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RESEARCH ARTICLE

Breakpoint Analysis of the Pericentric Inversion Distinguishing Human Chromosome 4 From the Homologous Chromosome in the Chimpanzee (*Pan troglodytes*)

Hildegard Kehrer-Sawatzki,[1*] Catharina Sandig,[1] Nadia Chuzhanova,[2,3] Violaine Goidts,[1] Justyna M. Szamalek,[1] Simone Tänzer,[4] Stefan Müller,[5] Matthias Platzer,[4] David N. Cooper,[2] and Horst Hameister[1]

[1]Department of Human Genetics, University of Ulm, Ulm, Germany; [2]Institute of Medical Genetics, University of Wales College of Medicine, Cardiff, United Kingdom; [3]Biostatistics and Bioinformatics Unit, University of Wales College of Medicine, Cardiff, United Kingdom; [4]Department of Genome Analysis, Institute of Molecular Biotechnology, Jena, Germany; [5]Institute of Anthropology and Human Genetics, University of Munich (LMU), Munich, Germany

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The study of breakpoints that occurred during primate evolution promises to yield valuable insights into the mechanisms underlying chromosome rearrangements in both evolution and pathology. Karyotypic differences between humans and chimpanzees include nine pericentric inversions, which may have potentiated the parapatric speciation of hominids and chimpanzees 5–6 million years ago. Detailed analysis of the respective chromosomal breakpoints is a prerequisite for any assessment of the genetic consequences of these inversions. The breakpoints of the inversion that distinguishes human chromosome 4 (*HSA*4) from its chimpanzee counterpart were identified by fluorescence in situ hybridization (FISH) and comparative sequence analysis. These breakpoints, at *HSA*4p14 and 4q21.3, do not disrupt the protein coding region of a gene, although they occur in regions with an abundance of LINE and LTR-elements. At 30 kb proximal to the breakpoint in 4q21.3, we identified an as yet unannotated gene, *C4orf12*, that lacks an homologous counterpart in rodents and is expressed at a 33-fold higher level in human fibroblasts as compared to chimpanzee. Seven out of 11 genes that mapped to the breakpoint regions have been previously analyzed using oligonucleotide-microarrays. One of these genes, *WDFY3*, exhibits a three-fold difference in expression between human and chimpanzee. To investigate whether the genomic architecture might have facilitated the inversion, comparative sequence analysis was used to identify an ~5-kb inverted repeat in the breakpoint regions. This inverted repeat is inexact and comprises six subrepeats with 78 to 98% complementarity. (TA)-rich repeats were also noted at the breakpoints. These findings imply that genomic architecture, and specifically high-copy repetitive elements, may have made a significant contribution to hominoid karyotype evolution, predisposing specific genomic regions to rearrangements. Hum Mutat 25:45–55, 2005. **© 2004 Wiley-Liss, Inc.**

KEY WORDS: pericentric inversion; breakpoints; human-chimpanzee; chimpanzee; *C4orf12; WDFY3*

INTRODUCTION

The comparison of the human and chimpanzee genomes promises to yield new insights into the molecular evolution of Hominoidea, and at the same time, potentiating advances in molecular medicine [Olson and Varki, 2003]. The DNA sequence divergence between humans and chimpanzees is only 1.24% if single nucleotide differences are considered [Chen and Li, 2001; Ebersberger et al., 2002; Fujiyama et al., 2002]. However, a variety of sequence rearrangements, both large and small, also contribute to the genomic divergence between the two species [Britten, 2002; Wildman, 2002; Liu et al., 2003; Locke et al., 2003a; Frazer et al., 2003]. Comparisons of the human and chimpanzee karyotypes have yielded to date a total of one fusion and nine pericentric inversions affecting chromosomes 1, 4, 5, 9, 12, 15, 16, 17, and 18 [Yunis and Prakash, 1982]. Since the majority of these inversions occurred in the chimpanzee lineage, it is presumed that the corresponding human and orangutan chromosomes broadly resemble those of the putative ancestral hominoid karyotype [Dutrillaux, 1979; Yunis and Prakash, 1982; Müller and Wienberg, 2001].

The Supplementary Material referred to in this article can be viewed at http://www.interscience.wiley.com/jpages/1059-7794/suppmat

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*Correspondence to: Dr. Hildegard Kehrer-Sawatzki, Department of Human Genetics, University of Ulm, Albert-Einstein-Allee 11, 89081 Ulm, Germany.
E-mail: hildegard.kehrer-sawatzki@medizin.uni-ulm.de

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While pericentric inversions are preponderant in the evolution of Hominidae and Pongidae, gibbon chromosomes are characterized by numerous translocations and fissions are quite frequent in the evolution of the Cercopithecidae [reviewed by Dutrillaux, 1979].

Several hypotheses have been put forward regarding the biological consequences of the pericentric inversions and their potential contribution to speciation in early hominids and chimpanzees [King and Wilson, 1975; Hey, 2003]. The inversions might either have disrupted or otherwise changed the structure of genes at the breakpoints or could instead have altered gene expression by modifying the local genomic environment. Chromosomal rearrangements including pericentric inversions have been proposed to constitute genetic barriers to gene flow between subpopulations, possibly by suppressing recombination [King, 1993; Rieseberg, 2001]. Navarro and Barton [2003] have recently shown, by comparing the ratio of nonsynonymous to synonymous substitutions of human and chimpanzee genes, that evolutionary changes occurred twice as fast in chromosomes that had undergone a gross rearrangement than in those that had remained colinear. It may be that the higher rate of amino acid substitutions manifested by rearranged chromosomes is a consequence of reduced gene flow between chromosomes heterozygous for the rearrangement.

A first step toward investigating the potential biological consequences of pericentric inversions is to characterize their breakpoints at the molecular level. In this study, we have analyzed the breakpoints of the pericentric inversion that distinguishes *Pan troglodytes* chromosome 3 (*PTR*3) from *Homo sapiens* chromosome 4 (*HSA*4). Previously, breakpoint-spanning YACs were identified [Nickerson and Nelson, 1998; Marzella et al., 2000], but the breakpoint regions were not sequenced. We therefore isolated breakpoint-spanning restriction fragments from chimpanzee BAC clones and compared their sequences with their human counterparts. The aim was both to determine whether we could identify genomic architectural features that might have mediated the inversion, and to ascertain whether the expression of specific genes in the vicinity might have been directly affected by the rearrangement. Further, we investigated whether the inversion breakpoints were involved in chromosomal rearrangements in other primate species.

MATERIALS AND METHODS

Filter Libraries and Southern Blot Hybridizations

The RPCI-43 male chimpanzee BAC library and BAC clones were obtained from the BACPAC Resource Center (www.chori.org/bacpac). For Southern blot analysis, BAC DNA was digested with restriction endonucleases, electrophoresed on 0.8% agarose gels, and transferred to nylon membranes (Amersham Pharmacia, Freiburg, Germany). BAC-filters and Southern blot membranes were hybridized in buffer containing 7% SDS, 0.5 M Na phosphate (pH 7.2), and 1 mM EDTA at 65°C overnight. For filter and Southern blot hybridizations, PCR products were labeled by random priming with hexamer-oligonucleotides and Klenow polymerase in the presence of ^{32}P-dCTP (Amersham Pharmacia, Freiburg, Germany). Zoo-blot hybridizations were performed with a ready-made zoo-blot of *Eco*RI-digested DNA (BioCat, Heidelberg, Germany). This blot was hybridized overnight at 56°C under low-stringency conditions with ^{32}P-dCTP-labeled PCR product cs3/4 amplified from human genomic DNA with primers cs3 and cs4 as listed in Supplementary Table S3 (available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat).

Cell Lines and Fluorescence In Situ Hybridization

The *Pan troglodytes* lymphoblastoid cell line *PTR*-EB176 (ECACC No. 89072704), the *Pan troglodytes* primary fibroblast culture CP132 (*PTR*, ECACC No. 91012416), the orangutan (*Pongo pygmaeus*) lymphoblastoid cell line *PPY*-EB185 (ECACC No. 8907275), and the lymphoblastoid cell line EB(JC) (ECACC No. 89072703) from *Gorilla gorilla* were purchased from the European Collection of Cell Cultures (www.ecacc.org.uk). The *Pan troglodytes* lymphoblastoid cell line *PTR*-L2008 was a generous gift from Dr. W. Schempp, Freiburg, Germany. The fibroblast cell line GM03446 of *Macaca fascicularis* (crab-eating macaque) was obtained from the Coriell Cell Repository (Camden, NJ). For expression analysis, two foreskin fibroblast cell lines and four Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines from human donors were used. The siamang gibbon lymphoblastoid line [*Symphalangus (Hylobates) syndactylus*], the lymphoblastoid cell line for the white-cheeked gibbon [*Nomascus (Hylobates) leucogenys*], and the white-handed gibbon (*Hylobates lar*) cell line HY35 were obtained or established as previously described [Kehrer-Sawatzki et al., 2002]. BAC or PAC DNA and PCR products used as fluorescence in situ hybridization (FISH) probes were labeled either with biotin-16-dUTP (Roche-Diagnostics, Mannheim, Germany) and detected with fluorescein isothiocyanate (FITC)-avidin and biotinylated anti-avidin (Vector, Burlingame, VT) or labeled with digoxygenin-11-dUTP (Roche-Diagnostics, Mannheim, Germany) and detected by mouse anti-digoxygenin coupled with Texas-Red and in a second step with anti-mouse antibody conjugated with Texas-Red (Dianova, Hamburg, Germany). Slides were counterstained with diamidinophenylindole (DAPI) and mounted with Vectashield antifade solution (Vector, Burlingame, VT).

Sequencing and Analysis of Breakpoint-Spanning BAC and PAC Clones

The breakpoint-spanning 4.3-kb *Pst*I-fragment of *PTR* BAC RP43-41D24 and the 6.8-kb *Pvu*II-fragment of BAC RP43-59P20 were cloned into pUC18 and the sequences determined from both ends. To complete their sequences, plasmids were fragmented by nebulization. DNA ends of the resulting fragments were polished by Klenow polymerase and T4 polynucleotide-kinase. Fragments were then ligated into pUC18 and sequenced using the ABI Prism Big Dye terminator kit and ABI Prism 377 and 3700 DNA sequencers (Applied Biosystems, www.appliedbiosytems.com). Sequence assembly and manual editing were performed with GAP4 software [Dear and Staden, 1991]. Alignments and homology searches were performed using the Wisconsin Package Version 10.2, Genetics Computer Group (GCG, Madison, WI) and the BLAST program at the NCBI server (www.ncbi.nlm.nih.gov). Sequence comparisons using the FASTA program of the GCG software package were performed to search for topoisomerase-II bindings sites in the breakpoint regions. Inverted repeats were sought using complexity analysis [Gusev et al., 1999] while

FIGURE 2. **Schematic representation of the pericentric inversion and the position of the inversion breakpoint-spanning BACs from human chromosome 4 (*HSA*4), BACs RP11-779N22 (AC110811.3), RP11-8N8 (AC108021.3), and those from chimpanzee (*Pan troglodytes*) chromosome 3 (*PTR*3), and BACs RP43-59P20 (AG185792) and RP43-41D24. IBF3p (AY335550.1) and IBF3q (AY335551.1) are the inversion breakpoint-spanning fragments on *PTR*3p and *PTR*3q, respectively. Schematic of the inversion breakpoint-spanning fragments IBF3p and IBF3q. In red are the regions homologous to BAC RP11-779N22 and in blue, those with homology to BAC RP11-8N8.**

repetitive elements were identified by *Repeatmasker* (www.repeatmasker.genome.washington.edu/cgi-bin/RepeatMasker).

Expression Analysis

Cytoplasmic RNA of lymphoblastoid and fibroblast cultures of chimpanzees and humans was isolated using the RNeasy kit (Qiagen, Hilden, Germany). First strand cDNA was prepared with 2 μg total RNA (as determined by absorbance), random hexamers, and the SuperScript Preamplification System (Invitrogen, Karlsruhe, Germany). RT-PCR analysis to confirm the structure of the *C4orf12* gene was performed with cDNA transcribed from human skeletal muscle-, placenta-, and brain-derived RNA (BD Biosciences, Heidelberg, Germany) with primer sequences available from the authors on request. Northern blot analysis was performed with the human MTN-Blots (7760-1, 7755-1, and 7793-1; BD Biosciences) using PCR product hs53/54 as probe, labeled by random priming with hexamer-oligonucleotides and Klenow-polymerase in the presence of ^{32}P-dCTP (Amersham Pharmacia, Freiburg, Germany). Product hs53/54 was amplified from human cDNA with primers hs53 (5′-GCTGAGAAAGGACAACTGGG-3′) and hs54 (5′-GACTTTTCTGTGCACTCCTGG-3′) and spans exons 1–3 of the *C4orf12* gene.

Real-time RT-PCR was performed using the DNA-binding dye SYBR green on a Lightcycler with the Quantitect SYBR Green PCR kit (Qiagen). We used relative quantification to normalize the target genes (*WDFY* and *C4orf12*) to an internal standard (hypoxanthine guanine phosphoribosyl transferase, *HPRT*). To determine the mRNA expression, a standard curve for each of the two fragments was generated. The change in gene expression was given as the ratio of the target gene to *HPRT* expression. Primers used for this analysis were hs61 (5′-GATGACACAGCAAGAGG-CAA-3′) and hs62 (5′-CTGCCCTACCGATCAACATT-3′) spanning exons 4–6 of *C4orf12*, 5′-AGGCCACAATCATTGCAGA-3′ and 5′-CAAGACGTTTAGAAACCTGGCTA-3′ for *WDFY*, and 5′-GCTGGTGAAAAGGACCTCT-3′ and 5′-CACAGGACTA-GAACACCTGC-3′ for *HPRT.*

RESULTS

Identification of BAC Clones Spanning the Inversion Breakpoints

FISH analysis was used to identify *HSA* BAC RP11-8N8, which spans the inversion breakpoint at 4q21.3 (Fig. 1A). A split FISH signal from this BAC was observed on the homologous chromosome 3 in the chimpanzee (Fig. 1B). *HSA* BAC RP11-779N22 spans the breakpoint at 4p14 and hybridizes to *PTR*3p and 3q (Fig. 1C, D). BLAST analysis with the sequence of *HSA* BAC RP11-779N22 indicated a high degree of homology with *PTR* BAC

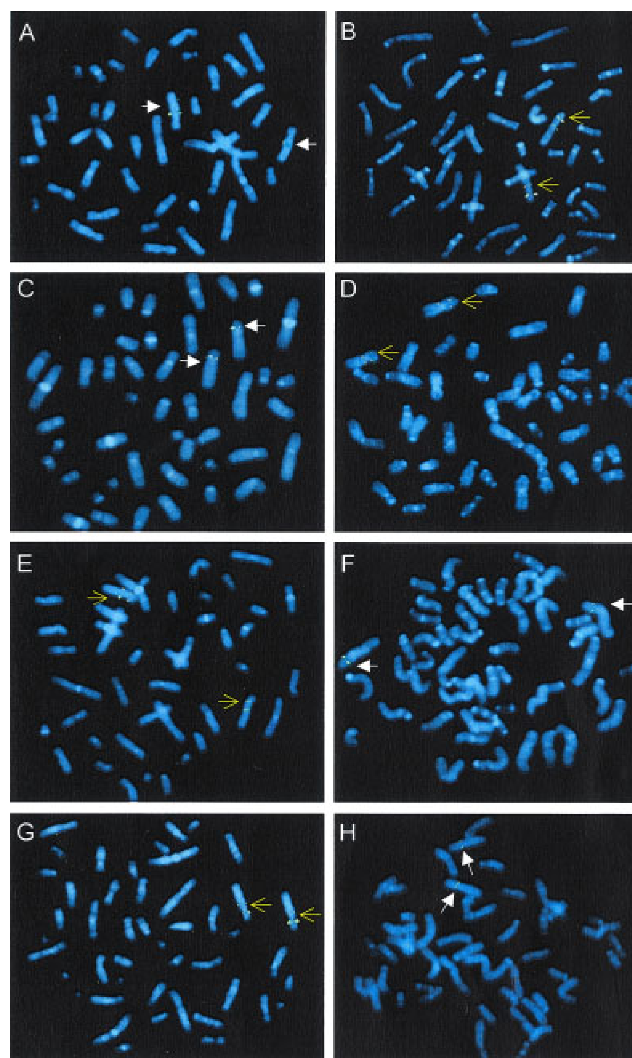
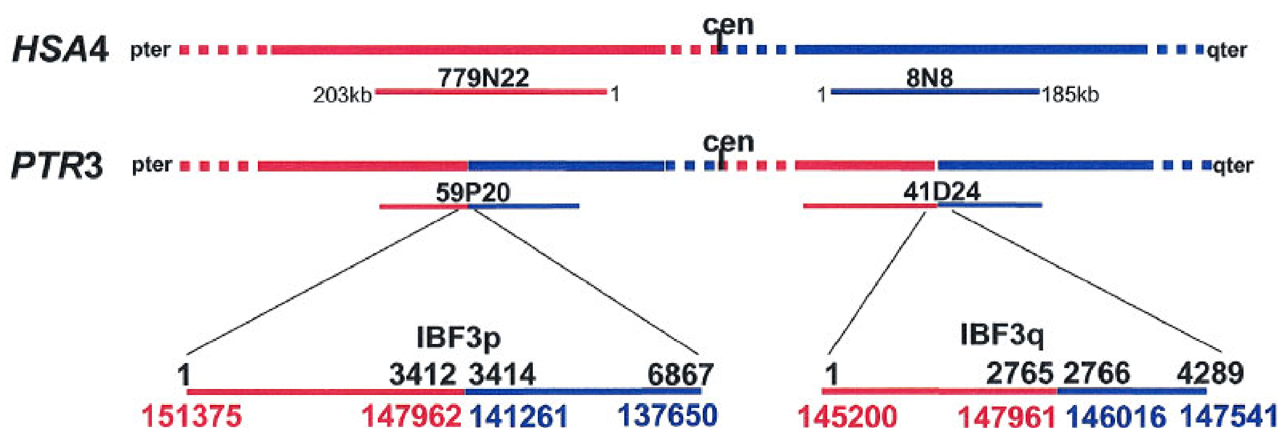


FIGURE 1. **FISH analysis of biotin-labeled BACs RP11-8N8 (A,B), RP11-779N22 (C,D), RP43-59P20 (E,F), and RP43-41D24 (G,H) that span the breakpoints of the pericentric inversion. A,C,E,G: hybridizations to human metaphase chromosomes. B,D,F,H: hybridizations to *PTR* metaphase chromosomes. Chromosomes with single hybridization signals are denoted by white arrows, split hybridization signals are indicated by yellow arrows.**



RP43-59P20. FISH analysis revealed that BAC RP43-59P20 spans the inversion breakpoint in *PTR*3p. Although a single FISH signal of BAC RP43-59P20 was seen on *PTR*3p (Fig. 1F), split signals were observed at *HSA*4q21.3 and 4p14 (Fig. 1E). To identify a chimpanzee BAC covering the breakpoint in *PTR*3q, the RP43 library was screened with labeled PCR product hs3/4 (Supplementary Table S1). In this way, breakpoint-spanning chimpanzee BACs RP43-41D24 and 16N8 were identified that hybridize to *PTR*3q (Fig. 1H), as well as to *HSA*4q21.3 and to 4p14 (Fig. 1G).

Refinement of the Breakpoint Regions by PCR

To determine the extent of sequence overlap between chimpanzee and human breakpoint-spanning BACs, PCR was performed with the primers listed in Supplementary Table S1 and designed by reference to the human BACs. The breakpoint in *HSA*4p14 was assigned to the region between positions 140061 and 149146 of *HSA* BAC RP11-779N22 (according to AC110811.3).

PCR analysis was also employed to refine the position of the breakpoint region in *HSA*4q21.3. Primers used in these experiments are listed in Supplementary Table S2. *PTR* BAC RP43-41D24 proved positive for the PCR products hs49/50 and hs77/78, indicating that the breakpoint was proximal to position 147065 of *HSA* BAC RP11-8N8 (AC108021.3). PCR products hs7/8 to hs5/6 were amplified from *PTR* BAC RP43-59P20 (Supplementary Table S2). It was therefore inferred that the breakpoint must be distal to position 139989 of *HSA* BAC RP11-8N8. To further narrow down the breakpoint region, PCR was performed with primers hs51/52, but neither the *PTR* BACs nor DNA isolated from chimpanzee cell lines were positive (results not shown). PCR 51/52, however, was positive using genomic DNA from gorilla, orangutan, and macaque as template. The deletional loss of the corresponding region in the chimpanzee genome was confirmed by Southern blot analysis with PCR product hs51/52 as a labeled probe. The deletion is thus specific to the chimpanzee.

Isolation of Inversion Breakpoint-Spanning Restriction Fragments

To isolate an inversion breakpoint-spanning restriction fragment (IBF) from *PTR* BAC RP43-41D24 (IBF3q), Southern blot hybridizations were performed with PCR product hs77/78 as labeled probe. This probe detects a 7.3-kb *Pst*I fragment of *HSA* BAC RP11-8N8, and a 4.3-kb fragment of *PTR* BAC 41D24 (IBF3q), which was cloned and sequenced.

The fragment spanning the breakpoint in *PTR*3p (IBF3p) was identified by Southern hybridization with probe hs73/74 (Supplementary Table S1), which detects a 4.9-kb *Pvu*II fragment of *HSA* BAC RP11-779N22, and the 6.9-kb IBF3p of *PTR* BAC RP43-59P20. Sequence comparisons between the IBFs and the human BACs then permitted the identification of the inversion breakpoints.

Sequence Alignments and Structure of the IBFs at *PTR*3p and *PTR*3q

IBF3q (AY335551.1) is homologous to human BAC RP11-779N22 (position 145200–147961 according to AC110811.3) (Fig. 2). From position 2766 up to the end of the sequence, IBF3q matches *HSA* BAC 8N8 (146016 to 147541). IBF3p (AY335550.1) exhibits homology to *HSA* BAC RP11-779N22 (positions 151375 to 147962) and to *HSA* BAC RP11-8N8 from positions 141261 to 137650. Thus, the region orthologous to 141262–146015 of *HSA* BAC RP11-8N8 is absent in chimpanzee, a finding which explains the failure to amplify PCR product hs51/52 from chimpanzee genomic DNA (Supplementary Table S2).

We then compared the sequence of the breakpoint-spanning fragments to the Chimpanzee Genome Draft Sequence (December 2003 release; www.ensembl.org) using the Ensembl browser. IBF3q corresponded exactly to scaffold 37561 and IBF3p to scaffold 37596. These comparisons further served to confirm the chimpanzee-specific 4.7-kb deletion, which leads to the loss of mainly repetitive sequences (as determined by Repeatmasker analysis based upon the human sequence). Owing to the occurrence of this deletion, the precise breakpoint of the inversion in *HSA*4q21.3 cannot be determined. Repeatmasker analysis also indicated that the inversion breakpoints occurred in regions rich in LINE elements. Indeed, some 50% of an ∼18-kb region around the 4p14 breakpoint consists of LINEs (Table 1). The median LINE density in both breakpoint regions is 42.15%. In contrast, the LINE density in eight randomly chosen 18-kb segments on chromosome 4 amounted to 9.55% (Supplementary Table S4).

We then screened the breakpoint regions for residual evidence of the features in the human genome that might once have facilitated the inversion in the chimpanzee lineage. An inexact inverted repeat of ∼5-kb was identified at *HSA*4p14 and *HSA*4q21.3. This repeat is made up of six subrepeats with complementarity ranging from 75 to 98% (Fig. 3). The breakpoint in *HSA*4p14 occurred within a $(TA)_n$-repeat that would have

TABLE 1. **Repeat Composition of 18-kb Around the Inversion Breakpoint Regions on Human Chromosomes 4 (*HSA*4) and Chromosome 17 (*HSA*17) as Determined by Repeatmasker Analysis**

	Repeat density (%) of the breakpoint regions in			
	***HSA*17p**	***HSA*17q**	***HSA*4p**	***HSA*4q**
SINEs	22.1	21.4	5.9	9.2
LINEs	11.4	19.1	50.2	34.1
LTR elements	6.9	5.3	17.1	18
DNA elements of MER1 and 2 type	6	11.6	0	0
Total interspersed repeats	46	57.2	73.2	61.8
%G+C	47.8	48.8	37.7	38.7

FIGURE 4. **Schematic diagram indicating the positions of genes flanking the inversion breakpoints on chromosome 4p14 (A; in red) and 4q21.3 (B; in blue). The position of the inversion breakpoint in *HSA*4p14 is marked by a vertical dotted lines while the transcriptional orientation of the respective genes is indicated by horizontal arrows. The map positions of the BACs indicated are depicted as horizontal bars. The deduced orientation and position of genes at the breakpoint in *PTR*3p (C) and *PTR*3q (D) are given. The GenBank accession numbers of the respective genes are: *FLJ39576* (AK096895.1), *FLJ13220* (NM_021927.1), *GNPDA2* (NM_138335.1), *GABRA2* (NM_000807.1), *GABRG1* (NM_173536.1), *NKX6-1* (NM_006168.1), *CDS1* (NM_001263.2), *WDFY3* (NM_014991.3), *C4orf12* (AY250185.1), *ARHGAP24* (NM_031305.1), and *MAPK10* (NM_002753.2). Genes marked in yellow are not differentially expressed in human and chimpanzees (data from Enard et al. [2002] and Karaman et al. [2003]).**

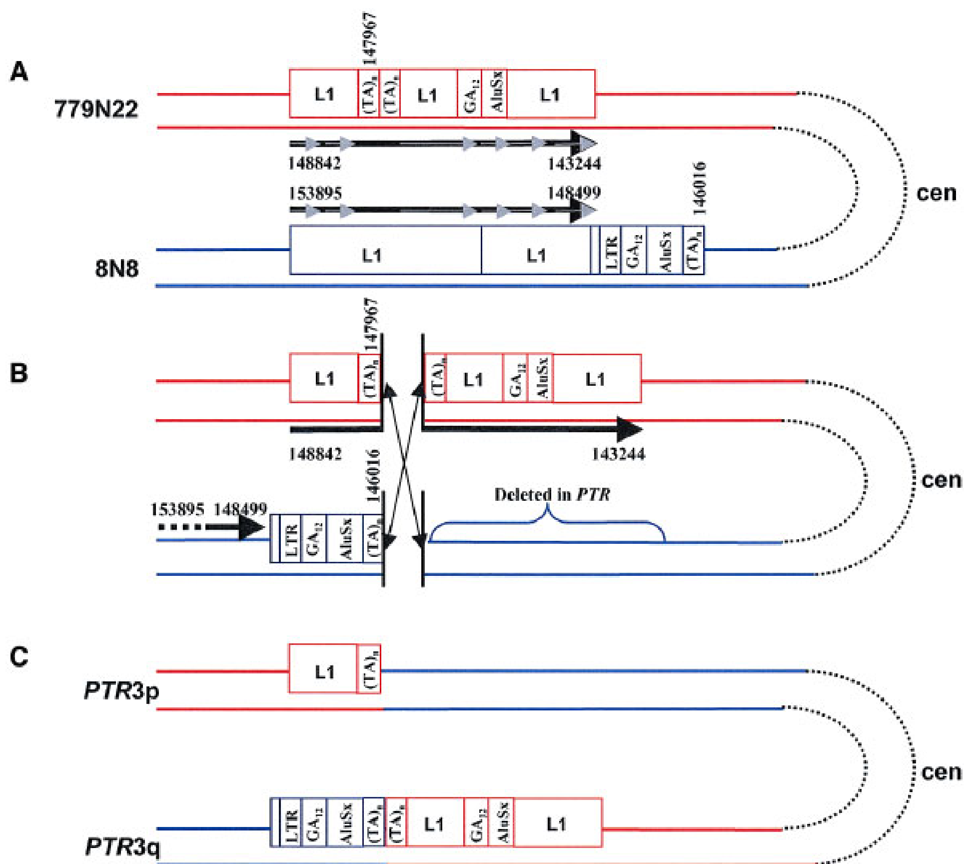
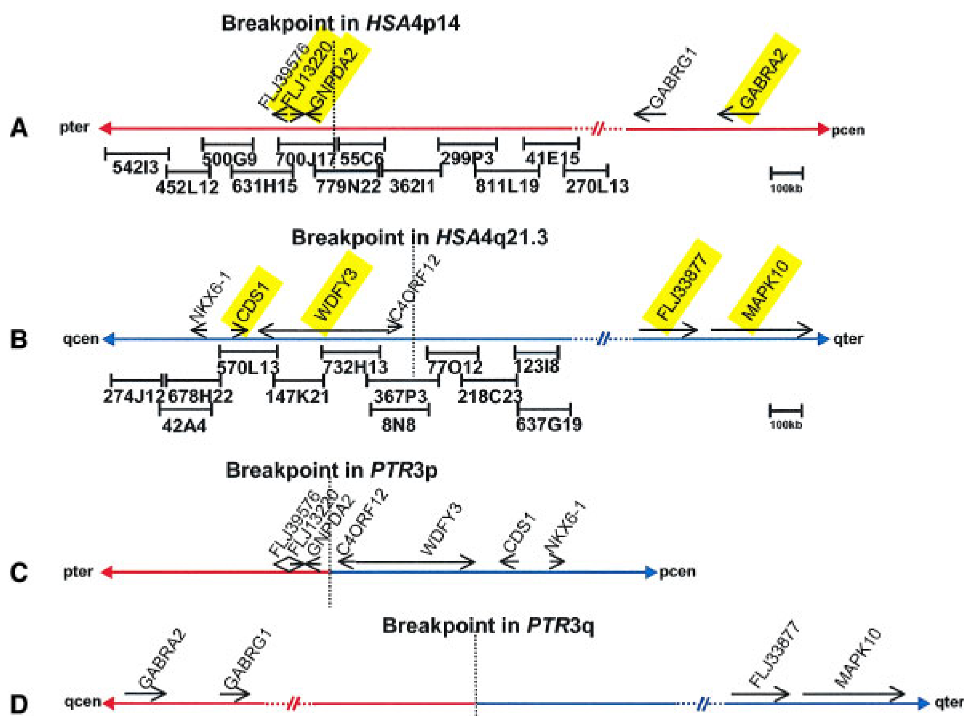


FIGURE 3. Schema proposed to explain the formation of the pericentric inversion by intrachromosomal misalignment between the inexact inverted repeats in 4p14 (red) and 4q21.3 (blue). **A:** The inverted repeats of ~5-kb are composed of six subrepeats indicated by gray arrows. Numerals denote the basepair positions according to the respective BAC sequences. **B:** In 4p14, the breakpoint occurred within the 5-kb imperfect repeat within a (TA)-rich segment. In 4q21, the suggested breakage and fusion site maps 2.5-kb proximal to the inverted repeat. **C:** Resulting *PTR*3 chromosome with pericentric inversion. The 4.7-kb deletion may have occurred contemporaneously with the inversion but could also have occurred independently.



been split by the inversion. Directly at this breakpoint, and within the $(TA)_n$-repeat, is a 16-bp segment that is palindromic to a segment located 8-bp distally. No evidence was found, however, for an unusually high density of topoisomerase binding sites that might have been suggestive either of the presence of matrix-attachment regions or of the involvement of topoisomerase II in mediating the breaks underlying the inversion.

Genes Flanking the Inversion Breakpoints in the Human and Chimpanzee Genomes

According to BLAST and EST analysis of the breakpoint regions, the *GNPDA2* (glucosamine-6-phosphate deaminase 2) gene maps ~83-kb distal to the breakpoint in *HSA*4p14 (Fig. 4A). Proximal to this breakpoint, at a distance of ~1.3-Mb, lies the *GABRG1* (gamma-aminobutyric acid receptor A, gamma 1) gene.

The breakpoint in *HSA*4q21.3 also fails to interrupt the protein coding region of a gene. Thus, the *WDFY3* (WD repeat and FYVE domain-containing protein 3) gene is found ~50-kb proximal to the breakpoint, while the *FLJ33877* (GenBank Accession No. AK091196.1) gene, encoding a putative GTPase-activating protein, maps 1.4-Mb distal to the breakpoint (Fig. 4B). About 30-kb proximal to the breakpoint, we identified an hitherto unannotated gene, *C4orf12* (chromosome 4 open reading frame 12) and determined its structure and expression profile by EST-PCR, RT-PCR, and Northern blot analysis (AY250185.1). A 7.3-kb mRNA transcript of *C4orf12* was detected in human skeletal muscle, a 4.3-kb transcript in several human brain regions (Fig. 5), and a 1.8-kb transcript in human pancreas. The size and structure of these transcripts are explicable in terms of a combination of alternative splicing and the usage of alternative polyadenylation sites. *C4orf12* comprises six exons that are encompassed by the IMAGE cDNA clone 3502957 (BC019264.1) (Fig. 6). Sequence comparisons with IMAGE clone 3351252 (BC033127.1) indicated that exons 3 and 5 are alternatively spliced. The *C4orf12* sequence may encode at the most, a short protein of 83 amino acids of unknown function. Similarities to other known genes or proteins were not detected. Comparison with the Chimpanzee Genome Draft Sequence (December 2003 release) and PCR analysis with primers designed by reference to the human sequence (Supplementary Table S3), suggest that the *C4orf12* gene is highly conserved between human and chimpanzee. Although the overall divergence between human and chimpanzee *C4orf12* sequences is 1.6%, no nucleotide substitutions were apparent within the open reading frame.

The *C4orf12* and *WDFY3* genes are localized head-to-head, separated by a bidirectional promoter. As is commonly observed for bidirectional promoters, a CpG-island of 1,720-bp, with a GC-content of 69%, overlapped with the first exons of both genes (Fig. 6A).

Assessment of the evolutionary conservation of the *C4orf12* gene was performed by Zoo-blot analysis; hybridization signals were detected in DNA of cow and dog, but not in mouse, rat, zebrafish, *Xenopus*, *C. elegans*, and yeast (data not shown). Further, BLAST analysis of the human *C4orf12* cDNA sequence against the genomic sequence of chicken, *Fugu*, zebrafish, mouse, and rat confirmed that the *C4orf12* gene does not possess an ortholog in these species. On mouse chromosome 5, however, in a region orthologous to human chromosome 4q21.3, five fragments with 59 to 81% identity to the human *C4orf12* gene were identified by unmodified BLAST analysis (Fig. 6B). The lack of any mouse or rat EST matching *C4orf12* further implies that *C4orf12* does not have an orthologous counterpart in Muridae. By contrast, *WDFY3*, located close to *C4orf12*, is highly conserved in the mouse (NM_172882.2).

The expression of *C4orf12* was investigated by real-time PCR comparison of human and chimpanzee fibroblast cell lines. A 33-fold higher level of expression of *C4orf12* was observed in human as compared to chimpanzee. In EBV-transformed lymphoblastoid cell lines, *C4orf12* was only very weakly expressed, so that these cell lines could not be used for comparative expression analysis. The *WDFY* gene was expressed at a three-fold higher level in human fibroblast cell lines as compared to chimpanzee fibroblast cells as determined by real-time PCR.

The pericentric inversion of *PTR3* served to place the 3′ end of the *C4orf12* gene proximal to the *GNPDA2* gene (Fig. 4C). As deduced from the available human genome sequence, and confirmed by the draft sequence of the chimpanzee genome, the inversion breakpoint in *PTR*3q is located in a region of more than 2-Mb that is devoid of any obvious identifiable gene (Fig. 4D).

GC-Content of the Inversion Breakpoint Regions on *HSA*4p14 and 4q21.3

The genome of warm-blooded vertebrates is composed of long stretches of DNA homogeneous in GC-content called isochores. According to their density, five classes of isochores have been defined: two light ones, L1 and L2, and three heavy ones, H1, H2, and H3, with GC contents of ~39, 41, 45, 49, and 53%, respectively [Bernardi, 2000]. The GC content of the ~600-kb region flanking the inversion breakpoints in *HSA*4p14 and *HSA*4q21.3 is relatively homogeneous with average values of 36 and 37.5%, respectively, indicative of their location in similar light isochores.

FISH Analysis of Breakpoint-Spanning BACs in Other Primate Species

To investigate whether one of the breakpoints described here may have been involved in more than one chromosomal rearrangement during primate evolution, FISH analysis was performed with the breakpoint-spanning BACs on metaphase chromosomes of gibbon species *Hylobates lar*, *H. leucogenys*, and *H. syndactylus*, whose karyotypes are characterized by multiple rearrangements [Müller et al., 2003]. However, none of the BACs spanning the inversion breakpoints described here was found to encompass any of the rearrangement breakpoints in these gibbon species (data not shown).

The homologous chromosomes of the four great apes and the macaque all differ from *HSA*4 by pericentric inversions [Yunis and Prakash, 1982; Nickerson and Nelson, 1998; Marzella et al., 2000]. Marzella et al. [2000] noted that YAC 695H10 mapped to *HSA*4q21 and spanned the inversion breakpoint in both *PTR* and macaque. This suggested that a common breakpoint might have been involved in both rearrangements. However, FISH analysis with *HSA* BAC 8N8 indicated that this BAC was not split by the inversion in the macaque. We may therefore surmise that the same breakpoint was not involved in the two independent instances of pericentric inversions in chimpanzee and macaque.

The pericentric inversion of the *PTR* chromosome homologous to human chromosome 4 is also evident in bonobo (*Pan paniscus*); split signals of breakpoint-spanning *HSA* BACs RP11-8N8 and RP11-779N22 were detected by FISH on bonobo chromosomes (data not shown).

Differentiating Between the Derived Forms of Human and Chimpanzee Homologs Distinguished by Pericentric Inversions

The findings of Marzella et al. [2000] implied that the pericentric inversion of *PTR3* represents the derived form and that *HSA*4 retained the ancestral chromosomal arrangement. It has previously been shown that the chimpanzee chromosome 19 homologous to *HSA*17 [Kehrer-Sawatzki et al., 2002] and *PTR*10, homologous to *HSA*12, both represent the derived forms [Nickerson and Nelson, 1998; Kehrer-Sawatzki et al., 2004]. The pericentric inversions of the chimpanzee chromosomes homologous to *HSA*15 [Locke et al., 2003b] and *HSA*16 also represent derived forms. In the latter case, this was determined by FISH analysis with two BACs flanking the inversion breakpoints, *HSA* BACs RP11-46D6 and RP11-20D16. Sequences corresponding to BAC 20D16 from *HSA*16p12–p11 are inverted to the q-arm in chimpanzee. In the orangutan, *HSA* BAC RP11-20D16 hybridized to the region homologous to *HSA*16p12–p11, as in human (data not shown). Thus the pericentric inversion in the chimpanzee is seen to be derived. Intriguingly, seven of the nine pericentric inversions that distinguish the human and chimpanzee karyotypes occurred in the chimpanzee lineage, with chimpanzee therefore exhibiting the derived form of the respective chromosome (Table 2).

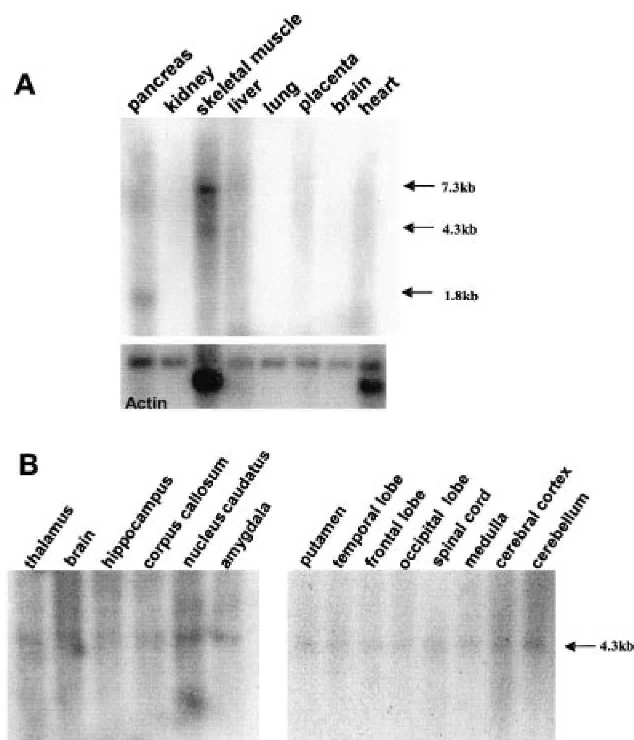


FIGURE 5. **Northern blot analysis of the human *C4orf12* gene using RNA derived from multiple adult human tissues (A) and different regions of adult human brain (B). Arrows indicate the three transcripts of 7.3-kb, 4.3-kb, and 1.8-kb that result from alternative splicing and usage of different polyadenylation sites.**

DISCUSSION

To gain insight into the nature of the generative mechanisms underlying the pericentric inversion of *PTR3* that occurred in the chimpanzee lineage at some time in the last 5–6 million years (since the divergence of human and chimpanzee) [Sibley and Ahlquist, 1987; Kumar and Hedges, 1998; Chen and Li, 2001], we compared the breakpoint regions in the human and chimpanzee genomes. FISH analysis with breakpoint-spanning BACs indicated that the bonobo also possesses the pericentric inversion. Thus, the inversion must have predated the separation of the bonobo lineage from that of *Pan troglodytes*, an event which is thought to have occurred $\sim$1.8 million years ago [Yoder and Yang, 2000]. It must, however, be remembered that the inversion occurred in an ancestral DNA sequence context that no longer exists and that the DNA sequences that originally mediated the rearrangement have inevitably decayed to some extent. This notwithstanding, the

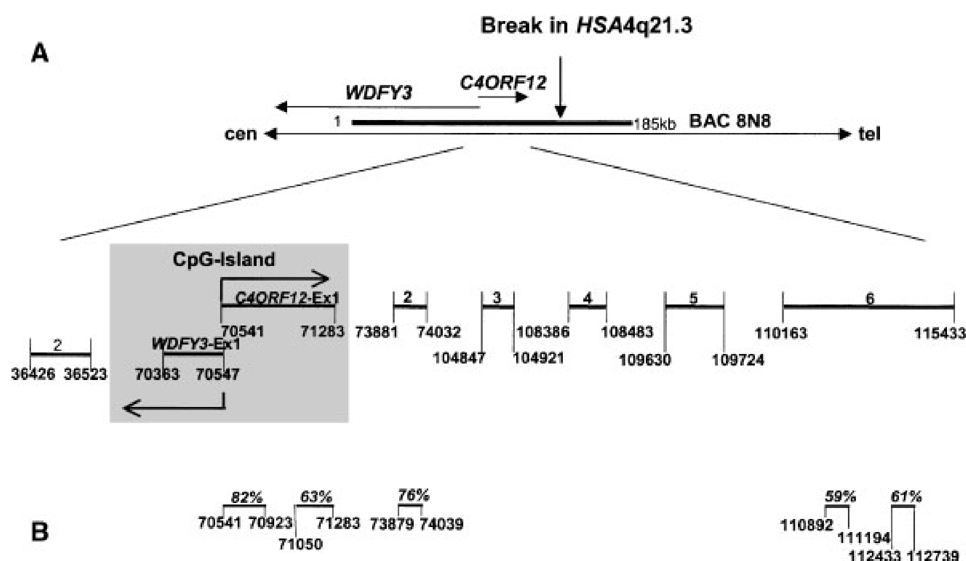


FIGURE 6. **A: Exon–intron structure of the *C4orf12* gene according to the IMAGE clone 3502957 (BC019264.1). This clone does not contain the complete 5′ and 3′UTRs of the *C4orf12* gene, which were characterized by RT-PCR (AY250185.1). Exons 1–6 are denoted by horizontal black bars. The *C4orf12* gene is expressed from a bidirectional promoter shared with the *WDFY3* gene. The opposing transcriptional orientations of *C4orf12* and *WDFY3* are indicated by horizontal arrows. B: Unmodified BLAST analysis of the human *C4orf12* genomic sequence revealed five fragments with the indicated identity (in percent) to the orthologous genomic region in the mouse. The positions of these fragments are indicated with respect to the basepair numbering of AC108021.3.**

TABLE 2. Evaluation of the Ancestral State of Primate Chromosomes That Differ by Virtue of Pericentric Inversions Between Human (*HSA*) and Chimpanzees (*PTR*)

HSA chromosome distinguished by a pericentric inversion from its chimpanzee orthologue	*HSA* with the derived (inverted) form	*PTR* with the derived (inverted) form
1	+[a]	
4		+[b,c,j]
5		+[d,e,j]
9		+[f]
12		+[g,j]
15		+[h,j]
16		+[c,j]
17		+[i]
18	+[d]	

[a]According to Weise et al. [2004].
[b]Shown by Marzella et al. [2000].
[c]This study.
[d]As determined by the analysis of G-banded human, chimpanzee, gorilla, and orangutan karyotypes according to Yunis and Prakash [1982].
[e]Müller and Wienberg [2001].
[f]Montefalcone et al. [1999].
[g]Nickerson and Nelson [1998].
[h]Locke et al. [2003a].
[i]Kehrer-Sawatzki et al. [2002].
[j]Reviewed in Dutrillaux [1979].

study of extant orthologous sequences represents the only available means to improve our knowledge of the causes and consequences of primate chromosome evolution and, more specifically, to attempt to infer the nature of the DNA sequences responsible for the genomic instability.

The breakpoints of the pericentric inversion between *HSA*4 and *PTR*3 are located in genomic segments characterized by a high abundance of repetitive elements from the LINE and LTR-families. Sequence comparisons reveal a chimpanzee-specific 4.7-kb deletion at the breakpoint in *PTR*3q that appears to have led to the loss of mainly repetitive sequences. Owing to the occurrence of the deletion, the position of the breakpoint in 4q21.3 cannot be determined with any degree of precision. We nevertheless screened the breakpoint regions for genomic features that could have mediated the inversion, and succeeded in identifying imperfect inverted repeats of $\sim$5-kb at *HSA*4p14 and *HSA*4q21.3 (Fig. 3). These inverted repeats could have facilitated the intrachromosomal pairing of the p- and q- arms at the breakpoints. The breakpoint in *HSA*4p14 occurred within one copy of this 5-kb repeat in a 109-bp $(TA)_n$-rich segment that is split by the inversion (Fig. 3B). A 16-bp motif within this $(TA)_n$ motif was found to be palindromic to a segment located 8-bp distally. These palindromic sequences may have facilitated the formation of a hairpin loop that blocked the progression of the replication fork, thereby generating free chromosome ends. Consistent with this model, a number of recurring translocations involved in human tumorigenesis possess breakpoints that are characterized by the presence of TA-rich palindromes [Kehrer-Sawatzki et al., 1997; von Lindern et al., 1992; Pomykala et al., 1994; Mimori et al., 1999; Ramakrishnan et al., 2000; Edelmann et al., 2001; Kurahashi and Emanuel, 2001; Kurahashi et al., 2003].

Both the breakpoints of the pericentric inversion analyzed in this study, and those of the inversion that distinguishes *HSA*17 from *PTR*19, lie in regions of abundant interspersed high-copy number repeats (Table 1; Supplementary Table S4) [Kehrer-Sawatzki et al., 2002]. All four breakpoint regions are devoid of low-copy repeats (LCRs). This is in stark contrast to other "evolutionary breakpoints," such as the fusion site in human chromosome 2q13–2q14.1, which is rich in LCRs [Fan et al., 2002]. Similarly, the gorilla-specific translocation t(4;19) occurred within an LCR-rich region and is associated with a gorilla-specific duplication of $\sim$250-kb. This duplication encompasses a genomic segment that is syntenic to a region in human that contains the proximal CMT1A (Charcot-Marie-Tooth)-LCR that is known to be involved in high frequency deletion/duplication events [Stankiewicz et al., 2001]. The breakpoints of the pericentric inversion that distinguishes *HSA*15 from the orthologous chromosome 16 in the chimpanzee also map to positions of segmental duplication [Locke et al., 2003b]. Segmental duplications in euchromatic regions of human chromosome 5 have been shown to have reshaped their region of insertion during primate evolution and could have caused ectopic rearrangements that contributed both to evolutionary and genomic instability [Courseau et al., 2003]. The human-specific pericentric inversion of chromosome 18, which distinguishes this chromosome from the homologous chromosomes of the great apes, was most probably mediated by an inverted segmental duplication of 19-kb [Dennehey et al., 2004; Goidts et al., 2004]. It may be therefore that the genomic architecture associated with these LCRs served to facilitate the inversions by promoting homologous recombination, thereby rendering these regions unstable. By contrast, the pericentric inversions characterizing chimpanzee chromosomes 3 and 19, homologous to *HSA*4 and *HSA*17, respectively, appear to belong to another category of evolutionary breakpoint. Since all four pericentric inversion breakpoints are located in regions with abundant high-copy repeats, it may be that evolutionary rearrangements of the ancestral hominoid genome have been facilitated not only by segmental duplications and low-copy repeats, but also by high-copy repeat-rich regions. Comparing human and mouse genomes, an association between primate-specific segmental duplications at the breakpoints of syntenic blocks was observed [Bailey et al., 2004]. However, segmental duplications do not necessarily cause rearrangements. Rather, a nonrandom model of chromosomal evolution may be assumed, with specific regions of the mammalian genome being predisposed to recurrent small-scale duplications and large-scale evolutionary rearrangements [Bailey et al., 2004].

Another feature of the pericentric inversions of the chimpanzee chromosomes homologous to *HSA*4 and *HSA*17 is that they did not disrupt protein coding regions and appear to be located in relatively gene-poor regions. Although no gene appears to have been directly affected by the *HSA*4 inversion, it is possible that this rearrangement served instead to alter the expression of genes in the vicinity of the breakpoints (Fig. 4). A 33-fold difference was observed when comparing the expression of *C4orf12* in human and chimpanzee fibroblast cell lines, the higher level of expression being found in human cells. As the expression of *C4orf12* is high in skeletal muscle (as adduced by Northern blot analysis; Fig. 5), it would be interesting to extend the expression analysis using primary muscle tissue of both species, together with that of other apes as an out-group. The *C4orf12* gene identified 30-kb proximal to the *HSA*4q21.3 breakpoint is unusual in that it belongs to the minority of genes that originated during mammalian evolution.

Seven of the 11 genes that mapped to the regions around the *HSA*4 inversion breakpoints are represented on the Affymetrix-U95Av2 oligonucleotide microarrays recently used to assess

differential gene expression in human and ape cultured fibroblasts [Karaman et al., 2003]. However, none of the seven genes displayed consistent expression differences between human and chimpanzee cells. These Affymetrix arrays were also employed by Caceres et al. [2003] to assess differential gene expression in samples of chimpanzee and human cerebral cortex. Interestingly, the *WDFY3* gene (also termed ALFY), displayed a four-fold difference in expression between human and chimpanzee [Caceres et al., 2003]. We observed a three-fold higher expression in human compared to chimpanzee fibroblast cell lines by real-time PCR. Further expression analysis of tissues from other primates lacking the inversion, and sequence comparisons of gene regulatory regions, are required to determine whether or not this expression difference is related to the adjacent inversion.

The traditional model of chromosomal speciation implies that chromosomal changes such as pericentric inversions can represent genetic barriers to gene flow between subpopulations that possess different arrangements of their respective chromosomes [Wu and Ting, 2004]. This interpretation would, however, be rendered implausible if possession of the rearrangement were to be detrimental to the carrier, a situation that would inevitably militate against its fixation [King, 1993; Rieseberg, 2001; Hey, 2003]. Recently, Navarro and Barton [2003] reported that nucleotide variability is slightly lower on rearranged chromosomes than on colinear chromosomes. The most convincing interpretation is that these rearrangements served to suppress recombination with consequent indirect effects on the rate of gene evolution. Determining the precise location of the inversion breakpoints thus assumes great importance for a test of this hypothesis because the selective pressure acting on flanking genes must be measured both within and without of the inverted segments [Clark et al., 2003].

The recombination suppression hypothesis requires that the reduced recombination mediated by a pericentric inversion in the heterozygous state should facilitate speciation in the absence of local separation (parapatric speciation), as was the case for early hominids and chimpanzees in East Africa. Navarro and Barton [2003] further suggested that the key genomic regions involved in speciation were those spanning the chromosomal rearrangements. If true, this could potentiate the identification of those genetic differences that could have accompanied or even driven the process of hominoid speciation. Given that the proposed primary effect of pericentric inversions would have been to allow the accumulation of incompatible alleles, thereby creating a reproductive barrier, one might assume that both human and chimpanzees would share a similar number of pericentric inversions. However, the majority of the chromosomal inversions that distinguish the two species occurred in the chimpanzee lineage whereas humans appear to have retained the karyotype that most resembles the ancestral form (Table 2).

Pericentromeric regions appear to have been particularly prone to rearrangement during primate evolution, being subject to amplification, duplication, deletion, and inversion events as well as translocations [Jackson et al., 1999; Eichler, 1999]. They have also frequently acquired sequences from remote genomic locations [Eichler, 1998]. This genetic instability could be related to the presence of specific repetitive sequences [Wöhr et al., 1996]. The potential evolutionary importance of pericentromeric regions can perhaps be gauged from Eichler's [1999] description of them as "recruitment stations for repeats" and "reservoirs for the accumulation of transposed genic segments."

Although chromosomal inversions in human pathology are nonrandomly distributed in the genome [Weichhold et al., 1990], it remains unclear as to whether this is due to interchromosomal differences or to clinical ascertainment bias [Dutrillaux et al., 1986]. Some of these inversions have nevertheless been characterized with sufficient precision to allow comparison with inversions that have occurred in the karyotypic evolution of the Hominoidea [Yunis and Prakash, 1982]. Indeed, Miró et al. [1992] claimed that 10 out of 20 of the evolutionary pericentric inversions in great apes coincided, albeit at low level of resolution, with known sites of pathological inversion. While this claim remains to be validated, if the sites of these chromosome inversions were also to be involved in cases of human chromosome pathology, it might be that these chromosomal regions possess sequence characteristics that predispose to this type of rearrangement.

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