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Reciprocal chromosome painting shows that the great difference in diploid number between human and African green monkey is mostly due to non-Robertsonian fissions

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Abstract. We used reciprocal chromosome painting with both African green monkey (*C. aethiops*) and human chromosome specific DNA probes to delineate homologous regions in the two species. Probes were derived by fluorescence-activated chromosome flow sorting and then were reciprocally hybridized to metaphase spreads of each species. Segments in the size range of a single chromosome band were identified, demonstrating the sensitivity of the approach when comparing species that diverged more than 20 million years ago. Outgroup analysis shows that the great difference in diploid numbers between the African green monkey (2n = 60) and humans (2n = 46) is mainly owing to fissions, and the direction of change is towards increasing diploid numbers. However, most break points apparently lie outside of the centromere regions, suggesting that the changes were not solely Robertsonian as has been previously assumed. No reciprocal translocations have occurred in the phylogenetic lines leading to humans or African green monkeys. The primate paints established here are a valuable tool to establish interspecies homology, to define rearrangements, and to determine the mechanisms of chromosomal evolution in primate species.

Introduction

Over the last decade, chromosome painting has been extensively used to study primate chromosome evolution. With this technique, homologies between karyotypes are established by hybridizing DNA probes specific to individual human chromosomes to metaphases of the species under comparison (Wienberg et al. 1990). Hybridization with human chromosome-specific probes outside of the primates provides the necessary outgroup comparison for drawing phylogenetic and taxonomic conclusions (reviewed in Wienberg and Stanyon 1995, 1997).

Chromosome banding and gene mapping studies have suggested that most Old World monkeys and especially Papionini (macaques, baboons, mandrills, and Cercocebus monkeys; 2n = 42) have remarkably conserved genomes (Fig. 1). This conclusion was confirmed by chromosome painting (Wienberg et al. 1992) and recently was extended to leaf-eating monkeys (Colobine; 2n = 44; Bigoni et al. 1997a, 1997b). However, Cercopithecus monkeys, which have been divided into 20 or 22 species (Fleagle 1988), show a high variability of diploid numbers, ranging from 2n = 48–72 (Dutrillaux et al. 1978; Dutrillaux 1979; Ponsà et al. 1980; Sineo et al. 1986).

The karyology of *Cercopithecus aethiops* has been the subject of numerous reports (Stock and Hsu 1973; Finaz et al. 1976; deGrouchy et al. 1977; Estop et al. 1978; Dutrillaux et al. 1978; Dutrillaux 1979; Ponsà et al. 1980; Sineo et al. 1986). Many chromosomes of *C. aethiops* show a close resemblance to single arms of human or Papionini banded chromosomes. The general assumption is that the karyological differences are mainly due to Robertsonian fusions or fissions (Stock and Hsu 1973; Dutrillaux et al. 1978; Clemente et al. 1990.). Molecular cytogenetics now provides the tools to test this assumption and to determine whether fissions or fusions are the primary mechanism responsible for the variability in Cercopithecus monkey chromosome evolution.

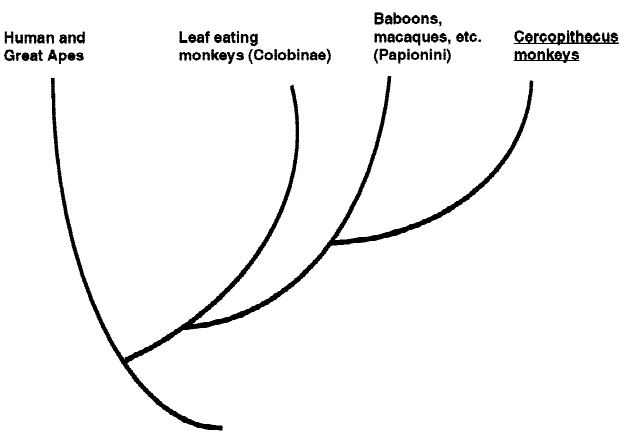

Fig. 1. A simplified phylogeny of Old World primates including humans and *Cercopithecus aethiops.*

We analyzed the karyotype of *C. aethiops aethiops* (2n = 60; fundamental number of FN = 98) by reciprocal painting. In reciprocal painting, chromosome-specific DNA probes from both species are used to paint karyotypes bi-directionally. Reciprocal painting (Arnold et al. 1996; Goureau et al. 1996; Wienberg et al. 1997) provides important additional information to one-way painting by helping to define sub-chromosomal homologies and breakpoints. We employed cross-species FISH with probes derived from flow-sorted metaphase chromosomes of human to "paint" homologous segments in African green monkey chromosomes. To confirm and extend these observations, DNA probes from flow-sorted African green monkey chromosomes were prepared and then used to "paint" human metaphase spreads.

Materials and methods

Cell samples and tissue culture. Cell cultures were established from kidneys of four *C. aethiops aethiops* males. Animals were housed at the

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Paul-Ehrlich Institut (Frankfurt, Germany). Metaphase chromosome spreads were prepared and stored as previously reported (Wienberg et al. 1992; Stanyon et al. 1992). Chromosome isolation for sorting was as previously reported (Rabbits et al. 1995; Wienberg et al. 1997; Stanyon et al. 1999) with staining by Chromomycin A3 and Hoechst 33258.

Flow cytometry. *Cereopithecus aethiops* chromosome preparations were sorted on a FACStar Plus flow sorter (Becton Dickinson) equipped with two 5-W argon ion lasers to allow bivariate flow sorting. Four hundred of each chromosome type where sorted directly into separate 500-μl PCR tubes containing 32 μl of sterile distilled water and then stored at −20°C.

Preparation of chromosome-specific paints. DOP-PCR amplification was a modification of the method described previously (Telenius et al. 1992). Briefly, reaction buffer (25 mM N-Tris (hydroxymethyl)-3-aminoproanesulfonic acid, 50 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, pH 9.00, detergent (0.05% polyoxyethylene ether W-1), deoxynucleotide triphosphates (each at 250 μM), primer 6-MW (5′CCG ACT CGA GNN NNN NAT GTG G 3′ where N = any base, 4 μM), and 2.5 units of *Taq* polymerase (NBS Biologicals) were added and gave a final reaction volume of 50 μl and were overlaid with 30 μl of mineral oil. After an initial denaturation for 9 min at 94°C, nine cycles of 94°C for 1 min, 30°C for 1.5 min, transition at 0.23°C per s to 72°C held for 3 min were followed immediately by 30 cycles of 94°C for 1 min, 62°C for 1 min, 72°C for 3 min. The final extension at 72°C was increased to 10 min.

Primary PCR products were labeled either with biotin-16-dUTP or digoxigenin-11-dUTP (Boehringer Mannheim) by taking 1 μl of the primary DOP-PCR product to a second round of PCR under the conditions described above, with the exception that only 25 cycles at the higher annealing temperature were performed and the dTTP concentration was lowered to 125 μM, and 100 μM of labeled dUTP was added. All PCR reactions were performed on a Trio thermal cycler (Biometra). The concentrations and the sizes of PCR products were determined by 1% agarose gel electrophoresis. Eventually, fragment size was adjusted to 300–600 bp by DNase I treatment.

Chromosome painting. Hybridization and detection were carried out by a modification of the procedure described previously (Cremer et al. 1988; Lichter et al. 1988; Pinkel et al. 1988). For single color, FISH 100–150 ng of biotinylated *C. aethiops*-specific paint and 1 μg of competitor DNA (human Cot-1 DNA, DRL) were made up to 12 μl with hybridization buffer (50% deionized formamide, 10% dextran sulfate, 2 × SSC), denatured at 68°C for 10 min, and preannealed by incubation at 37°C for 30 min. When performing two-color FISH, we combined paints by mixing 150 ng of differently labeled DNA for each probe. Denaturation and preannealing of probes were performed as described above. Slides were denatured by incubation in 70% formamide in 2 × SSC at 68°C for 1 m 20 s, quenched in ice-cold 70% ethanol, and dehydrated through an ethanol series. The preannealed paints were applied on two slides and allowed to hybridize overnight at 37°C. Post-hybridization washes, with high stringency, and detection were performed as previously described (Lichter et al. 1988). After detection, chromosomes were counter-stained with 0.08 μg/ml 4′,6-diamidino-2-phenylindae (DAPI) solution in 2 × SSC for 5 min and mounted in antifade AF1 (Citifluor).

Detection of hybridized signals, microscopy, and image processing. Digital images were taken with a cooled CCD camera (Photometrics NU200 series equipped with a Kodak KAF 1400 chip) coupled to a ZEISS Axiophot epifluorescence microscope. Camera control and digital image acquisition (8-bit gray scale) employed an Apple Macintosh Quadra 950 computer and NU200 software (Photometrics). The DAPI, TRITC, and FITC images were merged with BDS Registration software.

Results

Characterization of the flow karyotype of C. aethiops. The *C. aethiops* flow karyotype was resolved into 32 peaks (Fig. 2). Chromosome paints were made from each peak, and FISH to metaphase spreads of the same species allowed us to identify the content of each peak in the flow karyotype. Almost all chromosomes were resolved in single peaks. However, Chrs 23 + 24 + 25 were found in one peak and 14 + 22 in another peak and could not be resolved. Other chromosomes were not found in single peaks but could be resolved. Chrs 8 + 21 displayed heterogeneous sorting of two polymorphic homologs of Chr 21, leading to one chromosome being present in two different peaks, and an enriched Chr 13 fraction was sorted from a "border" of the mixed peak of 4 + 13. Some polymorphic chromosomes were sorted in two different peaks (Chrs 17, 19, 21, 26, 28). Thus, from 31 different African green monkey chromosomes we obtained 28 paint probes. The border sort of Chr 13 allowed us to treat the combined peak 4 + 13 as a single chromosome paint for Chr 4. Therefore, 26 different paints informative for single, one paint specific for two, and one for three monkey chromosomes were obtained.

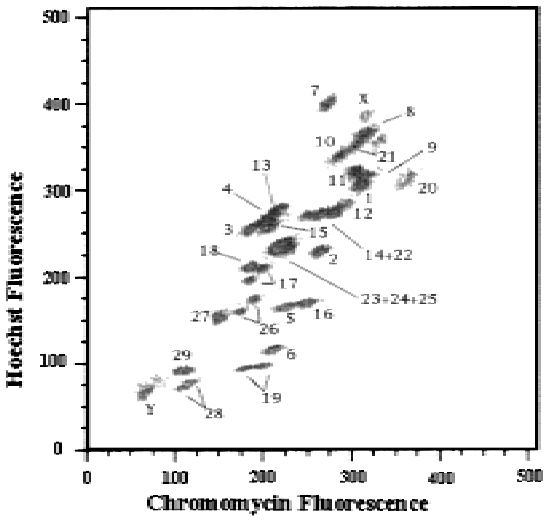

Fig. 2. Bivariate flow karyotype of African green monkey chromosomes from a primary fibroblast culture of a male. Painting probes established by DOP-PCR from flow-sorted chromosomes and in situ hybridization to African green monkey metaphase spreads allowed the chromosomal assignment of each peak. Note the nomenclature for African green monkey follows an ordering of chromosomes by which metacentrics are numbered first, then submetacentrics and acrocentrics (Sineo et al., 1986). Thus, Chrs 7, 20, and the X are the largest in the karyotype, a finding which is also reflected by the chromosome-sorting results. Two peaks showed more than one chromosome (14 + 22 and 23, 24, 25), while from the 4 + 13 peak an enriched sort for Chr 13 was possible. Note that chromosomes 21, 26, and 28 showed polymorphisms that caused the homologs to be found in two separate peaks.

Reciprocal chromosome painting. Reciprocal chromosome painting was carried out with human chromosome-specific probes on *C. aethiops* chromosomes and vice versa. Both human and *C. aethiops* paints gave intensive cross-species signals, as previously reported for human vs. other Old World monkey genomes (Wienberg et al. 1992; Bigoni et al. 1997a, 1997b). Examples of painting experiments are given in Fig. 3. The results obtained from reciprocal hybridizations are internally consistent in every case. When the *C. aethiops* paints contained multiple chromosomes, the reciprocal approach allowed us to assign homologies for all human and monkey chromosomes.

Painting African green monkey probes to human chromosomes. The 28 different chromosome paints produced 39 signals on the human karyotype. Eleven *C. aethiops* autosomal paints (paints derived from Chrs 1, 3, 5, 6, 8, 9, 11, 12, 16, 18 and 19) hybridized to an entire human homolog. *C. aethiops* probe 2 painted two entire human chromosomes (human Chrs 20 and 21). The other 16 *C. aethiops* paints gave signals on subregions of human chromosomes. Paints for *C. aethiops* chromosomes 4, 7, 10, 13, 17, 20, 26,

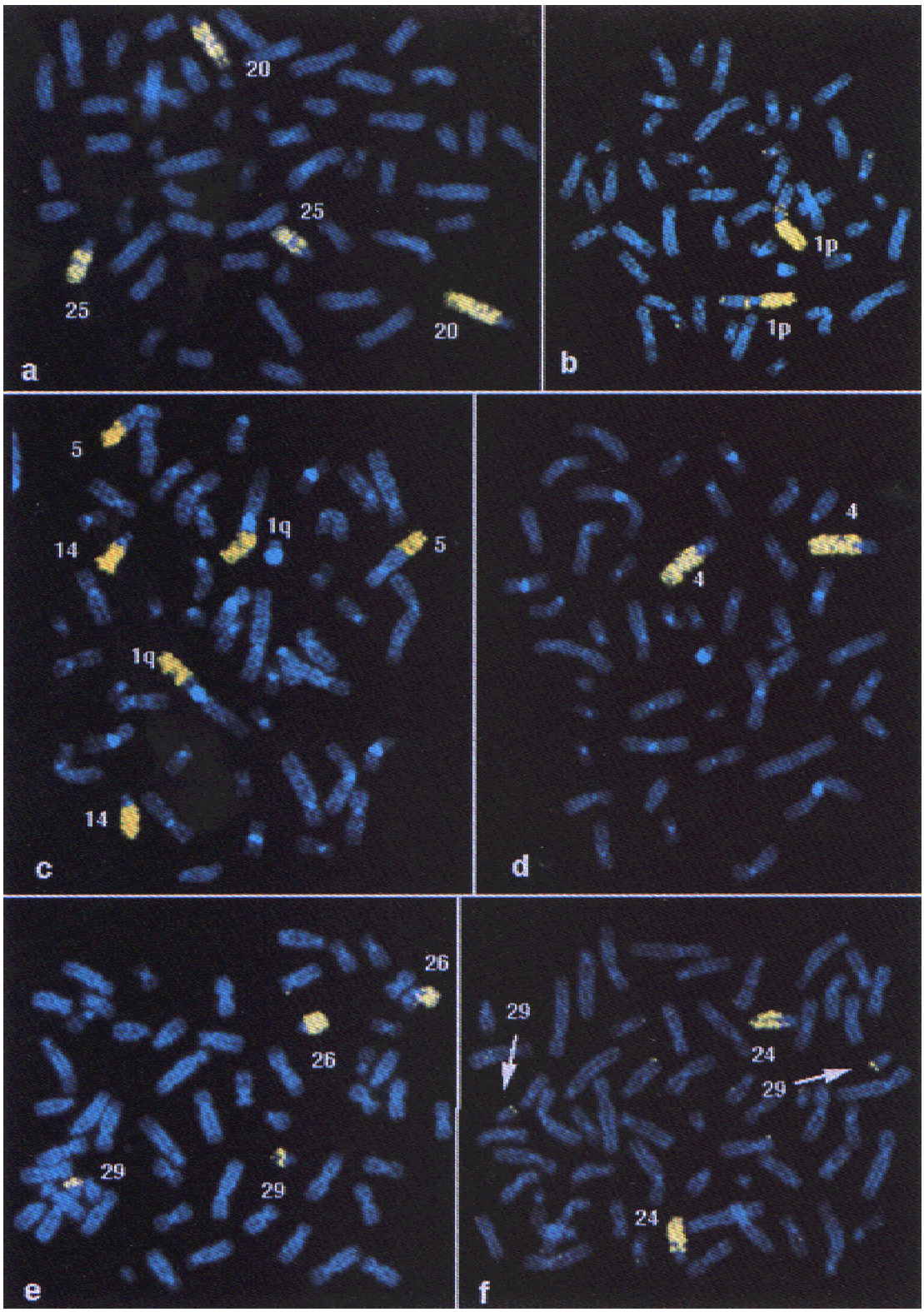

Fig. 3. Examples of in situ hybridizations of DOP-PCR-generated African green monkey paints hybridized to human chromosomes and vice versa. **a)** shows the human chromosome paint 1 on African green monkey Chrs 20 and 25, while **b)** is the reverse painting of African green monkey paint 20 to human Chr 1p and to a small band on the long arm. **c)** shows a hybridization with a paint containing *C. aethiops* Chrs 23, 24, and 25. The chromosome probe 23 paints part of the long arm of human Chr 5, monkey probe 24 paints most of human Chr 14, and 25 hybridizes to the long arm of human Chr 1. A small band on human Chr 14 is not painted and is homologous to material specific for African green monkey Chr 29 (see also f). **d)** African green monkey paint 7 delineates the entire long arm of human Chr 4. **e)** human Chr 15 paints two African green monkey chromosomes, 26 and 29. **f)** human Chr 14 paints African green monkey Chrs 24 and 29 (arrows).

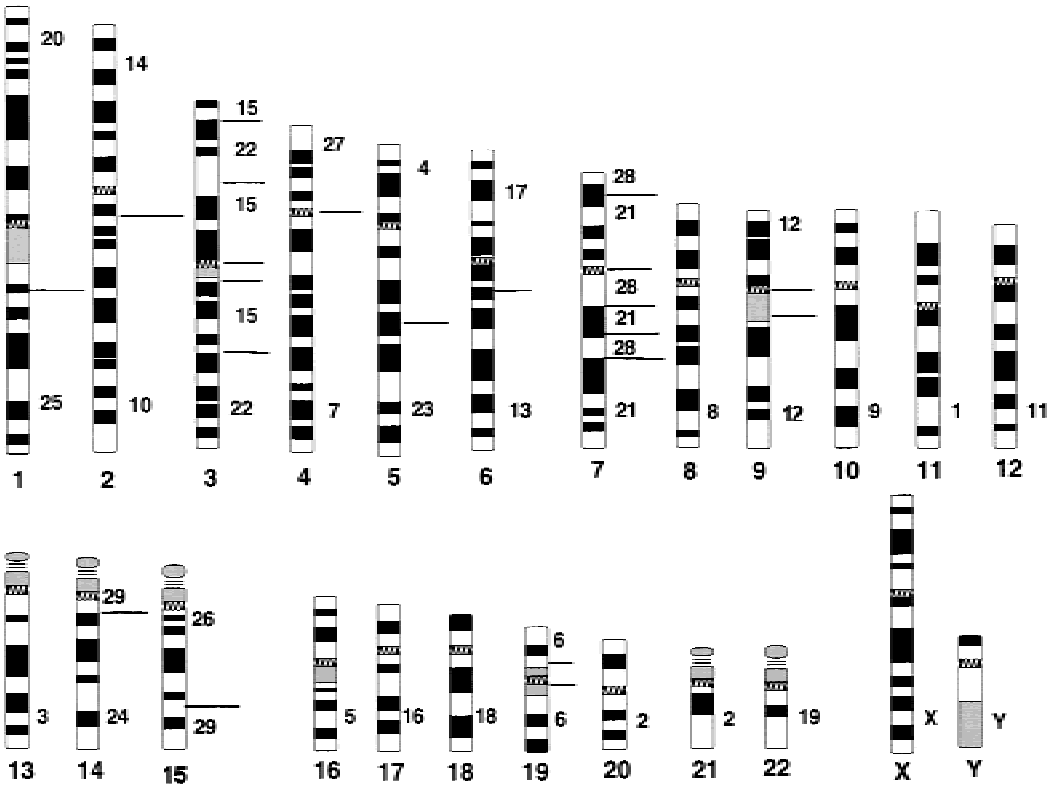

Fig. 4. The idiogram summarizes the in situ hybridization experiments painting African green monkey probes on human chromosomes. The hybridization sites and the number of the African green monkey painting probes are given on the right side of each chromosome. The horizontal lines indicate the limits of the hybridization signals. Pericentromeric areas were generally without hybridization signals. The exact assignments for probes containing African green monkey probes 14 + 22 and 23, 24, 25 were deduced from the reciprocal results painting human on African green monkey chromosomes.

and 27 gave one; 15, 22, and 29 gave two; and paints 21 and 28 gave three signals each. The mixed paint for Chrs 23 + 24 + 25 gave three signals on different human chromosomes 1, 5, and 14. The mixed monkey paint 14 + 22 hybridized to two regions on human Chr 3, to the entire short arm of Chr 2, and to the proximal region of 2q. The sex chromosome paints hybridized to their homologs. A summary of the results is shown in Fig. 4.

Painting human probes to African green monkey chromosomes. The total number of signals obtained with the 24 human chromosome-specific DNA paints on *C. aethiops* metaphases was 33. Thirteen of 22 human autosome paints (paints 8–13, 16–22) gave one signal. All of these, with the exception of 21 and 22, which together form *C. aethiops* Chr 2, hybridized one entire *C. aethiops* homolog each. Seven human autosomal probes (paints 1, 2, 3, 4, 5, 6, and 7) gave two signals each and hybridized to two entire *C. aethiops* chromosomes. Paints 14 and 15 both hybridized to a separate, entire *C. aethiops* chromosome (chromosomes 24 and 26 respectively). In addition, they both recognized subregions on *C. aethiops* Chr 29. The human sex chromosome probes hybridized to their *C. aethiops* homologs. The summary of the hybridizations is shown in Fig. 5.

Discussion

Transfer of gene mapping data between human and African green monkey. The African green monkey is an important animal model in biomedical research; however, the gene map is poorly developed. Only 29 genes are mapped both in human and this monkey. A further seven genes are in various unassigned linkage groups (O'Brien, 1993, for review; Fig. 5). This low number reflects the labor-intensive techniques involved in classical gene mapping approaches. Further, polymorphic markers are of little use in comparing genetic maps between species, since they are often not very well conserved. All the previous assignments of homologies are in accordance with the present painting results. Recent reciprocal chromosome painting has shown that FISH can be used effectively to transfer gene mapping data from densely mapped species to those with poorly developed maps. It has been shown that the accuracy of this transfer is more than 90% of genes (Goureau et al. 1996; Wienberg et al. 1997; Stanyon et al. 1999). For instance, we can immediately propose three previously identified but unassigned linkage groups to specific chromosomes of *C. aethiops:* U1 = AK1 to CAE12; U2 = HEXA, MPI, NP, and PKM2 to CAE29; U3 = CKBB to CAE24 (Fig. 5). In the 13 cases where human chromosomal synteny is maintained (Chrs 8–13 and 16–22), genes already assigned to these individual human chromosomes can be transferred to individual green monkey chromosomes with a better than 90% confidence. In the nine cases (human Chrs 1–7, 14, and 15) where synteny is not conserved between species reciprocal painting can permit a provisional sub-chromosomal assignment of mapping. For example, genes mapped on human Chr 1p can be assigned to *C. aethiops* Chr 20, which is homologous to this human chromosome arm. Sub-chromosomal assignments can be proposed for nine individual human chromosomes that are found fragmented in the monkey karyotype: 1, 2, 3, 4, 5, 6, 7, 14, and 15. In this way, literally thousands of genes assignments of human chromosomes or human chromosomal subregions can now be transferred to individual monkey homologs (Fig. 4 and 5).

Mechanisms of chromosome evolution in C. aethiops. Compared with other Old World monkeys (Wienberg et al. 1992; Bigoni et al. 1997a, 1997b), the karyotype of *C. aethiops* seems highly derived,

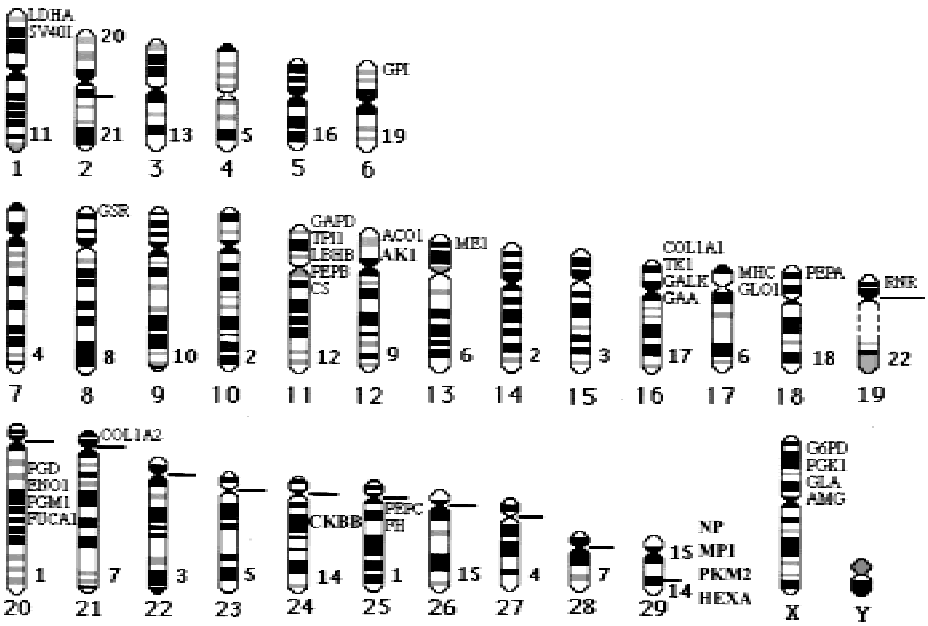

Fig. 5. The idiogram summarizes the in situ hybridization experiments painting human probes on African green monkey chromosomes. The hybridization sites and the number of the human painting probes are given on the right of each chromosome. The horizontal lines indicate the limits of the hybridization signals. In addition, gene mapping data (O'Brien 1993) are included. All gene mapping data are consistent with the chromosome painting results. We have also included in bold number three previously identified but unassigned linkage groups to specific chromosomes of *C. aethiops.*

but is still much more conserved than those of gibbons (Jauch et al. 1992; Koehler et al. 1995a, 1995b), which are even more closely related to humans. These differences suggest that chromosome painting may not be sensitive enough to identify small translocations in more distantly related species. However, in the present experiments a number of hybridization signals of *C. aethiops* paints on human chromosomes were about the size of single chromosome bands: CAE chromosome paints 21 and 28 on HSA7, and CAE paint 29 on HSA14. These hybridizations demonstrate the sensitivity of the approach even when comparing species that diverged more than 20 million years ago. We conclude that chromosome painting clearly demonstrated large differences in frequencies of chromosome rearrangements over evolutionary time in various phylogenetic lines and that there is no simple molecular clock for karyotype reshuffling (Wienberg and Stanyon 1997).

Outgroup analysis including New World monkeys, prosimians, and several non-primate mammals indicates that the direction of changes in the karyotype of African green monkey is towards increasing diploid number (that is, fission). On analysis of the position of hybridization signals, the breakpoints can be mapped in most instances outside the centromeric regions. This result suggests that the changes were not solely Robertsonian, as has been commonly assumed (Dutrillaux et al. 1978; Estop et al. 1978; Ponsà et al. 1980). This conclusion can be made for the breakpoints of the monkey homologs to human Chrs 1, 3, 5, 6, 14, and 15, whereas hybridization data for homologs to human Chrs 4 and 7 may include breakpoints within the centromeres. This hypothesis was not previously clear, because the exact borders of homologous subregions were not well defined in the banding comparisons of *C. aethiops.*

Further outgroup comparisons show that there are a number of other derived chromosome rearrangements in both human and African monkey karyotypes. The well-known human Chr 2 fusion is reflected by two homologs in *C. aethiops.* The chromosome association homologous to human 14/15 is ancestral to primates and other mammalian orders, but derived in both species. This chromosome association was independently fissioned in both humans and African green monkey, leading to two and three homologs respectively. In *C. aethiops* Chrs 26, 24, 29 have resulted from two fissions of this ancestral chromosome. The hybridization pattern demonstrates, that the breakpoints differ from those in human Chrs 14 and 15 (Fig. 3 e, f). Finally, the association between human Chr 20 and 21 homologs found in *C. aethiops* Chr 2 is a derived trait, since both chromosomes are independent in the ancestral Catarrhine karyotype. The various combinations found for the human NOR-bearing chromosome homologs to 21 and 22 in different Old World monkeys definitely show that these chromosomes were independent in the last common ancestor (Wienberg et al. 1992; Stanyon et al. 1995; Bigoni et al. 1997a, 1997b).

Generally, chromosome painting delineates interchromosomal rearrangements such as fusions and fissions and translocations. There is no evidence for any reciprocal translocation in the phylogenetic lines leading to humans or *C. aethiops.* However, the multiple hybridization patterns of some *C. aethiops* paints on human Chrs 3 and 7 suggest that inversions have occurred in the phylogenetic lines leading to human and African green monkey. Further chromosome painting to other Old World monkeys and great apes will clarify the exact rearrangements involved.

The origin of Cercopithecus monkeys is still under discussion. Comparisons of the hybridization pattern in other Old World monkeys shows that all Papionini share two derived chromosome associations, 7/21 and 20/22. These associations appear to link all Papionini after the divergence of Cercopithecus monkeys. However, it would be helpful to have more chromosome painting data from a number of Cercopithecus species to define the exact origin of this genus. The paint set of *C. aethiops* will be helpful in defining the chromosomal rearrangements in the genus Cercopithecus; however, it could also be used to establish interspecies homology and establish chromosomal phylogenetic evolution in other Old World monkeys, New World monkeys, and prosimians. Such research could help analyze the origin and evolution of primate chromosomes as will finally help to establish the ancestral karyotypes of all primates.

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