

Comparative High-Resolution Mapping of Human and Primate Chromosomes by Fluorescence *in Situ* Hybridization

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A molecular cytogenetic approach that facilitates high-resolution comparative mapping of defined human genes in different primate species is presented. Fluorescence *in situ* hybridization and digital imaging microscopy were used to visualize human DNA probes on simultaneously banded or "painted" metaphase chromosomes of great apes (*Pan troglodytes*, *Gorilla gorilla*, *Pongo pygmaeus*), hylobatids (*Hylobates lar* and *Hylobates syndactylus*), and Old World monkeys (*Macaca fuscata* and *Cercopithecus aethiops*). Using a series of DNA probes, chromosomal rearrangements in the karyotypes of primates were readily detected at the molecular cytogenetic level. This approach should contribute considerably to the understanding of primate phylogeny and evolution. © 1993 Academic Press, Inc.

INTRODUCTION

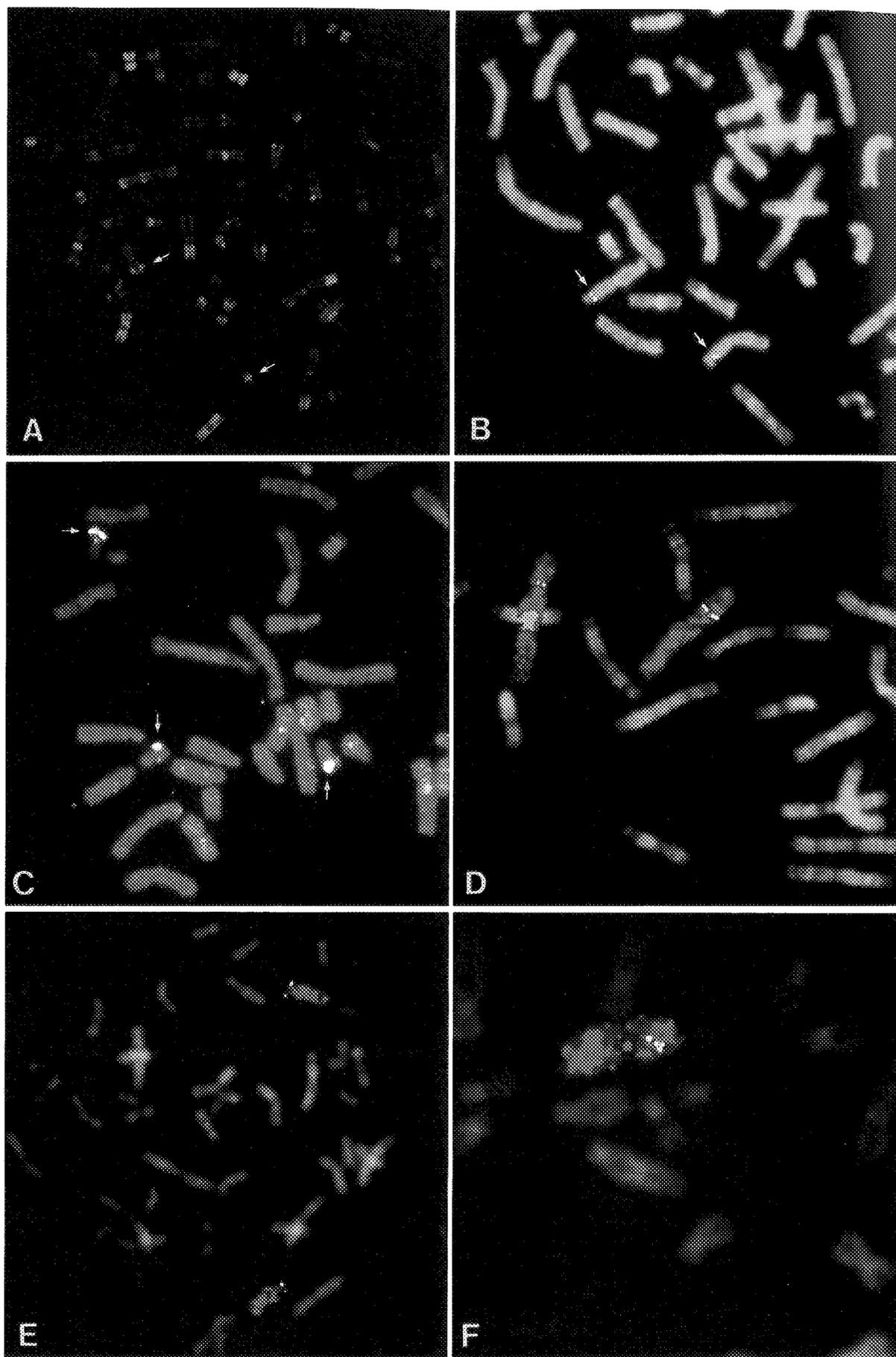
Fluorescence *in situ* hybridization (FISH) of DNA probes to chromosomes has become an important tool in the analysis of the human genome. Currently, composite DNA probe sets for delineating whole chromosomes, chromosomal subregions, or gene-specific loci are available (for reviews see Raap *et al.*, 1990; Lichter *et al.*, 1991; McNeil *et al.*, 1991). FISH makes mapping efforts extremely rapid compared to classical techniques, such as genetic linkage, cell hybrid analysis, or isotopic *in situ* hybridization. In general, DNA probes are haptenized enzymatically and visualized via reporter molecules conjugated to fluorophores. The chromosomal map position can be determined by fractional length measurements, which reveal the map locus with respect to the total chromosomal length (Lichter *et al.*, 1990a), and by assigning the probes to classical cytogenetic bands, identified by karyotype analysis (Baldini and Ward, 1991; Lawrence *et al.*, 1990; Arnold *et al.*, 1992).

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Comparative mapping of genes revealed only patchy information about homologies between human and primate chromosomes, which is mainly due to the labor-intensive procedures employed. In general, analysis of panels of primate-rodent hybrid cell lines leads to the identification of homolog chromosomes but not to further homologization of chromosome bands. Thus, intrachromosomal rearrangements are difficult to analyze with somatic cell hybrid techniques. On the other hand, comparative chromosome banding analysis may be subjective and unreliable when species with pronounced chromosomal reorganizations are examined (e.g., hylobatids; for review see O'Brien *et al.*, 1988).

The molecular homology of entire primate chromosomes was recently demonstrated by *in situ* hybridization using chromosome-specific DNA libraries derived from flow-sorted human chromosomes (Jauch *et al.*, 1992; Stanyon *et al.*, 1992; Wienberg *et al.*, 1990, 1992). However, these chromosome-specific DNA libraries did not permit the demonstration of homology of subchromosomal regions or the reconstruction of intrachromosomal rearrangements, which are supposed to be prominent in the evolution of human and great ape karyotypes (Dutrillaux, 1979; Seuànez, 1979; Yunis and Prakash, 1982; Stanyon and Chiarelli, 1983). Here we present a molecular cytogenetic approach that allows a high-resolution comparative mapping of plasmid, phage, and cosmid clones of defined human genes by FISH to chromosome spreads of different primate species. DNA probes from the human muscular dystrophy gene (Ried *et al.*, 1990), the c-myc oncogene (Ried *et al.*, 1992a), the human thyroglobulin gene (Baas *et al.*, 1985), and probes specific for the Down syndrome region on human chromosome 21q22 (Lichter *et al.*, 1990b) were hybridized to chromosomes of hominoids and Old World monkeys. In each case, the chromosomal map position in the primate was unambiguously determined. In addition, the chromosome 21-specific probe set revealed a trisomy of the human chromosome 21 homolog in one of the two orangutans investigated. Cohybridization of chromosome "painting" probes (Pinkel *et al.*, 1988; Cremer *et al.*, 1988) and single-copy probes also was used to delineate translocation events in the gibbon (*Hylobates lar*) genome.



MATERIALS AND METHODS

Primate cell lines and chromosome preparations. Metaphase spreads were prepared from peripheral blood lymphocytes (*Cercopithecus aethiops*), from skin fibroblasts from *Macaca fuscata*, and from a *Pongo pygmaeus* with morphological features of Down syndrome (a male orangutan named Sahib, Duisburg Zoo, Germany; the cells were kindly provided by Dr. A. Wirtz, Munich). In addition to our own cell lines, lymphoblastoid cell lines (*Pan troglodytes*, *Gorilla gorilla*, *Pongo pygmaeus*, *Hylobates syndactylus*, *H. lar*) were kindly provided by Drs. J. Kidd (New Haven), T. Ishida (Tokyo), and M. Schmid (Würzburg). Cell culture and chromosome preparations followed standard procedures.

DNA probes. The following DNA clones were used. Cosmid cpt1 and PERT87.1, kindly provided by Dr. G.-J. van Ommen (Leiden) and Dr. T. Monaco (London), contain parts of the Duchenne muscular dystrophy (DMD) gene and map to Xp21 (Ried *et al.*, 1990). The phage clones for the c-myc region were a gift of Dr. M. Lipp (Munich) and map to 8q24 (Ried *et al.*, 1992a). The plasmid clones (PCHT16/8.0, HT0.96) for the human thyroglobulin gene were purchased from ATTC (Cat. Nos. 57437 and 57337) and map to 8q24 (Baas *et al.*, 1985). The chromosome 21 cosmid probes (cos519, cos523) were kindly provided by Dr. P. Lichter (Heidelberg) and map to 21q22 (Lichter *et al.*, 1990b).

The *Alu*-PCR products used for hybridization banding were generated as described by Baldini and Ward (1991). The chromosome X- and 15-specific plasmid libraries, pBS X and pBS 15 (Collins *et al.*, 1991), were a generous gift of Dr. J. Gray (San Francisco).

In situ hybridization and detection. *In situ* hybridization followed the protocols described elsewhere with minor modifications (Wienberg *et al.*, 1990; Ried *et al.*, 1992a,b). Briefly, cosmid clones (50 ng), phage clones (80 ng), or plasmid clones (100 ng) and the chromosome X- and 15-specific libraries (250 ng) were precipitated with 5 μ g salmon sperm DNA and 5 μ g yeast t-RNA, together with 10 μ g total human competitor DNA, and then resuspended in 10 μ l 50% formamide, 2 \times SSC, and 10% dextran sulfate. Probe DNAs were denatured at 75°C for 5 min, allowed to preanneal to the human competitor DNA for 30 min at 37°C, and applied to the denatured chromosome specimens; a coverslip was added and sealed with rubber cement. The chromosomes were denatured separately in 70% formamide, 2 \times SSC, for 2 min at 80°C, followed by a dehydration step through an ethanol series. After overnight incubation at 37°C, the coverslips were removed and the slides were washed three times at 42°C in 50% formamide, 2 \times SSC, followed by three washes at 60°C in 0.5 \times SSC. After a blocking step (in 4 \times SSC, 3% BSA and for 30 min at 37°C), the biotinylated probes were detected using avidin conjugated to fluorescein (Vector

Laboratories) or avidin conjugated to Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA). The digoxigenin-labeled probes were detected with a rhodamine- or fluorescein-labeled anti-digoxigenin IgG (Boehringer Mannheim). Dinitrophenol-11-dUTP (Novagen, Madison, WI)-labeled probes were detected with a monoclonal rat anti-dinitrophenol antibody (Novagen) and visualized with a goat anti-rat antibody coupled with fluorescein (Sigma). For some biotinylated probes the signals were amplified using a biotinylated anti-avidin antibody (Sigma) followed by an additional layer of avidin-fluorescein (Pinkel *et al.*, 1986). 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, 150 ng/ml final concentration) or propidium iodide (200 ng/ml, final concentration) was used as a chromosome counterstain. Finally, the slides were mounted in a medium consisting of 9 parts glycerol and 1 part 1 M Tris-HCl, pH 7.5, containing 2% 1,4-diazabicyclo[2,2,2]octane (DABCO) as an antifade agent.

Fluorescence microscopy and digital imaging. Conventional light microscopy was performed with a Zeiss Axiophot epifluorescence microscope. Photographs were taken with Agfachrom 1000 color slide film. Digital images were obtained using a Zeiss Axioskop epifluorescence microscope coupled to a cooled CCD camera (Photometrics PM512, Tucson, AZ). Camera control and digital image acquisition (8-bit gray scale) employed an Apple Macintosh IIx computer. The images were then separately pseudocolored and merged using software developed by Timothy Rand. For a more detailed description, see Ried *et al.* (1992b). Photographs were taken with a Agfa-matrix procolor slide printer using Kodak 100 HC color slide film.

RESULTS

To detect the genetic loci on primate chromosomes that correspond to known human genes we used FISH with human DNA probes together with human *Alu*-PCR products to generate fluorescent signals on R-banded primate metaphase chromosomes. All of the DNA probes used gave strong hybridization signals. The R-banding pattern produced by the human *Alu*-PCR products excluded the centromeric C-bands in all species analyzed, as well as terminal C-bands in the African ape chromosomes. The lack of labeled centromeric and telomeric C-bands suggests that these chromosomal subregions contain few, if any, elements of the *Alu*-repeat family. Staining with DAPI gave a G-banding pattern that

FIG. 1. Several examples of *in situ* hybridization experiments of different human DNA probes to primate chromosomes. All images were acquired with a cooled CCD camera mounted on an epifluorescence microscope and processed with custom computer software, except for C, which was photographed directly through the microscope. (A) The hybridization of an X-chromosome-specific cosmid clone for a subregion of the human dystrophin gene, hybridized to chromosomes of a female *Gorilla gorilla*. In human, the probe maps to Xp21. Cohybridization with a *Alu*-PCR-generated probe, which is detected with green fluorescence, readily allows the assignment of the clone signals (detected with red fluorescence) with respect to cytogenetically defined bands. Note that terminal C-bands on various gorilla chromosomes are not stained with the *Alu*-PCR products. Both X chromosomes are labeled on both chromatids on the homologous gorilla chromosome band (arrows). (B) The hybridization of a plasmid clone for the human thyroglobulin gene to gorilla chromosomes. The chromosomal map position in human is 8q24. In great apes the probe mapped to the homologous chromosome in band 7q24 (arrows). The chromosomes were identified by DAPI banding, resulting in a G-banding pattern. Note the terminal C-bands that are prominent when DAPI stain is used on gorilla chromosomes. (C) The hybridization pattern of a cosmid probe for the terminal band of human chromosome 21 (21q22), the region involved in the development of Down syndrome. Three signals can be detected after hybridization to the metaphase spread of one of the orangutans analyzed in PPY 22q22 (arrows). This individual shows clinical features of Down syndrome. Note that both chromatids of the target chromosomes are not always labeled. (D) The chromosomal assignment of the probe specific for the human dystrophin gene (yellow) on the X chromosome of *Hylobates syndactylus*. The probe was cohybridized with a human chromosome X-specific DNA library to "paint" the homologous chromosome (red). (E) A "painting" of a human chromosome 15-specific DNA library (red) and a cosmid clone specific for chromosome 21q22 (yellow) hybridized to a chromosome preparation from *Hylobates lar*. The hybridization pattern confirms a previously assumed fusion (see text) between human chromosomes 15 and 21. The cosmid signals hybridized to the telomeric region of the long arm of gibbon chromosome 15, indicating the orientation of chromosomes in the fusion event. (F) The hybridization pattern of two cosmids (yellow spots) that are found about 800 kb apart in the human dystrophin gene map on chromosomes of *Cercopithecus aethiops*. For both clones a distinct signal is found on both chromatids, demonstrating the high-resolution mapping capacity of FISH even in lower primates. The X chromosome (red) is painted with the X-specific DNA library.

TABLE 1
Chromosomal Mapping of Various Human DNA Clones in Different Primate Species

	cpt1	c-myc phages	pCHT16/8.0	cos519/523
<i>Pan troglodytes</i>	PTR Xp21	PTR 7q24	PTR 7q24	PTR 22q22
<i>Gorilla gorilla</i>	GGO Xp21	GGO 7q24	GGO 7q24	n.d. ^a
<i>Pongo pygmaeus</i>	PPY Xp21	PPY 6q24	PPY 6q24	PPY 22q22
<i>Hylobates lar</i>	HLA Xp	n.d.	n.d.	HLA 15qter ^b
<i>Hylobates syndactylus</i>	HSY Xp	n.d.	n.d.	n.d.
<i>Cercopithecus aethiops</i>	CAE Xp	n.d.	n.d.	n.d.
<i>Macaca fuscata</i>	MFU Xp	n.d.	n.d.	MFU 2pter ^c

^a n.d., not determined.

^b According to the idiogram published by Jauch *et al.*, 1992.

^c According to the idiogram published by Small *et al.*, 1985, for *M. mulatta*.

further assisted in the unambiguous identification of primate chromosome. Since the probe signal and the banding patterns are detected with different fluorophores, the comparative band assignment is facilitated considerably. In some experiments we also used human chromosome-specific libraries in combination with gene-specific probes to delineate the homologous primate chromosomes. Examples of the experiments with different DNA probes are given in Fig. 1, and a summary of FISH mapping data is given in Table 1.

FISH of Human DNA Clones to Great Apes

In great apes all the probes examined mapped to the primate chromosomes, which are assumed to be homologous to the corresponding human chromosomes, according to classical chromosome banding techniques (Dutrillaux, 1979; Yunis and Prakash, 1982). The primate chromosome assignments were confirmed by *in situ* hybridization of human chromosome-specific DNA libraries to primate chromosomes (Wienberg *et al.*, 1990, 1992; Jauch *et al.*, 1992).

The cosmid probes for parts of the human muscular dystrophy gene mapped to a band homologous to human Xp21 in all great apes. Figure 1A shows the hybridization pattern of the DMD probe on the chromosomes of a female gorilla. This assignment was confirmed by a cohybridization experiment with a human chromosome X-specific DNA library and the cosmid clone specific for the dystrophin gene (not shown).

The hybridization signal of a pool of three overlapping phage clones specific for the c-myc oncogene was found in a band homologous to human chromosome 8q24 in all great apes (not shown). This approach to evolutionary gene mapping is sensitive enough to visualize even low-complexity probes. This is exemplified by the plasmid clone for the human thyroglobulin gene, which contains only 7 kb of DNA insert. Figure 1B shows this probe hybridizing to gorilla chromosomes.

The human chromosome 21-specific cosmids hybridized to the band on great ape chromosome 22, which is assumed to be homologous to human 21q22. The subtelo-meric localization of these probes in the gorilla homolog (data not shown) is due to terminal C-bands (Wienberg

et al., 1990). One of the orangutan specimens examined came from an individual that revealed clinical characteristics comparable to Down syndrome in humans. In this case three hybridization signals were observed on small acrocentric chromosomes (Fig. 1C), identified as orangutan chromosome 22 by DAPI-banding.

FISH of Human DNA Clones to Chromosomes of Lesser Apes

In *H. lar* and *H. syndactylus* metaphase chromosomes, the hybridization signal of the DNA probe for the human muscular dystrophy gene is found on the X chromosome. Except for additional telomeric C-banding in *H. syndactylus*, the gibbon X chromosome is similar to that found in humans and great apes. The probe can be assigned to the short arm of this particular chromosome as shown by "chromosome painting" and simultaneous *in situ* hybridization banding with *Alu*-PCR products. Figure 1D shows the chromosome painting of the gibbon X chromosome with the human X chromosome library composite probe and the cosmid cpt1.

The autosomal chromosomes of the gibbon, however, do not show any homology in the chromosome banding pattern to human and great ape chromosomes. The cosmid probes specific for the human Down syndrome region are found on the long arm of *H. lar* chromosome 15 close to the telomere (according to the idiogram published by Jauch *et al.*, 1992). In an additional experiment, we cohybridized both the chromosome 21 cosmids and a human chromosome 15-specific DNA library. The results show that the gibbon chromosome 15 is composed of segments that are homologous to both human chromosomes 15 and 21 (Fig. 1E).

FISH of Human DNA Clones to Chromosomes of Old World Monkeys

The human chromosome 21-specific cosmids were hybridized to macaque chromosomes (*Macaca fuscata*). The hybridization signal was found on the short arm of macaque chromosome 2 (not shown), according to the idiogram published by Small *et al.* (1985). The hybridization signal intensity on the macaque chromosomes was as strong as that observed on human metaphase spreads,

suggesting that a high degree of sequence conservation is retained among primates in spite of the chromosomal rearrangements (Wienberg *et al.*, 1992). In contrast, the chromosomal map position of two cosmids derived from the human muscular dystrophy gene remained on the X chromosome in the African green monkey and was assigned to the short arm of the X chromosome by chromosome painting. These cosmids are known to be about 800 kb apart from each other in the human. In about 50% of the green monkey metaphase spreads four independent hybridization signals were observed (Fig. 1F).

DISCUSSION

The present work shows that FISH with plasmid, phage, or cosmid clones of defined human genes can be used as a simple tool for high-resolution comparative mapping of primate chromosomes. Chromosomes or chromosome segments that were rearranged can be readily identified. This is of particular practical significance for the molecular analysis of chromosomes from the lesser apes, which have experienced massive chromosome rearrangements during evolution (Van Tuinen and Ledbetter, 1983; Stanyon *et al.*, 1987; Jauch *et al.*, 1992).

In all species analyzed the probe for the human muscular dystrophy gene mapped to the short arm of the X chromosome. This is in agreement with comparative banding analysis and gene mapping data of other sex-linked genes (O'Brien *et al.*, 1992) and further corroborates Ohno's hypothesis that this chromosome has been highly conserved during mammalian evolution (Ohno, 1973).

The probes specific for the *c-myc* oncogene and the human thyroglobulin gene both mapped to the assumed homolog of the human band 8q24 in great apes. The conservation of this band has been demonstrated even in lesser apes and macaques by comparative band mapping using a micro-DNA library established from the human band 8q24 after microdissection (Lengauer *et al.*, 1991). This microlibrary was shown to map to the long arm of gibbon chromosome 9 and to chromosome 8 in the macaque karyotype. These results suggest that the synteny of the bulk sequences of human chromosome band 8q24 has been conserved for more than 20 million years. The present data prove that this is also true for specific genes originating from the 8q24 band.

Great apes that show trisomy for chromosome 22 exhibit phenotypic features similar to those of humans with Down syndrome (McClure *et al.*, 1969; Andrieu *et al.*, 1979; de Boer *et al.*, 1982). Indeed, human chromosome 21-specific cosmids, derived from 21q22 (the region implicated in the Down syndrome phenotype), hybridize to chromosome 22 of the great apes. The same probes revealed three specific hybridization signals on an orangutan metaphase chromosome preparation derived from an individual showing clinical features of human Down syndrome. Therefore, these hybridization experiments demonstrate, on the molecular level, that Down syn-

drome in human and that in great ape are most likely homologous.

Recent *in situ* hybridization experiments with whole chromosome libraries have revealed various rearrangements in lesser apes. The 22 autosomal human chromosome complements have been divided into 52 segments composing the 21 autosomal chromosomes of *H. lar* (Wienberg *et al.*, 1990; Jauch *et al.*, 1992). The segments were easily identified with the painting approach. However, chromosome-specific libraries provide information of regional homologies only and provide a limited resolution with no information with respect to gene order. The complete reconstruction of the karyotype is further complicated, since the patterns of only a few chromosome bands are impossible to compare between species. Subregional, gene-specific probes like the one used in the present experiments provide additional information about homologies, as shown with the human chromosome 21-specific cosmid probes. The assignment of human chromosome 21 probes to the gibbon chromosome 15 is in agreement with previous *in situ* hybridizations using human specific painting probes (Jauch *et al.*, 1992). Here we present evidence that the band homologous to human 21q22 is the terminal light band in gibbon chromosome 15. We expect that subregional, locus-specific DNA probes will finally clarify chromosome shuffling in lesser apes at a high-resolution level.

FISH of the human chromosome 21-specific cosmids gave strong signals on the macaque chromosome 2, with no detectable decrease in signal intensity compared to hybridizations to chromosomes of great apes. Again, this map position is in agreement with recent results obtained with chromosome painting of macaque chromosomes with the human chromosome 21-specific DNA library (Wienberg *et al.*, 1992). The spatial resolution of two hybridized cosmids from the human dystrophin gene to the African green monkey X chromosomes revealed a resolution close to that found in *in situ* hybridization experiments with human chromosomes as a target. Lawrence *et al.* (1990) were able to resolve probes from the same gene, which were separated by about 500 kb. As in the present experiment, the signals were not separated along the length of the chromosome, but revealed four distinct spots almost perpendicular to the chromosome axis. The similarity in the hybridization pattern suggests that the dystrophin gene in African green monkeys should have a length comparable to that of the homologous gene in humans.

In this paper we demonstrate that FISH can detect chromosomal rearrangements in the karyotypes of primates at the molecular cytogenetic level and with high-resolution using human DNA probes. As the progress of the human genome project continues, an increasing number of DNA probes will become available for comparative gene mapping studies of any region of interest. We expect that comparative mapping by FISH of both chromosome region- and gene-specific DNA probes will allow a detailed description of rearrangements and therefore will contribute considerably to the understanding of primate phylogeny and evolution.

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