993; Smith 1991; Smith and Hagen 1996; Smith et al. 1991), specifically *A. mellifera* (Franck et al. 1998; Garnery et al. 1995; Smith and Brown 1990). The honeybee mtDNA has been completely sequenced (16,343 bp) (Crozier and Crozier 1993).

A limited number of other Hymenopteran species have been studied in similar ways. Restriction fragment length polymorphism (RFLP) methods were used (Sappal et al. 1995) to differentiate several species of *Trichogrammatidae.* DNA sequences of the cytochrome *b* locus, as well as 16S and 28S ribosomal RNA, were used to determine the phylogeny of some Braconidae species (Dowton and Austin 1994; Dowton et al. 1998; Gimeno et al. 1997). Dowton et al. (1998) also included two members of the same Aculeate Hymenoptera genera we sampled (*Apis* and *Polistes*). Danforth et al. (1998) distinguished two cryptic species of *Halictus* using sequence differentiation of another mtDNA locus, cytochrome oxidase I. Koulianos (1999) and Koulianos and Schmid-Hempel (2000) inferred phylogenetic relationships within the bumblebees, *Bombus,* based on cytochrome *b* and cytochrome oxidase I sequences.

The purpose of this experiment was to see if a group of restriction endonucleases (*Ava*I, *Bgl*II, *Eco*RI, *Hin*dIII, *Hin*fI, *Xba*I) that have been used with *A. mellifera* (Crozier and Crozier 1993; Sheppard et al. 1996; Smith and Brown 1988) would be useful for distinguishing other Hymenopteran species of the mideastern United States.

Materials and Methods

Insect Collection

Hymenoptera were collected from several locations near Columbia, MD, during the months of August and September 1997. The samples were killed by freezing at 5°C and stored in an ultracold freezer (–80°C) at the Bee Research Laboratory, USDA, ARS, Beltsville, MD. Species collected were identified by local experts as *Vespa crabro,* the European hornet; *Bombus impatiens,* a bumblebee; *Vespula germanica,* the German yellow jacket; *Polistes fuscatus,* a paper wasp; *Halictus ligatus,* an alkali bee; and an unspecified *Megachile,* a leafcutting bee. Voucher specimens are being held at the Bee Research Laboratory.

DNA Extraction and PCR Procedures

MtDNA was extracted using a modified phenolic extraction method (Sheppard and McPheron 1991). For each extraction, one thorax (or one-half thorax for the large species) was ground in a 1.5 ml microcentrifuge tube with 250 μl of lysis solution. The extracted samples were amplified using the polymerase chain reaction (PCR) on a Perkin-Elmer Cetus DNA Thermal Cycler for 35 cycles at 94°C for 1 min, 46°C for 1 min 15 s, 64°C for 2 min, and then held at 0°C. The reaction was performed using 7 μl of sterile water, 5 μl of reaction buffer, 8 μl of dNTPs mixture (200 μM final concentration of each), 2.5 μl of each primer (1 μM final), 0.5 μl of *Taq* polymerase, and 1 μl of template DNA. We used the *Bgl*II primer pair (Crozier and Crozier 1993), which amplifies a section of cytochrome *b* that includes polymorphic restriction sites for *A. mellifera.* The primer sequences are as follows (Sheppard et al. 1994):

BGL II Forward

5′-TAT GTA CTA CCA TGA GGA CAA ATA TC-3′

BGL II Reverse

5′-ATT ACA CCT CCT AAT TTA TTA GGA AT-3′

The PCR products (15 μl) were digested with each of the restriction enzymes separately, then electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and visualized under ultraviolet (UV) light.

Purification and Sequencing

A representative sample from each species was chosen, PCR was performed as before, and the product was electrophoresed and visualized to assess quality and quantity. Thirty microliters of the PCR product were purified for sequencing using a Nucleo-Trap PCR purification kit (CLONTECH Laboratories Inc., Palo Alto, CA). The purified sample was sent to the Center for Agricultural Biotechnology, University of Maryland, College Park, MD, and sequenced in both directions using dye-primer sequencing reactions and an AB1377 sequencing machine (PE Biosystems).