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An initial genetic linkage map of the rhesus macaque (*Macaca mulatta*) genome using human microsatellite loci

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Abstract

Rhesus macaques (*Macaca mulatta*) are the most widely used nonhuman primate species in biomedical research. To create new opportunities for genetic and genomic studies using rhesus monkeys, we constructed a genetic linkage map of the rhesus genome. This map consists of 241 microsatellite loci, all previously mapped in the human genome. These polymorphisms were genotyped in five pedigrees of rhesus monkeys totaling 865 animals. The resulting linkage map covers 2048 cM including all 20 rhesus autosomes, with average spacing between markers of 9.3 cM. Average heterozygosity among those markers is 0.73. This linkage map provides new comparative information concerning locus order and interlocus distances in humans and rhesus monkeys. The map will facilitate whole-genome linkage screens to locate quantitative trait loci (QTLs) that influence individual variation in phenotypic traits related to basic primate anatomy, physiology, and behavior, as well as QTLs relevant to risk factors for human disease.

Keywords: Rhesus monkey; Genetic linkage; Recombination; Linkage mapping; Microsatellite; Macaque

Introduction

The study of rhesus macaques (*Macaca mulatta*) is critically important to progress in a wide variety of scientific disciplines. Rhesus monkeys have been the subjects of ecological, behavioral, and demographic analyses for many years [1–4] and as such have contributed significantly to our knowledge of basic primate biology. However, most research with this species involves its use as an animal model for studies related to human health and disease. Rhesus macaques are the most widely used nonhuman primate species in biomedical research [5]. They are used extensively in studies of physiology, endocrinology, and metabolism, including studies directly related to diabetes, obesity, cardiovascular disease, and hypertension [6,7]. These animals are also important in neuroscience, in developmental psychobiology, and in research related to addiction or alcoholism [8–12]. The development and assessment of candidate vaccines against HIV and AIDS depend heavily on rhesus monkeys because infection of these animals with simian immunodeficiency virus (SIV), and the resulting AIDS-like syndrome, is the best model available for the human disorder [13–15]. Other research fields as diverse as reproductive biology, aging, and pharmacology also depend on extensive use of rhesus macaques.

This species, *M. mulatta,* is a member of the Old World monkeys, the primate superfamily Cercopithecoidea that also includes cynomolgus macaques (*M. fascicularis*), baboons (*Papio hamadryas*), African green monkeys (*Chlorocebus aethiops*), and other commonly studied species. Old World monkeys are closely related to humans, sharing a common ancestor approximately 25 million years ago [16]. No group of primates other than the anthropoid apes (superfamily Hominoidea) is more closely related to humans than are the

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Old World monkeys. The fundamental similarity of the biology of rhesus monkeys and humans is one reason this species is so widely used as an animal model of normal human physiology as well as the mechanisms and risk factors for human disease. Genetics and genomics are now integral to all aspects of biomedical and evolutionary research. Because rhesus monkeys have been extensively studied in such a wide range of disciplines, an improved understanding of the genomics of this species can have broad impact. Due to their wide availability and the substantial amount of existing data, researchers are likely to conduct many functional genomic studies of normative primate biology and disease in this species. Recognition of the potential significance of genomic and genetic analyses in rhesus macaques led to the designation of this species as a high-priority organism for whole-genome sequencing by the National Human Genome Research Institute [17]. Substantial progress has been made toward a draft quality sequence of the rhesus macaque genome [18,19]. Assembly and publication of the draft rhesus sequence will likely have an immediate and significant impact on primate research, since it will facilitate genomic approaches to many research questions that have thus far been studied using less effective methods.

However, despite the growing amount of DNA sequence available, other types of genetic information for this species remain quite limited. A radiation hybrid panel for physical mapping is available [20], but only 35 loci have been mapped to two macaque chromosomes using this resource. Specific tools for assessing gene expression in this species are being developed [21], and a major cDNA sequencing effort is now underway [22], but the amount of genomic information for rhesus monkeys remains less than what is available for several other mammalian species. The only previously published linkage mapping results for rhesus monkeys report pairwise linkage between tightly linked blood group markers [23] and genes within the MHC complex [24].

To create new opportunities for genetic and genomic research using rhesus macaques, we constructed a first-generation genetic linkage map of the rhesus genome. Linkage maps for nonhuman primates facilitate whole-genome linkage scans for quantitative trait loci related to basic physiology [25] or risk factors for specific diseases [26–28]. Linkage maps also permit preliminary comparisons of genome structure, organization, and evolution. Whereas the anatomy, physiology, immunology, neuroscience, and behavior of rhesus monkeys have been studied extensively for many years, the functional genomics of rhesus monkeys has not, and as a result, many opportunities exist for significant new analyses.

Results

Microsatellite polymorphisms

We tested 300 human microsatellites that were previously mapped in baboons, *P. hamadryas* [29], and found that 154 met our criteria for genotyping and linkage analysis in the full pedigree of 865 rhesus individuals. We also screened microsatellites previously found to be polymorphic in rhesus [30–33] and other randomly selected human microsatellites from published human linkage maps [34]. These latter two approaches yielded a total of 158 additional loci that are informative in the rhesus pedigrees we studied. Table 1 presents a summary of markers tested and mapped by chromosome (see [35] for discussion of rhesus chromosome nomenclature).

A total of 312 polymorphisms were genotyped in the 865 study animals and 241 of those could be placed into unique relative locations within the rhesus genome with relative

Table 1
Summary of microsatellite loci investigated and mapped in rhesus macaques

Rhesus chromosome	Human chromosome	Total loci examined	Loci mapped in unique order	Total length (cM)	Average gap (cM)	Gaps ≥20 cM	Average heterozygosity (mapped loci only)
1	1	30	23	185.6	8.44	0	0.72
2	3	17	15	122.3	8.74	0	0.72
3	7/21	20	18	176.5	10.38	1	0.79
4	6	28	23	156.3	7.10	0	0.74
5	4	13	11	102.6	10.26	0	0.71
6	5	17	12	110.4	10.04	0	0.73
7	14/15	15	13	118.6	9.88	0	0.78
8	8	20	12	128	11.64	0	0.73
9	10	15	12	109	9.91	0	0.72
10	20/22	11	9	95.6	11.95	1	0.72
11	12	12	12	87.5	7.95	0	0.74
12	2q	19	5	36	9.00	0	0.75
13	2p		7	51.8	8.63	0	0.71
14	11	14	13	97.8	8.15	0	0.67
15	9	19	14	104.2	8.02	0	0.73
16	17	8	7	66.4	11.07	0	0.70
17	13	12	7	56.8	9.47	0	0.79
18	18	15	10	88.4	9.82	1	0.76
19	19	14	11	92.9	9.29	0	0.73
20	16	13	7	61.2	10.20	0	0.66
Total		312	241	2047.9	9.27	3	0.730

likelihoods of at least 1000:1. The average heterozygosity of the 241 mapped loci is 0.730 and the distribution of heterozygosity values (Fig. 1) shows a substantial number of the markers have heterozygosity greater than 0.80. Additional information about each polymorphic microsatellite, including observed number of alleles, the measured size range for alleles, PCR conditions for each locus, and suggested groupings of markers for genotyping in multilocus panels, is available on the website of the Southwest National Primate Research Center [36].

The number of loci mapped to unique relative locations varied from 23 markers on rhesus chromosome 1 (homologous to HSA1) to 5 markers on rhesus chromosome 12 (HSA2q). Multipoint linkage maps for each rhesus chromosome are illustrated in Fig. 2. The total length of the current map is 2047.9 Kosambi centimorgans and the average spacing among the 241 loci is 9.3 cM. Previous studies [35,37] have shown that each of three rhesus autosomes is homologous to two different human chromosomes: MML3 consists of sequences homologous to HSA7 and HSA21, MML7 is homologous to HSA14 and HSA15, and MML10 is equivalent to HSA20 and HSA22. Our results illustrate the same rhesus–human homologies. Prior studies [38,39] also indicate that human chromosome 2 is a recent species-specific fusion of two ancestral primate chromosomes and, as expected, we find two separate linkage groups in rhesus monkeys consisting of microsatellite loci mapped either to HSA2p and HSA2q. Nine chromosomes show conservation of locus order between humans and rhesus monkeys for the specific loci mapped in this study, whereas seven chromosomes show one simple inversion that distinguishes the two species. One rhesus chromosome (MML10) shows two inversions relative to humans and one chromosome (MML16) differs from its human homologue in the position of just one locus. In two cases (MML2, homologous to HSA3, and MML15, homologous to HSA9), the order among loci on the rhesus chromosomes differs from that of their human counterparts by multiple inversions or translocations. Among all 241 loci mapped to date, and among the other 71 loci placed into syntenic groups but not mapped to unique locations, there are no microsatellites that map to chromosomes different from that predicted on the basis of the previous whole-chromosome hybridization or painting studies [35,37].

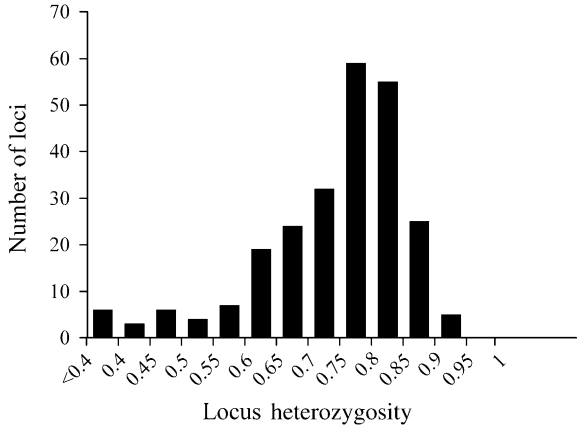


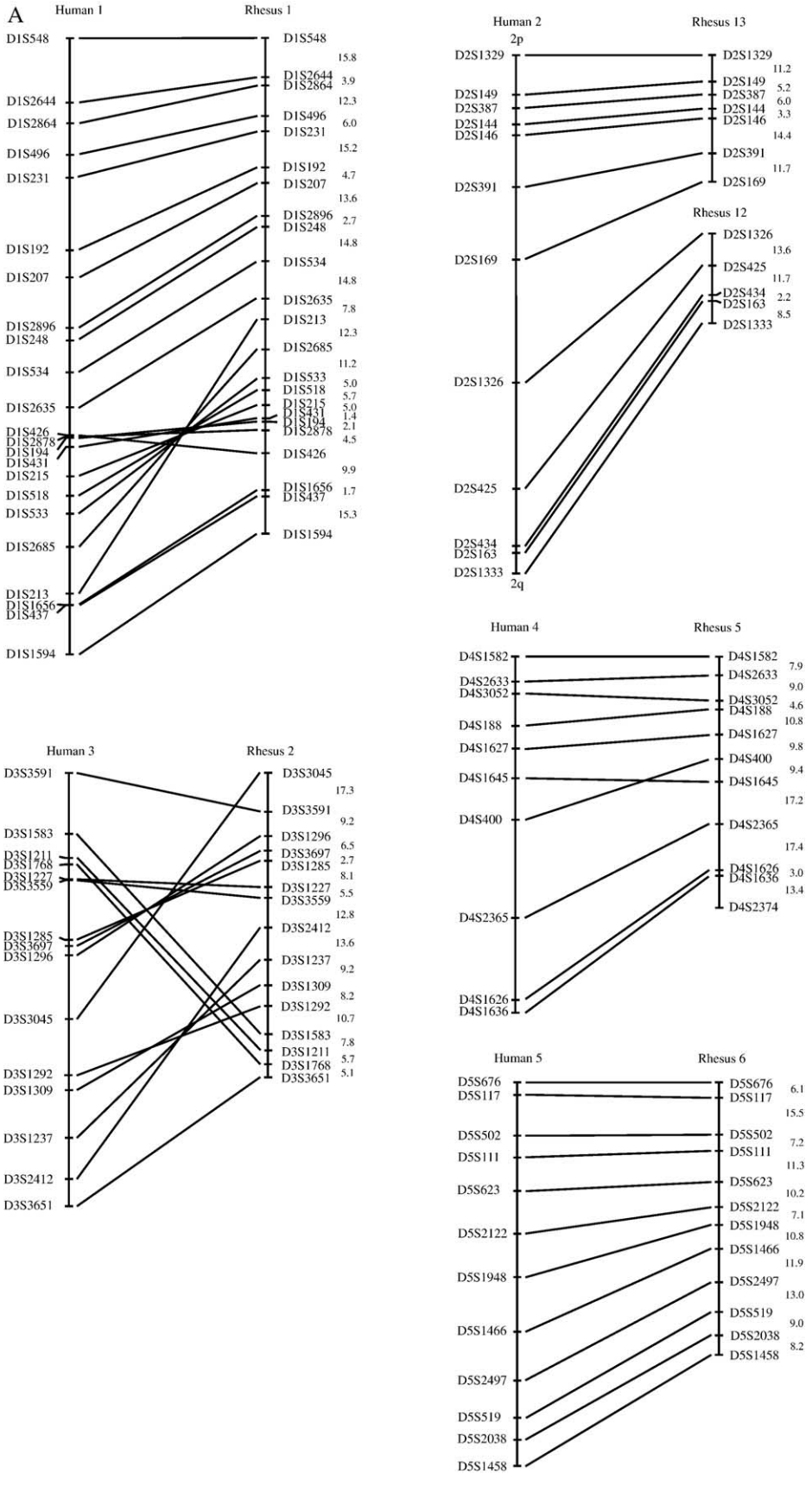
Fig. 1. Distribution of heterozygosity for the 241 microsatellite loci mapped to unique locations in the rhesus genome.

Discussion

Given the continuing progress toward an assembly of a draft sequence of the rhesus genome, and the significant utilization of this species in a wide range of biomedical and evolutionary studies, it is likely that rhesus macaques will be among the most intensively studied nonhuman primates in the area of functional genomics. Two other factors, the ongoing effort to increase the number of rhesus monkeys available for study and the parallel effort to develop multigeneration pedigrees of rhesus macaques at various breeding centers, suggest that the opportunity to use these animals for quantitative genetic and gene mapping studies will continue to grow.

Previous work has identified homologies between the chromosomes of rhesus monkeys and humans [35,37]. At the level of resolution provided by chromosome painting methods, most chromosomes are conserved between the two species and the difference in chromosome number can be explained by simple fissions or fusions. Little information has been available concerning the order of loci within rhesus chromosomes, although chromosome banding studies do suggest specific inversions and rearrangements. The one published study of locus order [20] used a radiation hybrid panel to determine order and relative physical distances among 35 loci on rhesus chromosomes 7 (HSA14 and 15) and 12 (HSA2q). Our linkage analysis provides information concerning order and recombinational distances among 241 loci and is consistent with the order of loci presented by Murphy et al. [20]. In addition, 71 other polymorphisms were identified. The locations of those markers could be established by analyzing additional rhesus families or expanding the present families with additional offspring. Detailed comparison of the linkage maps from rhesus macaques and baboons [29] will be published elsewhere.

This linkage map covers 2047.9 Kosambi centimorgans in the rhesus genome. We used available human linkage maps [40] and the known homologies with rhesus to estimate the proportion of the rhesus genome that is not covered by our markers. There are 35 rhesus chromosome ends for which the order of loci is (based on this initial mapping effort) conserved between the two species. For these chromosome ends, we determined the recombinational distance in humans between the human telomere and the first locus mapped onto the homologous rhesus chromosome. The results suggest that the initial rhesus map reported here does not cover approximately 500 cM at the 35 ends of those rhesus chromosomes. This figure does not include the five chromosomal ends in which inversions or translocations have rearranged locus order in rhesus relative to humans. However, for purposes of genomic scans to locate quantitative trait loci, analyses using this map will be able to detect the effects of genes that are located some distance beyond the last microsatellite marker mapped here. Consequently, genomic linkage screens using this first generation


A
Human 1
Rhesus 1
D1S548
D1S2644
D1S2864
D1S496
D1S231
D1S192
D1S207
D1S2896
D1S248
D1S534
D1S2635
D1S426
D1S2878
D1S194
D1S431
D1S215
D1S518
D1S533
D1S2685
D1S213
D1S1656
D1S437
D1S1594
15.8
3.9
12.3
6.0
15.2
4.7
13.6
2.7
14.8
14.8
7.8
12.3
11.2
5.0
5.7
5.0
1.4
2.1
4.5
9.9
1.7
15.3
Human 2
2p
Rhesus 13
Rhesus 12
D2S1329
D2S149
D2S387
D2S144
D2S146
D2S391
D2S169
D2S1326
D2S425
D2S434
D2S163
D2S1333
2q
11.2
5.2
6.0
3.3
14.4
11.7
13.6
11.7
2.2
8.5
Human 3
Rhesus 2
D3S3591
D3S1583
D3S1211
D3S1768
D3S1227
D3S3559
D3S1285
D3S3697
D3S1296
D3S3045
D3S1292
D3S1309
D3S1237
D3S2412
D3S3651
17.3
9.2
6.5
2.7
8.1
5.5
12.8
13.6
9.2
8.2
10.7
7.8
5.7
5.1
Human 4
Rhesus 5
D4S1582
D4S2633
D4S3052
D4S188
D4S1627
D4S1645
D4S400
D4S2365
D4S1626
D4S1636
D4S2374
7.9
9.0
4.6
10.8
9.8
9.4
17.2
17.4
3.0
13.4
Human 5
Rhesus 6
D5S676
D5S117
D5S502
D5S111
D5S623
D5S2122
D5S1948
D5S1466
D5S2497
D5S519
D5S2038
D5S1458
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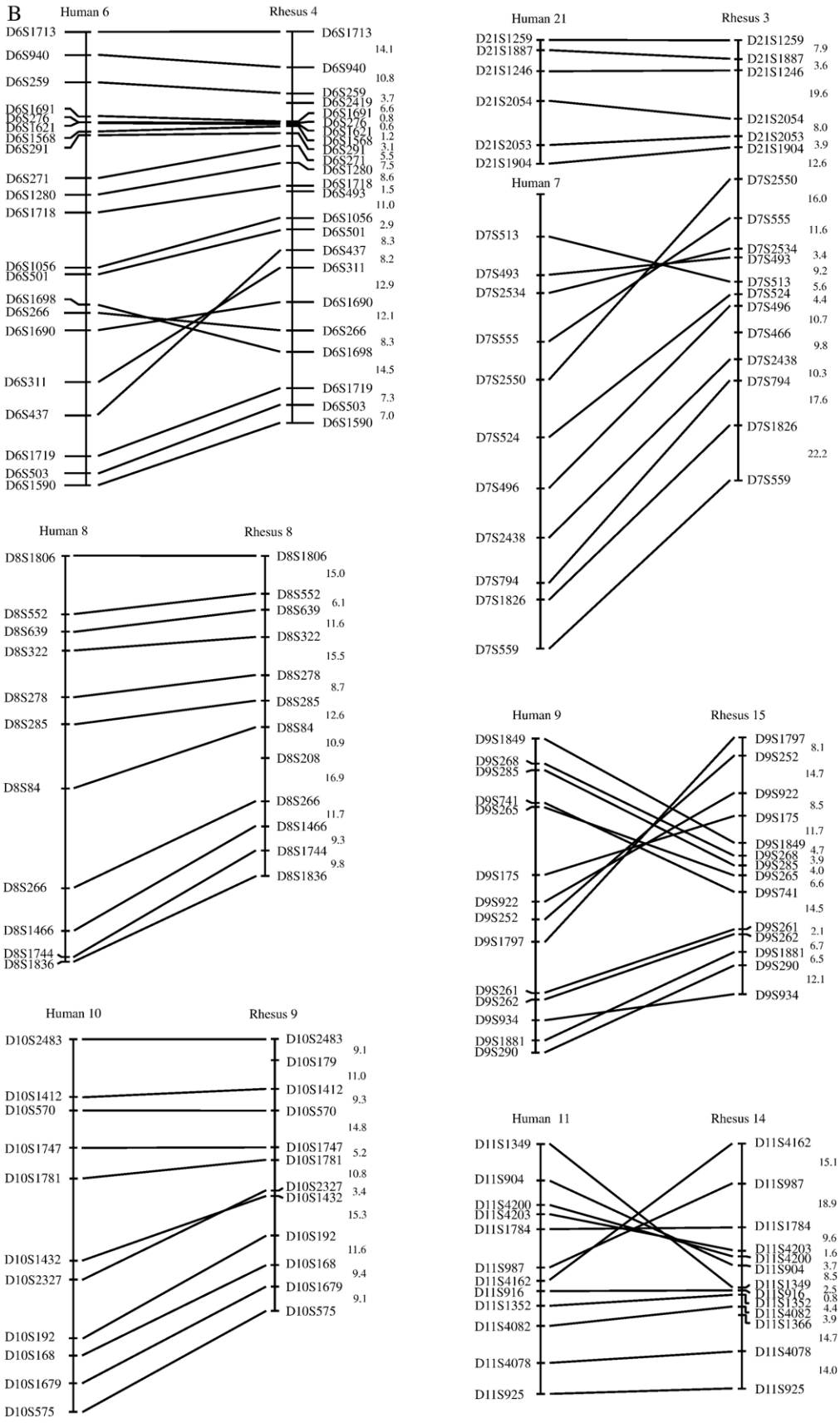
10.2
7.1
10.8
11.9
13.0
9.0
8.2


B
Human 6
Rhesus 4
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D6S940
D6S259
D6S1691
D6S276
D6S1621
D6S1568
D6S291
D6S271
D6S1280
D6S1718
D6S1056
D6S501
D6S1698
D6S266
D6S1690
D6S311
D6S437
D6S1719
D6S503
D6S1590
14.1
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3.7
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0.8
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1.2
3.1
5.5
7.5
8.6
1.5
11.0
2.9
8.3
8.2
12.9
12.1
8.3
14.5
7.3
7.0
Human 21
Rhesus 3
D21S1259
D21S1887
D21S1246
D21S2054
D21S2053
D21S1904
7.9
3.6
19.6
8.0
3.9
12.6
Human 7
D7S513
D7S493
D7S2534
D7S555
D7S2550
D7S524
D7S496
D7S2438
D7S794
D7S1826
D7S559
D7S2550
D7S555
D7S2534
D7S493
D7S513
D7S524
D7S496
D7S466
D7S2438
D7S794
D7S1826
D7S559
16.0
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3.4
9.2
5.6
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9.8
10.3
17.6
22.2
Human 8
Rhesus 8
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D8S552
D8S639
D8S322
D8S278
D8S285
D8S84
D8S208
D8S266
D8S1466
D8S1744
D8S1836
15.0
6.1
11.6
15.5
8.7
12.6
10.9
16.9
11.7
9.3
9.8
Human 9
Rhesus 15
D9S1849
D9S268
D9S285
D9S741
D9S263
D9S175
D9S922
D9S252
D9S1797
D9S261
D9S262
D9S934
D9S1881
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D9S1797
D9S252
D9S922
D9S175
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D9S268
D9S285
D9S265
D9S741
D9S261
D9S262
D9S1881
D9S290
D9S934
8.1
14.7
8.5
11.7
4.7
3.9
4.0
6.6
14.5
2.1
6.7
6.5
12.1
Human 10
Rhesus 9
D10S2483
D10S1412
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D10S192
D10S168
D10S1679
D10S575
D10S2483
D10S179
D10S1412
D10S570
D10S1747
D10S1781
D10S2327
D10S1432
D10S192
D10S168
D10S1679
D10S575
9.1
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3.4
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11.6
9.4
9.1
Human 11
Rhesus 14
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D11S904
D11S4200
D11S4203
D11S1784
D11S987
D11S4162
D11S916
D11S1352
D11S4082
D11S1366
D11S4078
D11S925
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D11S4078
D11S925
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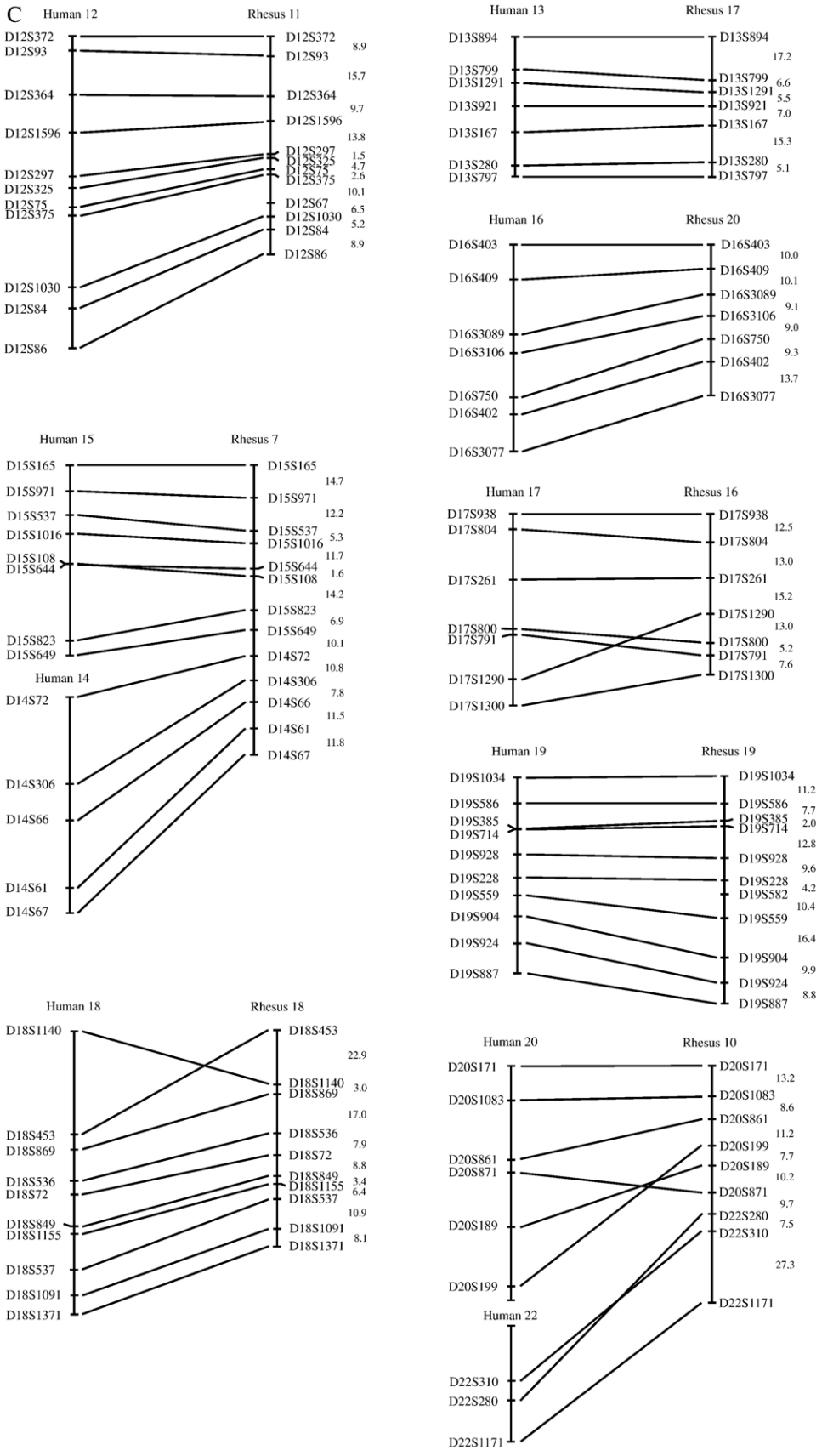

Fig. 2. Comparative maps of rhesus monkey and human autosomal chromosomes. The order among loci shown for each rhesus chromosome is 1000 times more likely than any alternative order. Distances are shown in Kosambi centimorgans as estimated by MultiMap [53]. See Moore et al. [35] for a discussion of the nomenclature for rhesus chromosomes.

map will cover more than 90% of the rhesus autosomal genome.

Rhesus monkeys are critically important for studies related to HIV infection and AIDS, neuroscience, reproductive biology, immunology, developmental psychology, and other fields. Construction of a whole-genome linkage map creates the opportunity to search for quantitative trait loci that influence individual variation in phenotypes significant to all these areas of research. For example, available evidence indicates that individual variation among rhesus monkeys in phenotypes known to be related to human psychopathology (e.g., temperament, neurotransmitter levels, and aspects of neuroanatomy) exhibits significant genetic heritability [12,41–43]. Progress in psychiatric genetics may benefit from the mapping and identification of genes in rhesus monkeys that influence phenotypes already known to be correlated with risk of psychiatric illness in humans.

The linkage map in Fig. 2 provides new information concerning comparative chromosome structure and evolution. The current level of resolution does not permit fine-grained comparisons, but it does indicate the approximate boundaries of chromosomal rearrangements that distinguish rhesus chromosomes from their human homologues. Eleven rhesus autosomes exhibit differences in locus order relative to humans, although this comparison cannot indicate whether the changes occurred in the evolutionary lineage leading to humans or to macaques. More detailed analyses of the breakpoints for these rearrangements would help determine whether random-breakage or fragile-breakage models best explain these evolutionary changes [44,45]. Multispecies comparisons using apes (e.g., chimpanzees and orangutans) plus other Old World monkeys and New World monkeys will eventually permit a more detailed reconstruction of the history of chromosomal change in primates. Such analyses will of course benefit tremendously from whole-genome sequencing of the chimpanzee, rhesus monkey, and marmoset (*Callithrix jacchus*) genomes and from sequencing or detailed physical mapping in other species.

Many of the microsatellite polymorphisms reported here will also be useful in other nonhuman primates. Several species that are closely related to rhesus macaques are also common research subjects, including pig-tailed macaques (*M. nemestrina*), cynomolgus macaques (*M. fascicularis*), and Japanese macaques (*M. fuscata*). Although our data concerning baboons and rhesus monkeys demonstrate that microsatellites that are polymorphic in one Old World monkey are not necessarily polymorphic or useful in another (see also [46]), the three species of macaques mentioned above are all more closely related to rhesus monkeys than rhesus monkeys are to baboons [16]. We expect that a substantial number of the polymorphisms described here will also be informative in other species of the genus *Macaca* and that a lesser number will be informative in more distantly related primates. We also note that as more whole-genome DNA sequence becomes available for rhesus macaques, it will be possible to use bioinformatics tools to search that sequence, identify potentially informative rhesus microsatellite loci, and design rhesus-specific PCR primers to amplify those rhesus-derived loci. That approach may be more efficient for identifying polymorphisms in rhesus macaques, and may generate loci that are more frequently useful in other Old World monkey species, than using human PCR primers to amplify loci originally identified in the human genome, as we did in the present study.

There are several areas for further research that should be addressed. First, recent studies indicate that meiotic recombination events are not randomly and evenly distributed along mammalian DNA sequences, but that mammalian chromosomes undergo recombination within local hotspots that are flanked by longer DNA segments that experience fewer recombinational events [47]. In addition, there is evidence that hotspots for recombination are not always conserved between humans and macaques [48], or even between humans and chimpanzees [49–51]. Comparisons of the numbers and locations of hotspots within various genomes, the average recombination rates per megabase and the intraspecies variation in those rates across multiple chromosomal regions may shed light on the factors that determine rates of recombination in a given genome and help explain evolutionary changes in those rates. Second, we have not yet established a linkage map of the rhesus X chromosome, although this work is currently underway. Third, we have not yet clearly defined the terminal ends of all rhesus chromosomes. Targeted efforts to identify additional polymorphisms as close as possible to the telomeres of rhesus chromosomes will address this issue. Finally, the density of loci on the rhesus map is currently sufficient to provide adequate power for whole-genome linkage screens for quantitative trait loci. However, the power of those screens and our ability to compare chromosome organization and evolution among species will be significantly enhanced by adding many more loci to this first-generation map.

Materials and methods

DNA sampling and pedigree structure

Whole blood or tissue was obtained from 865 pedigreed rhesus macaques housed at the Oregon National Primate Research Center in Beaverton, Oregon, and from the Southwest National Primate Research Center in San Antonio, Texas. Genomic DNA was isolated from white blood cells or liver using standard phenol/chloroform extraction methods. The five rhesus families used for linkage analysis varied in size from 93 to 210 individuals. All five families consist of three-generation pedigrees.

Identification of polymorphic microsatellites

To identify informative microsatellites in rhesus macaques, we used lists of published microsatellite polymorphisms for rhesus monkeys [30–33] and also tested 300 human microsatellite loci that were previously mapped by linkage in baboons, *P. hamadryas* [29]. Screening of these two sets of potential polymorphisms employed DNA samples from eight breeding males who had sired substantial numbers of offspring in the study pedigrees. We tested each fluorescently labeled primer pair using standard PCR conditions [50 ng genomic DNA, 1× buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl^{2+}$), 0.80 μM each primer, 200 μM each dNTP, 0.25 U TaKaRa *Taq* polymerase] and touchdown PCR temperature protocols (minimum temperature from 48 to 52°C). PCR products were analyzed on ABI 377 Automated DNA Sequencers using ABI GeneScan Analysis (V. 3.1.2) and Genotyper (v. 2.5) software. Microsatellite markers that reliably produced genotypes that could be

scored with standard methods (see below), that produced three or more alleles among the eight males tested, and that did not produce spurious amplification products that interfere with effective genotyping were selected for genotyping in the full pedigrees. After testing those primer pairs, we selected microsatellite primer pairs from published human linkage maps [34]. These human loci (not previously known to be polymorphic in rhesus monkeys) were tested using the same procedure and criteria for inclusion as above.

Initial genotyping

Polymorphic loci (n = 154) were fluorescently labeled with TET, FAM, or HEX and optimized for PCR amplification using a range of magnesium concentrations from 1.5 to 3.0 mM and annealing temperatures from 46 to 54°C. We employed the following amplification protocol in a 6 μl total volume in 96-well PCR plates (all reagents from TaKaRa): 50 ng genomic DNA, 1× buffer [10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl^{2+}$], 0.80 μM each primer, 200 μM each dNTP, 0.25 U *Taq* polymerase, and additional magnesium as required for optimized protocols requiring >1.5 mM $MgCl^{2+}$. The thermal cycling method followed a touchdown procedure consisting of an initial denaturing step at 95°C for 5 min, followed by 10 cycles of denaturing at 94°C for 40 s, annealing starting 10°C above the final annealing temperature for 30 s and decreasing 1°C per cycle, and extension at 72°C for 30 s. Following the initial 10 cycles, the method included 25 cycles of standard amplification at the final annealing temperature and ending with extension at 72°C for 7 min and then holding at 4°C. All PCR was performed on ABI (Applied Biosystems, Inc.) 9600 and 9700 thermal cyclers, and the PCR product was visualized by running panels of four to six markers with TAMRA-labeled MapMarker Low (BioVenture, Inc.) internal size standard on ABI 377 sequencers. Fragment size analysis was performed using GeneScan Analysis (V. 3.1.2) and Genotyper (v. 2.5) software.

Initial linkage analysis

Genotypes for pedigreed rhesus were analyzed for Mendelian inheritance in the known pedigrees and incompatible genotypes were rechecked. Final genotype data were organized by chromosome and pairwise linkage analysis performed using CRI-MAP linkage analysis software (Green, unpublished data; [52]). Following pairwise analysis to confirm linkage groups, framework maps were developed for sets of linked loci using CRI-MAP (v. 2.41 m) linkage analysis software and MultiMap (v. 2.0) map construction software [53]. Additional loci placed in unique order and supported by lod scores >3.0 were added to this framework map. Loci that could not be placed in unique order with sufficient confidence were located within broader, estimated map regions. The FLIPS function within CRI-MAP was used to assess the likelihood of alternative orders. This function inverts sets of adjacent loci (sets of two or more) and then compares likelihoods among alternative arrangements. Only when a given order was 1000 times more likely than all others was it accepted.

Targeted screening for filling of gaps

Following initial mapping with known baboon and rhesus markers, gaps larger than 20 cM between loci within rhesus chromosomes were targeted for further study by identifying the homologous position within the human linkage map. Sets of six microsatellites that mapped into that region in humans [34] were selected and those six primer pairs were screened for polymorphism as described above with eight male rhesus as the screening panel. This targeted screening of human microsatellites used the PCR protocols described above. To increase efficiency, DNA amplification and visualization of PCR products at this stage were performed using proprietary ABI 5-dye technology and reagents in 384-well PCR plates. The thermal cycling method used for genotyping the full pedigrees at this second stage of mapping consisted of an initial denaturing step at 95°C for 12 min followed by 10 cycles of denaturing at 94°C for 15 s, annealing at an optimal temperature for 15 s, and extension at 72°C for 30 s. The remaining 20 thermal cycles used denaturing temperature of 89°C, annealing at the same optimal temperature, and extension at 72°C, for the same time periods. Amplification reactions concluded with a final extension at 72°C for 10 min and a final 4°C hold.

Final mapping analysis

After the initial mapping and subsequent addition of loci into gaps, all genotypes were checked for previously undetected errors using SimWalk282 [54]. Data problems detected by SimWalk were corrected by blanking the minimum number of genotypes necessary. Final multipoint linkage maps were then constructed using Multi-Map (v 2.0) and CRI-MAP, including the FLIPS function.

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