Trabajo 4

Implication of fragile sites and intrachromosomal telomeric (TTAGGG)n sequences in Primate chromosome evolution

Ruiz-Herrera A, García F, Giulotto E, Attolini C, Egozcue J, Ponsà M, Garcia M

Implication of fragile sites and intrachromosomal telomeric (TTAGGG)n sequences in Primate chromosome evolution.

Aurora Ruiz-Herrera, Francisca García, Elena Giulotto, Carmen Attolini, Josep Egozcue, Montserrat Ponsà, Montserrat Garcia.

ABSTRACT

The concentration of evolutionary breakpoints in Primate karyotypes in some particular regions or chromosome bands suggests that these chromosomal regions are more prone to breakage. In this work our aim is to test whether fragile sites (FSs) and intrachromosomal telomeric sequences (TTAGGG)n are implicated in the evolutionary process of Primate chromosomes. For this purpose, we have analyzed: (a) the cytogenetic expression of aphidicolin-induced FSs at two different aphidicolin concentrations (0.1μmol/L and 0.2μmol/L) in three specimens of *Cebus apella* and *Cebus nigrivittatus* (F. Cebidae, Platyrrhini) and four specimens of *Mandrillus sphinx* (F. Cercopithecidae, Catarrhini), and (b) intrachromosomal telomeric sequences (ITSs) by fluorescent *in situ* hybridization with a synthetic (TTAGGG)n probe in *C. apella* chromosomes. The use of a multinomial FSM statistical model allowed us to identify 53 FSs in *C. apella*, 16 FSs in *C. nigrivittatus* and 50 FSs in *M. sphinx*. As expected, all telomeres hybridized with the probe, and 55 intrachromosomal loci were also detected. About 40% of the fragile sites detected in the Primate species studied are conserved in the corresponding homologous human chromosomes and that a high number of evolutionary chromosomal reorganizations is located in chromosome bands that express fragile sites and/or contain intrachromosomal telomeric sequences.

INTRODUCTION

Comparative cytogenetic analysis is a useful tool to establish chromosome homologies among different species and to identify the chromosomal changes that have taken place during the evolutionary history of chromosomes. About the chromosomal regions implicated in evolutionary reorganizations, some questions remain unclear. Are there chromosome *loci* or DNA sequences with a higher tendency to break and reorganize? Are these chromosomal regions more frequently

implicated in the evolutionary process? Is there any relationship among evolutionary chromosomal breakpoints, fragile sites and intrachromosomal telomeric sequences in mammalian genomes?

Specific chromosome *loci*, which are expressed as breaks and gaps when cells are exposed to specific culture conditions or to some chemical agents, have been defined as fragile sites (FSs) (Sutherland, 1979). The cytogenetic expression of these *loci* is a consequence of genome instability at specific chromosome *loci* and may be involved in chromosome breakage and recombination events (Glover and Stein, 1988; Svetlova et al., 2001). The implication of fragile sites in the chromosome evolutionary process has long been suggested (Miró et al., 1987; Clemente et al., 1990; Ruiz-Herrera et al., 2002a), because fragile sites can be considered unstable regions and probable targets for chromosome breakage.

The hexanucleotide (TTAGGG)n repeat sequence conserved among vertebrate telomeres, in addition to its location at telomeric sites, is also found in non-telomeric sites, called intrachromosomal telomeric sequences (ITSs) (Meyne et al., 1989; 1990). The study of the distribution of ITSs observed in more than 50 vertebrate species, such as fish, reptiles, amphibians, birds and mammals, opened the way to study the chromosomal reorganizations that have been leading the eucaryotic genomic evolution through the study of the distribution of the telomeric sequences in the karyotype of different species (Nanda et al., 2002). ITSs have been considered as remnants of ancestral chromosomal rearrangements (inversions and fusions) produced during karyotype evolution (Ijdo et al., 1991; Lee et al., 1993; Vermeesch et al., 1996; Thomsen et al., 1996; Metcalfe et al., 1997, 1998; Fagundes and Yonenaga-Yassuda, 1998; Pellegrino et al., 1999; Finato et al., 2000; Ruiz-Herrera et al., 2002b) and amplification of these conserved telomeric sequences would provide alternative sites for telomere formation, thus allowing great flexibility for chromosomal changes (Meyne et al., 1990). Azzalin et al. (2001) performed an extensive analysis of the ITS organization in the human genome and identified three types of sequences: i) short, ii) subtelomeric and iii) fusion ITSs. It is likely that the same sequence organization is present in other Primate species.

The expression of fragile sites has been studied in a reduced number of Primate species [great apes (Schimd et al., 1985; Smeets and Van de Klundert, 1990), New World monkeys (Fundia et al., 1991; 2000) and a macaque species (Ruiz-Herrera et al., 2002a)], while the distribution of ITSs has been studied in humans (Azzalin et al., 1997), lemurs (Meyne et al., 1990; Go et al., 2000), great apes (Hirai, 2001) and one macaque species (Ruiz-Herrera et al., 2002b). For this reason, a comparative study of the distribution of intrachromosomal telomeric sequences and common fragile site expression in a higher number of Primate species is especially interesting

in order to define the role of these chromosomal regions in Primate chromosome evolution and their implication in evolutionary chromosomal rearrangements.

Three Primate species with different chromosomal evolutionary processes have been chosen in this study due to their karyological features: one Old World monkey (*Mandrillus sphinx*) and two New World monkeys (*Cebus apella* and *Cebus nigrivittatus*). The Tribe Papionini (F. Cercopithecidae, Catarrhini) includes four genera (*Macaca*, *Mandrillus*, *Papio* and *Cercocebus*), with highly conserved karyotypes, both in diploid number (2n=42) and G-banding patterns (Rubio-Goday et al., 1976; Dutrillaux et al., 1979; Brown et al., 1986; Ponsà et al., 1986; Stanyon et al., 1988). The almost complete homology of the banding patterns among these species and with the human karyotype (Wienberg et al., 1992; Ruiz-Herrera et al., 2002a) makes the comparison of location of common fragile sites and ITSs based on G-banding possible. Otherwise, the genus *Cebus* (Family Cebidae, Platyrrhini) includes four species (Napier and Napier, 1985) with some different chromosomal morphologies and diploid numbers, 2n=54 (*C. apella*, *C. albifron*s and *C. capucinus*) and 2n=52 (*C. nigrivittatus*). Comparative cytogenetic studies of the chromosome homologies between *Cebus* and other Primate species suggest that *Cebus* has maintained a primitive karyotype (Dutrillaux and Couturier, 1981; Clemente et al., 1990; García et al., 2000; 2002). For this reason, a comparative study of the distribution of intrachromosomal telomeric sequences and common fragile site expression among the different Primate species studied so far (including man) plus *M. sphinx* and the *Cebus* species studied in this work, is especially interesting.

The main goal of this study is to establish a relationship among breakpoints implicated in chromosomal evolution in Primates, the location of fragile sites and the distribution of intrachromosomal telomeric sequences. For this purpose, the expression of aphidicolin-induced fragile sites in two species of New World monkeys (*Cebus apella* and *Cebus nigrivittatus*) and one species of Old World monkey (*Mandrillus sphinx*), and the distribution of interstitial telomeric (TTAGGG)n repeats in the karyotype of *Cebus apella* have been studied.

MATERIAL AND METHODS

Blood samples and metaphase spread preparations.

Heparinized, peripheral blood samples were obtained from two females and one male brown capuchin (*Cebus apella*, CAP, 2n=54) from Argentina (Parque Zoológico Fauna Corrientes), Colombia (Proyecto DAMA, Universidad Nacional de Colombia, Bogotá) and

Venezuela (Parque Zoológico Bararida, Barquisimeto), two females and one male weeper capuchin (*Cebus nigrivittatus*, CNI, 2n=52) from Venezuela (Parque Zoológico Bararida, Barquisimeto and Parque Zoológico El Pinar, Caracas) and three females and one male mandrill (M. sphinx, MSP, 2n=42) from Parc Zoològic de Barcelona (Barcelona, Spain). Samples from these *Cebus* species were processed in the Departamento de Biologia, GIBE (Grupo de Investigación de Biologia Evolutiva), Universidad de Buenos Aires (Buenos Aires, Argentina), Instituto de Genética, Universidad Nacional de Colombia (Bogotá, Colombia) and BIOEVO (Grupo de Biologia Evolutiva), Universidad Simón Bolívar (Caracas, Venezuela).

Fragile site analysis.

RPMI-1640 medium, supplemented with phytohaemagglutinin, lectin, 25% fetal bovine serum, L-glutamine, penicillin, streptomycin and Hepes buffer, was used for the peripheral blood cultures. A volume of 0.5 ml from each blood sample was cultured in 5 ml of medium for 96 h at 37ºC. For fragile site induction, 25 μl and 50 μl of aphidicolin (0.02 μmol/L dissolved in DMSO) were added 24 h before harvesting to each 5 ml of medium to give a final concentration of 0.1 μmol/L and 0.2 μmol/L, respectively. Untreated cultures of the same blood samples were used as controls. Cultures from peripheral blood samples were processed under standard conditions.

Between 32 and 495 metaphases were analyzed per specimen for each aphidicolin concentration. All metaphases analyzed were stained homogeneously for the detection of chromosome aberrations (breaks and gaps). Sequential G-banding and fragile site analysis were performed as previously described by Ruiz-Herrera et al. (2002a). An MS-DOS statistical program, FSM (Version 995) was used (Böhm et al. 1995; McAllister and Greenbaum, 1997) to determine which chromosome bands in *C. apella*, *C. nigrivittatus* and *M. sphinx* karyotypes could be regarded as fragile sites, as previously described by Ruiz-Herrera et al. (2002a).

Fluorescence *in situ* hybridization.

FISH was performed as previously described (Azzalin et al., 1997; Ruiz-Herrera et al., 2002b). Chromosomes were hybridized with a non-commercial biotin-labeled telomeric probe, i.e., a mixture of synthetic (TTAGGG)$_n$ polynucleotides. Prior to hybridization, slides were treated with RNase (100μgr/ml 2xSSC at 37ºC), pepsin digestion (0.005% in 10mM HCl at 37ºC), post-fixed (4% paraformaldehyde in PBS + 50mM MgCl$_2$) and denatured in 70% formamide/2xSSC at 75ºC. *In situ* hybridization with the probe was carried out overnight at 37ºC and the slides were washed three times in 25% formamide/4xSSC, three times in 2xSSC and one time 4xSSC/Tween20 0.05% at 37ºC. Detection was performed and results were interpreted using the

criteria of Azzalin et al. (1997) and Ruiz-Herrera et al. (2002b). Metaphases were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and observed with a Zeiss Axioplan microscope with the appropriate filter sets, using a cooled CCD camera system. The G-banding pattern was generated using the DAPI DNA counterstain. Only double spots (hybridization signals with the telomeric probe on both chromatids) were scored.

RESULTS

Aphidicolin-induced fragile sites analysis.

New World monkeys: *C. apella* and *C. nigrivittatus*

The chromosome abnormalities detected (gaps and breaks, Figure 1) were scored as single events in the FSM statistical program. The results of the expression of aphidicolin-induced fragile sites in *C. apella* and *C. nigrivittatus* are shown in Table 1 and Figures 2a and 2b. A total of 3276 metaphases were analyzed, 2498 from *C. apella* cultures and 778 from *C. nigrivittatus* cultures.

a) *Cebus apella* fragile sites: In control cultures, the total number of breaks/gaps detected was 76, located in 24 different chromosome bands. In cultures treated with aphidicolin, at a 0.1μmol/L dose, the total number of breaks/gaps detected was 449, located in 83 different chromosome bands, whereas in cultures treated with 0.2μmol/L aphidicolin, the total number of breaks/gaps detected was 1311, mapped to 123 different chromosome bands. With the higher dose of aphidicolin (0.2μmol/L), the proportion of chromosome abnormalities scored per metaphase increased two-fold, with respect to the dose of 0.1μmol/L aphidicolin, and almost ten-fold, with respect to control cultures (Table 1).

In cultures treated with 0.1μmol/L aphidicolin, the number of fragile sites detected by the FSM statistical program per specimen ranged from 4 to 18, and a total of 22 sites was considered fragile. In cultures treated with 0.2μmol/L aphidicolin, the number of fragile sites detected per specimen ranged from 21 to 37, and a total of 51 sites was considered fragile. Taking into account that the expression of fragile sites has an inter-individual variability, the use of the FSM statistical program allowed for the location of 53 fragile sites in the *C. apella* karyotype (Figure 2a).

b) *Cebus nigrivittatus* fragile sites: In control cultures, a total of 30 breaks/gaps was detected in 16 different chromosome bands. In cultures treated with aphidicolin, at a 0.1μmol/L dose, the number of breaks/gaps detected was 75 mapped to 24 different chromosome bands. In cultures treated with 0.2μmol/L aphidicolin, the total number of breaks/gaps detected was 233 in 53 different chromosome bands. With the higher dose of aphidicolin (0.2μmol/L), the proportion

of chromosome abnormalities scored per metaphase increased by almost 2.5-fold, with respect to the dose of 0.1µmol/L aphidicolin and almost 12-fold with respect to control cultures (Table 1).

Table 1: Number of metaphases analyzed, chromosomal breaks (gaps included) and breaks /metaphase (gaps included) observed in aphidicolin-treated and control lymphocyte cultures from peripheral blood samples of *Cebus apella* (CAP), *Cebus nigrivittatus* (CNI) and *Mandrillus sphinx* (MSP).

Specimens	Control			Aphidicolin-treated 0.1µmol/L			Aphidicolin-treated 0.2µmol/L		
	Metaphases	Breaks	Breaks/ Metaphase	Metaphases	Breaks	Breaks/ Metaphase	Metaphases	Breaks	Breaks/ Metaphase
CAP 1	202	13	0.064	136	51	0.375	258	462	1.791
CAP 2	288	48	0.166	495	317	0.640	482	622	1.290
CAP 3	104	15	0.144	160	81	0.506	281	227	0.807
Total	594	76	0.128	791	449	0.568	1113	1311	1.178
CNI 1	138	11	0.080	154	62	0.402	81	76	0.938
CNI 2	130	8	0.061	-	-	-	122	130	1.065
CNI 3	88	11	0.125	32	13	0.406	33	27	0.818
Total	356	30	0.084	186	75	0.403	236	233	0.987
MSP 1	102	2	0.02	91	9	0.10	262	560	2.14
MSP 2	112	9	0.08	99	33	0.33	-	-	-
MSP 3	80	3	0.04	-	-	-	50	100	2.0
MSP 4	222	5	0.02	161	23	0.14	-	-	-
Total	516	19	0.04	351	65	0.18	312	660	2.11

In cultures treated with 0.1µmol/L aphidicolin, the number of fragile sites detected by the FSM statistical program per specimen ranged from 1 to 6, and a total of 6 sites was considered fragile. In cultures treated with 0.2µmol/L aphidicolin, the number of fragile sites detected per specimen ranged from 6 to 10, and a total of 14 sites was regarded as fragile. The use of the FSM statistical program allowed for the location of 16 fragile sites in the *C. nigrivittatus* karyotype (Figure 2b). By using the well established chromosome homologies between *C. apella* and *C. nigrivittatus* karyotypes (García et al., 2002), 10 fragile sites were located in homologous chromosome bands in both species (CAP1p14, CAP2q13, CAP2q26, CAP5p14, CAP6q26/CNI6p21, CAP11q15, CAP12q32, CAP15q24, CAP15q32 and CAP18q23) (Table 2, Figures 2a and 2b).
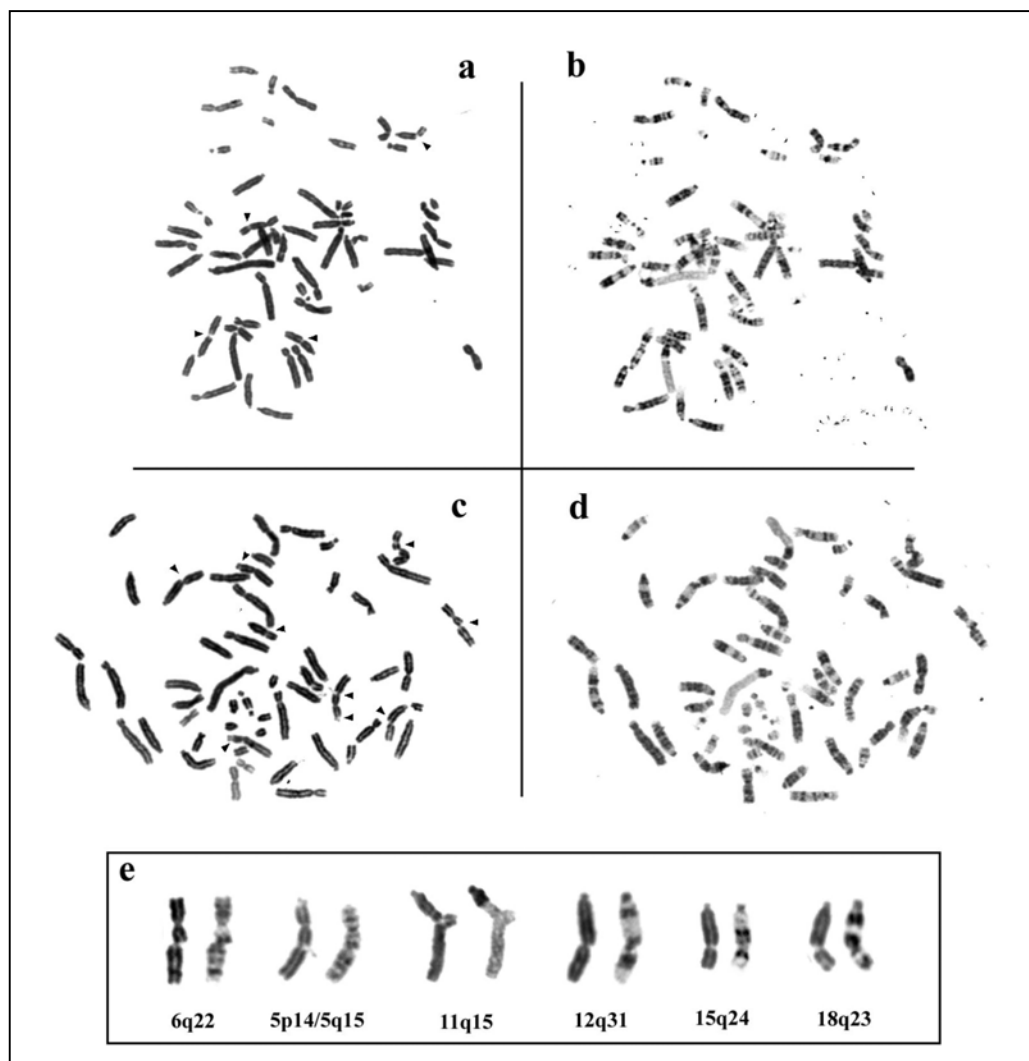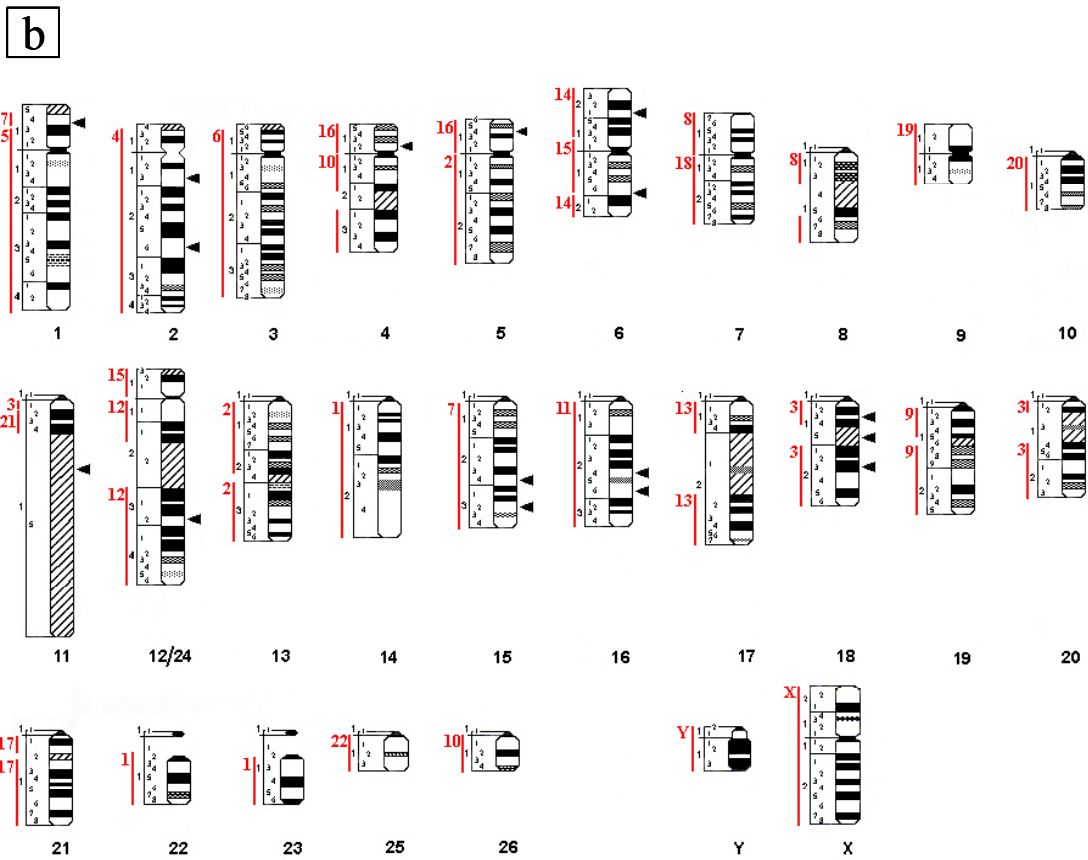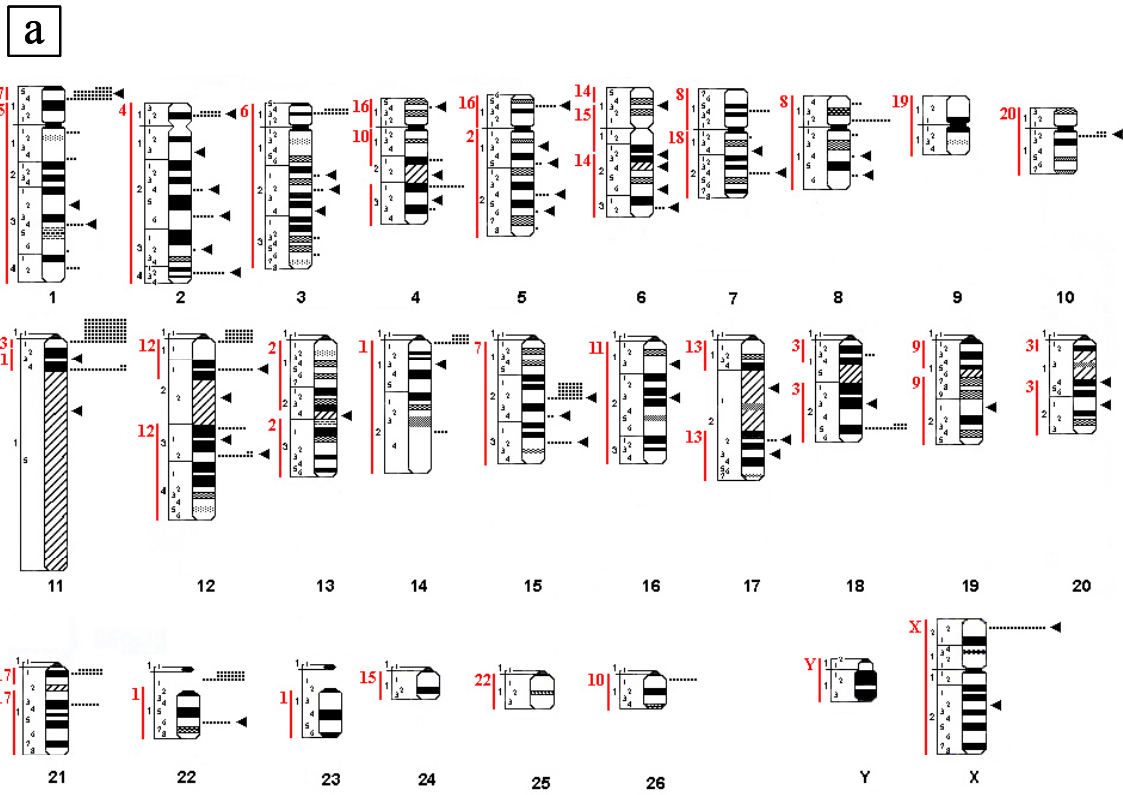
Figure 1: Homogeneously (**a,c**) and G-banded (**b,d**) sequentially stained metaphase chromosome spreads from *C. apella* showing chromatid and chromosome breaks and gaps (arrowheads). (**e**) Some examples of fragile sites expression in different *C. apella* chromosomes; gaps (5q15 and 15q24), breaks (12q31, 6q22, 5p14 and 18q23) and a trirradial figure (11q15).
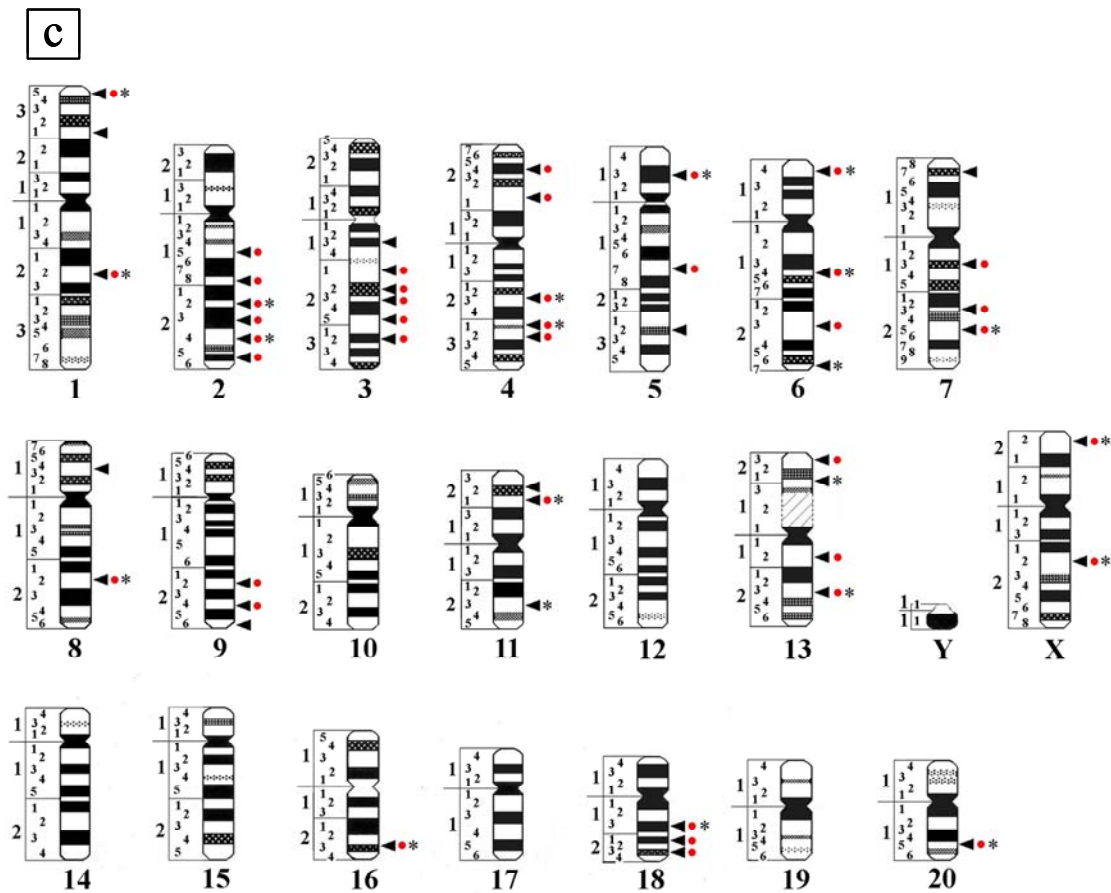
Figure 2: Ideograms of *Cebus apella* (CAP) (**a**), *Cebus nigrivittatus* (CNI) (**b**), and *Mandrillus sphinx* (MSP) (**c**) chromosomes illustrating the location of fragile sites (arrowheads). Homology with human chromosomes is shown in red to the left of the *Cebus* chromosomes. The distribution of intrachromosomal telomeric sequences (ITSs) is also represented in the CAP ideogram (black dots). Asterisks indicate those fragile sites conserved in the human karyotype and red dots indicate those *M. sphinx* fragile sites conserved in the *M. fascicularis* karyotype**.**

Old World monkey: *M. sphinx*

A total of 1179 metaphases obtained from peripheral blood samples of four *M. sphinx* specimens was analyzed. In control cultures, the total number of breaks and gaps detected was 19 located in 9 different chromosome bands. In cultures treated with 0.1μmol/L aphidicolin, the number of breaks and gaps detected was 65, mapped to 33 different chromosome bands, whereas in cultures treated with 0.2μmol/L aphidicolin, 660 breaks and gaps were detected in 68 different chromosome bands. With the higher dose of aphidicolin (0.2μmol/L), the proportion of breaks/gaps per metaphase increased by almost 12-fold, with respect to the dose of 0.1μmol/L, and almost 53-fold, with respect to control cultures (Table 1).

The expression of fragile sites is variable and this variability depends on the specimen studied. In cultures treated with 0.1µmol/L aphidicolin, the number of fragile sites detected per specimen ranged from 1 to 11, and a total of 12 chromosome bands was considered fragile. In cultures treated with 0.2µmol/L aphidicolin, the number of fragile sites detected in the two specimens was 11 and 45, and a total of 46 chromosome bands was considered fragile. The use of the FSM statistical program permitted the location of 50 aphidicolin-induced fragile sites in the M. sphinx karyotype (Figure 2c).

Interstitial telomeric sequence (ITS) distribution.

A total of 366 metaphases obtained from peripheral blood samples of the three *C. apella* specimens (210 metaphases from Specimen 1, 81 metaphases from Specimen 2 and 75 metaphases from Specimen 3) was analyzed. As expected, all telomeres hybridized with the telomeric probe and 55 intrachromosomal loci with different hybridization frequencies were detected in all of the chromosomes, with the exception of chromosomes CAP9, CAP13, CAP16, CAP19, CAP20, CAP23, CAP24, CAP25 and CAPY. The distribution of double spots observed is shown in Fig. 2a, and examples of hybridization images can be seen in Fig. 3.

Hybridization signals were located with a high frequency in six different chromosome bands: 1p14 (40 times), 11q11 (88 times), 12q11 (38 times), 14q11 (20 times), 15q22 (38 times) and 22q11 (28 times). Frequent signals, which hybridized more than five times, were located in 22 different chromosome bands: 1q34, 2p12, 2q26, 2q42, 3p13, 4q31, 5p14, 7p15, 7q24, 8p12, 10q12, 11q14, 12q21, 12q31, 12q32, 15q32, 18q25, 21q11, 21q14, 22q16, 26q11, Xp22. Rare signals, which hybridized less than five times with the telomeric probe, were found in 27 different chromosome bands (Fig. 2a). The analysis of the distribution of intrachromosomal telomeric sequences in the three specimens of *C. apella* shows that the double spots detected had the same hybridization intensity, except for the ITSs located at the 11q11 locus. In all metaphases analyzed from Specimen 2, a large telomeric signal at 11q11 is observed, in contrast with Specimens 1 and 3, where the signals are weak (Figure 3e).

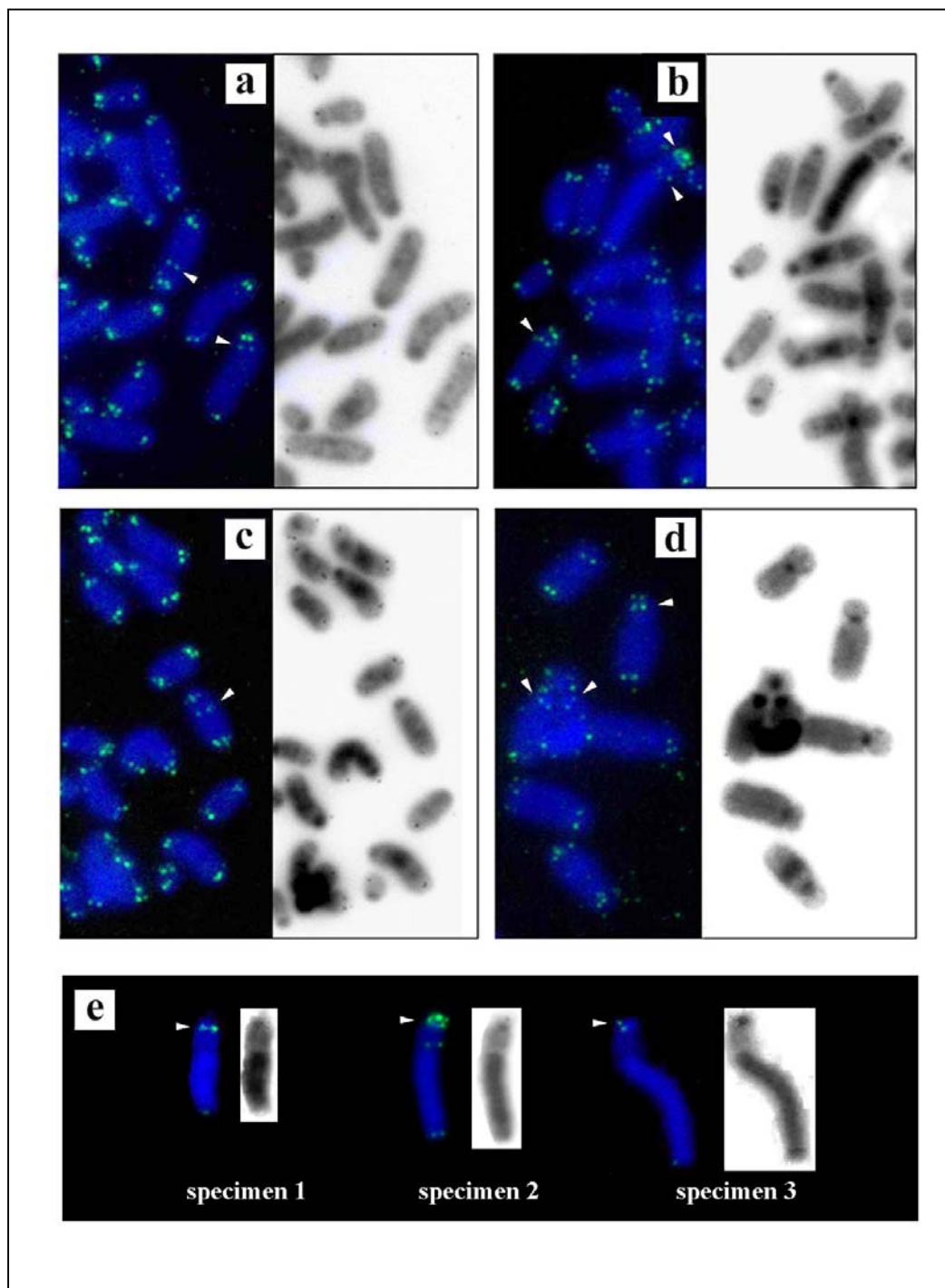**Figure 3: (a, b, c, d)** Partial metaphases images of *C. apella* showing FISH hybridization signals with the (TTAGGG)n probe (images left) and DAPI bands (images right). Arrowheads indicate double spots at intrachromosomal locations. **(e)** *C. apella* chromosome 11 from the three specimens studied showing different hybridization signal intensity in the ITS located in the 11q11 loci.

DISCUSSION

Chromosomal homologies among the Primate species analyzed in this study (*C. apella*, *C. nigrivittatus* and *M. sphinx*) and man based on G-banding and Zoo-FISH results are well established; this allows for a reliable comparison of fragile sites and ITSs location. Based on the present results, as well as those already published in other Primate species, the evolutionary implications of these two characteristics (fragile sites and ITSs) are discussed.

Evolutionary co-localization of aphidicolin-induced fragile sites in Primates.

The analysis of fragile site expression in *C. apella*, *C. nigrivittatus* and *M. sphinx* karyotypes has shown that the three species maintain the same response to aphidicolin, that is, the proportion of chromosomal abnormalities/metaphase at two aphidicolin doses with respect to control cultures, is equivalent. However, *M. fascicularis*, a Primate species that also belongs to Tribe Papionini (Ruiz-Herrera et al., 2002a), has a different behavior in front of aphidicolin effects.

Comparative cytogenetic analysis has revealed a conservation of the localization of aphidicolin-induced fragile sites among the different species studied. If fragile site localization between evolutionary related species is compared, as is the case of *C. apella* /*C. nigrivittatus*, and *M. fascicularis*/*M. sphinx*, some differences can be observed. A high percentage of *M. sphinx* fragile sites (80%) corresponds to *M. fascicularis* fragile sites that are localized at homologous chromosome bands, whereas *C. apella* only shares 18.87% of fragile sites with *C. nigrivittatus*. Therefore, there are fragile sites that are species-specific: *C. apella* has 43 de novo fragile sites, *C. nigrivittatus* has 6 de novo fragile sites and *M. sphinx* has 10 de novo fragile sites. Between *M. fascicularis* and *M. sphinx* there is a higher coincidence of fragile sites than between *C. apella* and *C. nigrivittatus*. This difference can be a consequence of the inter-individual variability in the expression of fragile sites, as well as of the different number of metaphases studied in each species. This inter-individual variability could also explain that, although *C. apella* and *C. nigrivittatus* share almost the same karyotype and both of them belong to the same genus, there are more fragile sites conserved between *C. apella* and *M. fascicularis* (37.73% of the *C. apella* fragile sites) than between *C. apella* and *C. nigrivittatus* (18.87% of the *C. apella* fragile sites) (Table 2).

When *C. apella, C. nigrivittatus* and *M. sphinx* are compared with humans, the percentage of fragile site co-localization detected between any of the species and man is considered high and is almost the same in all three species: 45.28% in *C. apella*, 37.50% in *C. nigrivittatus* and 42% in *M. sphinx*. However, the proportion of *C. apella* fragile sites conserved in the homologous human chromosome is higher than *C. apella* fragile sites conserved in the homologous *C. nigrivittatus* chromosomes (45.28% and 18.87%, respectively).

As can be observed in *C. apella, C. nigrivittatus, M. fascicularis, M. sphinx* and man, four fragile sites are expressed in the corresponding homologous chromosome bands: HSA4q31, HSA7q22, HSA7q32.3 and HSA16q22.1 (Table 2). If *C. nigrivittatus* (the species with the lowest number of metaphases analyzed) is excluded from the comparison, the fragile sites conserved are ten: HSA1p22, HSA4q27, HSA4q31, HSA7q22, HSA7q32.3, HSA8q22, HSA14q24.1, HSA16q22.1, Xp22.31 and Xq22.1.

Previous cytogenetic studies performed in human, gorilla, chimpanzee and orangutan chromosomes (Smeets and Van de Klundert, 1990) have shown that eight fragile sites (HSA1p22, HSA4q31, HSA7p13, HSA7q32.3, HSA16q22.1, HSA16q23.2, HSAXp22.31, HSAXq22.1) were located in the same chromosome band in all of these species, and also in *M. fascicularis* (Ruiz-Herrera et al., 2002a), and six fragile sites (HSA1p22, HSA4q31, HSA7q32.3, HSA16q22.1, HSAXp22.31, HSAXq22.1) were conserved in these species and in *M. sphinx*. It is important to notice that, with the exception of Xp22.1, the conserved fragile sites mentioned above are among those with the highest percentage of expression in *M. fascicularis* and *M. sphinx* chromosomes (those with more than the 2% of total abnormalities detected). Seven human fragile sites (HSA1q44, HSA2q37.3, HSA6p25, HSA7p22, HSA11p14, HSA14q24.1, HSA22q12) are conserved in the gorilla and chimpanzee (but are absent in orangutan) (Smeets and Van de Klundert, 1990), and in at least one of the *Cebus* and/or Papionini species compared here. Taking into account previous studies of the mouse karyotype (Djalali et al., 1987), eight mouse fragile sites are conserved in the human karyotype as well as in the Primate species included in this comparative study, like *M. fascicularis* and *M. sphinx* (Table 2): HSA1p32, HSA2q33, HSA7p13, HSA7q32.3, HSA10q22, HSA11p13, HSA22q12, HSAXq22.1.

Since common fragile sites are part of the normal chromosome structure (Handt et al., 2000), we have evidence to consider that these loci have been conserved along the evolutionary process. Among the eight Primate species compared (man included), three fragile sites are conserved in the homologous chromosome bands: HSA4q31, HSA7q32.3 and HSA16q22.1. From these fragile sites, one of them, HSA7q32.3 is also detected in other mammalian species, i.e., the mouse. Among the Primate species studied, *Cebus* has maintained a primitive karyotype, and 67.92% of *C. apella* fragile sites are conserved in at least one of the Primate species compared (Table 2). Regarding the rest of the fragile sites not conserved among Primates, we cannot discard an important fragile site feature: they are not expressed in 100% of the metaphases analyzed. In contrast with what happens with human fragile sites, studies on fragile site induction in non-human Primates are restricted to a low number of species analyzed. For this reason we can suppose that

there are probably more fragile sites not detected up to now that can be conserved in other Primate species.

Fragile sites and evolutionary chromosome reorganizations.

The chromosome homologies among *C. apella*, *C. nigrivittatus* and humans based on G-banding comparison and ZOO-FISH as well as the evolutionary reorganizations that explain these, homologies have recently been described (García et al., 2000; 2002). The comparative study has shown that at least 30% of evolutionary breakpoints detected in the *C. apella* karyotype, when it is compared with human chromosomes, express fragile sites (Table 2). When the *C. apella* karyotype is compared with other Primate species (*Cebus* species, *Ateles belzebuth hybridus*, *Lagothrix lagothricha*, Cercopithecidae species and great apes), some evolutionary reorganizations are located within chromosome bands that express fragile sites (Table 2, Figure 4). There are fragile sites located at fusion/fission points (CAP1p14, CAP2q13, CAP2q24, CAP3q22.1, CAP6q23, CAP13q24, CAP14q13, CAP15q24 and CAP15q32), at inversion points (CAP4q32, CAP5q13, CAP7q14, CAP6p13, CAP8q14, CAP10q12, CAP12q22, CAP15q22, CAP16q13 and CAP19q21) and other fragile sites that correspond to centromeric shifts (CAP2q32, CAP5q15 and CAP17q23). In conclusion, at least, 41.51% of *C. apella* fragile sites are located in evolutionary breakpoints when compared with some Platyrrhini and Catarrhini species. It is important to note that more than 60% of these *C. apella* fragile sites correspond to human fragile sites in the corresponding homologous chromosomes, and more than 35% correspond to *M. fascicularis* fragile sites.

From the five fragile sites conserved in *C. apella*, *M. fascicularis*, *M. sphinx*, great apes (gorilla, chimpanzee and orangutan) and man, three of them (HSA1p22; HSA4q31 and HSA7q32.3) correspond to fission/fusion points of two chromosomes in two Platyrrhini species (*Ateles belzebuth hybridus* and/or *Lagothrix lagothricha*) (Table 2, Figure 5). The fact that, in all seven species, these chromosome bands express a fragile site suggests that these loci can be considered "targets" for evolutionary reorganizations, because of their tendency to break (Ruiz-Herrera et al., 2002a). The situation of fragile sites HSA4q27 (conserved in *C. apella*, *M. fascicularis*, *M. sphinx*) and HSA7q22 (conserved in *C. apella*, *C. nigrivittatus*, *M. fascicularis*, *M. sphinx*) correspond to the fission points of two *Ateles belzebuth hybridus* and *Cercopithecus aethiops* chromosomes, respectively (Table 2).

Table 2: Co-localization of human (HSA) (Human Gene Mapping, 1991), *Cebus apella* (CAP), *Cebus nigrivittatus* (CNI), *Macaca fascicularis* (MFA) (Ruiz-Herrera *et al.*, 2002a) and *Mandrillus sphinx* (MSP) aphidicolin-inducible common fragile sites (FS) and the relationship between these *loci* and the chromosome bands involved in chromosomal evolution. The evolutionary reorganizations have been defined taking the putative ancestral Primate karyotype into account (Müller et al., 1999).

CAP FS	CNI FS	HSA FS	MFA FS	MSP FS	Evolutionary change	Reference
-	-	1p32 [b]	1q32	-	cen CNIc7	Ruiz-Herrera et al., 2002a
14q13	-	1p22 [a]	1q22	1q22	fis LLA9/LLA28	Ruiz-Herrera et al., 2002b
					fis ABH2/ABH4	García et al., 2002
-	-	1q31	-	-	fus/fis CAP22/CAP23	Ruiz-Herrera et al., 2002b
					fus/fis LLA25/LLA26	Stanyon et al., 2001
-	-	-	-	1p31	inv HSA1	Ruiz-Herrera et al., 2002b
-	-	1q44 [a]	1p35	1p35	-	-
5q15	-	2p13	9q12	-	cen LLA2	Miró et al., 1987
						Ruiz-Herrera et al., 2002a
5q24	-	-	9q22	9q22	-	-
5q26	-	-	9q24	9q24	-	-
5q13	-	2q11.2	-	-	inv CAP5	García et al., 2000
-	-	2q13	-	-	fus CAP5/CAP13	García et al., 2000
					fus LLA2/LLA15	Stanyon et al., 2001
					fus two Hominidae chrs	Yunis and Prakash, 1982
					fus two Cercopithecidae chrs	Clemente et al., 1990
					fus two *Ateles* chrs	García et al., 2002
13q24	-	2q31	-	-	fis ABH3/ABH14	García et al., 2002
-	-	2q21.3	-	-	cen Cercopithecidae	Clemente et al., 1990
					cen PTR2, GGO2	Yunis and Prakash, 1982
-	-	2q33 [b]	15q21	-	-	Ruiz-Herrera et al., 2002a
-	-	2q37.3 [a]	15q25	-	-	Ruiz-Herrera et al., 2002a
-	-	-	3q21	3q21	-	-
-	-	-	3q22	3q22	-	-
-	-	-	3q23	3q23	-	-
-	-	-	3q25	3q25	-	-
-	-	-	3q32	3q32	-	-
18q23	18q23	-	-	-	-	-
2q43	-	4p16.1	-	-	-	-
-	-	-	4p24	4p24	-	-
2q32	-	-	4p21	4p21	cen HSA4	García et al., 2000
2q26	2q26	-	-	-	-	-
2q24	-	4q27	4q23	4q23	fis ABH2/ABH9	García et al., 2002
2q13	2q13	4q31 [a]	4q31	4q31	fis ABH2/ABH15	García et al., 2002
					fis LLA14/LLA24	Stanyon et al., 2001
-	-	-	4q32	4q32	-	-
1p14	1p14	-	-	-	fus HSA5/HSA7	García et al., 2000
-	-	5p14	5p13	5p13	-	-
-	-	5p13	5p12	-	-	-
1q32	-	-	5q17	5q17	-	-
-	-	5q31.1	5q31	-	inv CAP1	García et al., 2000
					fis ABH5/ABH9	García et al., 2002
					fis LLA3/LLA11	Stanyon et al., 2001
-	-	6p25 [a]	-	6q27	-	-
3q22.1	-	6q13	-	-	fis ABH7/ABH10	García et al., 2002
					fis CAE13/CAE17	Finelli et al., 1999
3q24	-	6q21	-	-	-	-
-	-	6q26	6p14	6p14	-	-

Table 2: continued.

CAP FS	CNI FS	HSA FS	MFA FS	MSP FS	Evolutionary change	Reference
-	-	6q15	6q14	6q14	-	-
-	-	-	6q23	6q23	-	-
15q22	-	7p22 [a]	-	-	inv HSA7	García et al., 2000
-	-	7p13 [a,b]	2q13	-	-	Ruiz-Herrera et al., 2002a
-	-	7p11.2	2q11	-	-	Ruiz-Herrera et al., 2002a
-	-	-	2q15	2q15	-	-
-	-	7q11	-	-	inv HSA7	Ruiz-Herrera et al., 2002b
-	-	-	2q18	2q18	inv HSA7	Ruiz-Herrera et al., 2002a
15q24	15q24	7q22	2q22	2q22	fis CAE21/CAE28	Finelli et al.,1999
-	-	-	2q23	2q23	-	-
15q32	15q32	7q32.3 [a,b]	2q24	2q24	fis LLA11/LLA16	Stanyon et al., 2001
-	-	-	2q25	2q25	-	-
-	-	-	-	8p14	inv CAP8	García et al., 2000
					inv ABH1	García et al., 2002
8q14	-	8q22	8q22	8q22	inv GGO8	Yunis and Prakash, 1982
						Ruiz-Herrera et al., 2002a
8q16	-	8q24.1	8q24	-	-	Ruiz-Herrera et al., 2002a
19q21	-	9q22.1	-	-	inv PTR9	Yunis and Prakash, 1982
-	-	10q22 [b]	10q14	-	inv MFA10	Ruiz-Herrera et al., 2002a
4q32	-	-	-	-	inv MFA10	-
					inv CAP4	García et al., 2000
-	-	10q25	10q23	-	-	Ruiz-Herrera et al., 2002a
-	-	11p13 [b]	11p21	11p21	-	-
-	-	11p14 [a]	-	11p22	-	-
-	16q26	11p11	-	-	inv CAP16	García et al., 2000
					inv MFA11	Ruiz-Herrera et al., 2002a
					inv ABH11	García et al., 2002
16q13	-	11q13	11p12	-	inv PSP11, EPA16, MTA15	Clemente et al., 1990
					inv CMC4, CPE8	Clemente et al., 1990
					inv PPY11	Yunis and Prakash, 1982
					inv MFA11	Ruiz-Herrera et al., 2002a
16q23	-	-	11q21	-	-	-
-	16q24	11q23	-	11q23	inv ABH11	Garcia et al., 2002
12q22	-	-	-	-	inv CAP12	García et al., 2000
					inv ABH2/ABH16	Garcia et al., 2002
12q32	12q32	-	-	-	-	-
-	-	-	14q22	-	inv MFA14	Ruiz-Herrera et al., 2002a
-	-	13q13.1	16q23	16q23	-	Ruiz-Herrera et al., 2002a
17q22	-	13q21.1	16q13	-	-	Ruiz-Herrera et al., 2002a
17q23	-	-	-	-	cen MFA16	-
-	-	13q32	16p13	-	-	Ruiz-Herrera et al., 2002a
6p13	-	-	-	-	inv HSA14/HSA15	García et al., 2000
					inv CAP6	García et al., 2002
6q23	-	-	-	-	fus/fis HSA14/HSA15	García et al., 2000
-	6q16	-	-	-	inv CNI6	García et al., 2002
-	-	-	7q13	7q13	-	-
-	-	14q21	7q21	-	-	Ruiz-Herrera et al., 2002a
-	-	-	7q23	7q23	-	-
6q24	-	14q24.1 [a]	7q25	7q25	-	Ruiz-Herrera et al., 2002a
6q26	6p21	-	-	-	-	-
5p14	5p14	16q22.1 [a]	20q15	20q15	-	Ruiz-Herrera et al., 2002a
4p14	-	16p13.1	-	-	-	-

Table 2: continued.

CAP FS	CNI FS	HSA FS	MFA FS	MSP FS	Evolutionary change	Reference
-	-	16q23.2 [a]	20q16	-	-	Ruiz-Herrera et al., 2002a
-	-	17q23.1	17q14	-	inv ABH13	García et al., 2002
-	-	18q12.2	18q13	18q13	-	-
-	-	-	18q21	18q21	-	-
7q14	-	-	-	-	inv CAP7	García et al., 2000
7q24	-	18q21.3	-	-	-	-
-	-	-	18q23	18q23	-	-
10q12	-	20p11.2	-	-	inv CAP10	García et al., 2002
-	-	20p12.2	13q23	13q23	inv HSA20	Ruiz-Herrera et al., 2002a
11q13	-	-	2p22	-	-	-
11q15	11q15	-	-	-	-	-
-	-	-	13q23	13p23	-	-
-	-	22q12 [a,b]	-	13p21	-	-
-	-	-	13q12	13q12	-	-
Xp22	-	Xp22.31[a]	Xp22	Xp22	-	Ruiz-Herrera et al., 2002a
Xq22	-	Xq22.1[a,b]	Xq22	Xq22	-	Ruiz-Herrera et al., 2002a

Abbreviations: cen, centromere; inv, inversion; fus, fusion; fis, fission; chrs, chromosomes; ABH, *Ateles belzebuth hybridus*; CNIc, *Cercopithecus nictitans*; CMC, *Cercopithecus mona campbelli*; CPE, *Cercopithecus petaurista*; CAE; *Cercopithecus aethiops*; EPA, *Erythrocebus patas*; GGO, *Gorilla gorilla*; LLA, *Lagothrix lagothricha*; MTA, *Miopithecus talapoin*; PTR, *Pan troglodytes*; PSP, *Papio sphinx*; PPY, *Pongo pygmaeus*.

[a] Homologous chromosome bands that express fragile sites in great apes (Smeets and Van de Klundert, 1990).
[b] Homologous chromosome bands that express fragile sites in the mouse genome (Djalali et al., 1987).

One of the fragile sites most affected by breakage in *C. apella* and in *C. nigrivittatus* is 18q23, with 24% of total abnormalities detected. *C. apella* chromosome 18 is homologous to a region of human chromosome 3 (Richards et al., 1996; García et al., 2000). Due to the highly complex evolution of the chromosomes homologous to human chromosome 3 during the evolution of Old World monkeys (Müller et al., 2000), the G-banding assignment of fragile site homology between *Cebus* and human based on ZOO-FISH results is difficult to establish. Fragile site 18q23 is the most expressed in all specimens analyzed and we assume that this fragile site should have played an important role in chromosome evolution.

Evolutionary co-location of intrachromosomal telomeric sequences in Primates.

A study of the correspondence between chromosome bands that contain ITSs in *C. apella*, *M. fascicularis* and human chromosomes has shown that there are 9 ITSs (16.36% of the *C. apella* ITSs detected) localized in the homologous chromosome bands which are represented in human chromosomes 1, 6, 7, 8, 10, 12 and 13 (Table 3). Between *C. apella* and man there are 18 ITSs (32.72% of the *C. apella* ITSs detected) located in homologous chromosome bands and between *C.*

apella and *M. fascicularis* there are 12 ITSs (21.82% of the *C. apella* ITSs detected) located in homologous chromosome bands (Table 3). These telomeric repeats probably have been conserved during karyotype evolution in chromosome bands that have not been affected by reorganizations during the evolutionary process. They could also be potential new telomeres for the establishment of future reorganizations and could be present in the putative ancestral Primate karyotype. This is the case of ITSs conserved in the chromosomes homologous to human chromosome 7, where the corresponding chromosomes of *C. apella* and *M. fascicularis* have an equivalent banding pattern that can be considered similar to the ancestral chromosome number 7 (Ruiz-Herrera et al., 2002b). The ITS located in human chromosome band 7q32.3 is conserved in the *M. fascicularis* and *C. apella* homologous chromosome bands (Table 3); in all three species, this chromosome band expresses fragile sites, and corresponds to the fission point for chromosomes LLA11/LLA16 (Table 2).

ITSs and their implication in chromosome evolution.

Cebus has been considered to have a primitive karyotype. For this reason, we consider the study of the distribution of ITSs in this species as highly interesting in order to better understand the evolutionary process in Primates. In the *C. apella* karyotype, ITSs have been detected in 55 different chromosome bands, less than in *M. fascicularis* (90 ITSs; Ruiz-Herrera et al., 2002b) and human (103 ITSs; Azzalin et al., 1997). These results show that, apparently, there is a tendency to increase the number of ITSs during evolution, supporting the hypothesis proposed by Meyne et al. (1990).

Some hypotheses that could explain the origin of ITSs found in the karyotype of different mammalian species have been reported. Among the different mechanisms possibly implicated (gene amplification, unequal crossing-over, insertion of telomeric repeats at sites of double-strand breaks during the repair by telomerase), ITSs can be considered the result of evolutionary reorganizations, such as fusions or intrachromosomal reorganizations, and/or unstable loci where chromosomal rearrangements might occur (Ruiz-Herrera et al., 2002b). This last possibility could provide alternative sites for telomere formation within chromosomes (Meyne et al., 1990). Experimental evidence in telomerase null mice (mTrec$^{-/-}$) have shown that chromosomes with eroded telomeres fuse to radiation-induce breaks (Latre et al., in press).
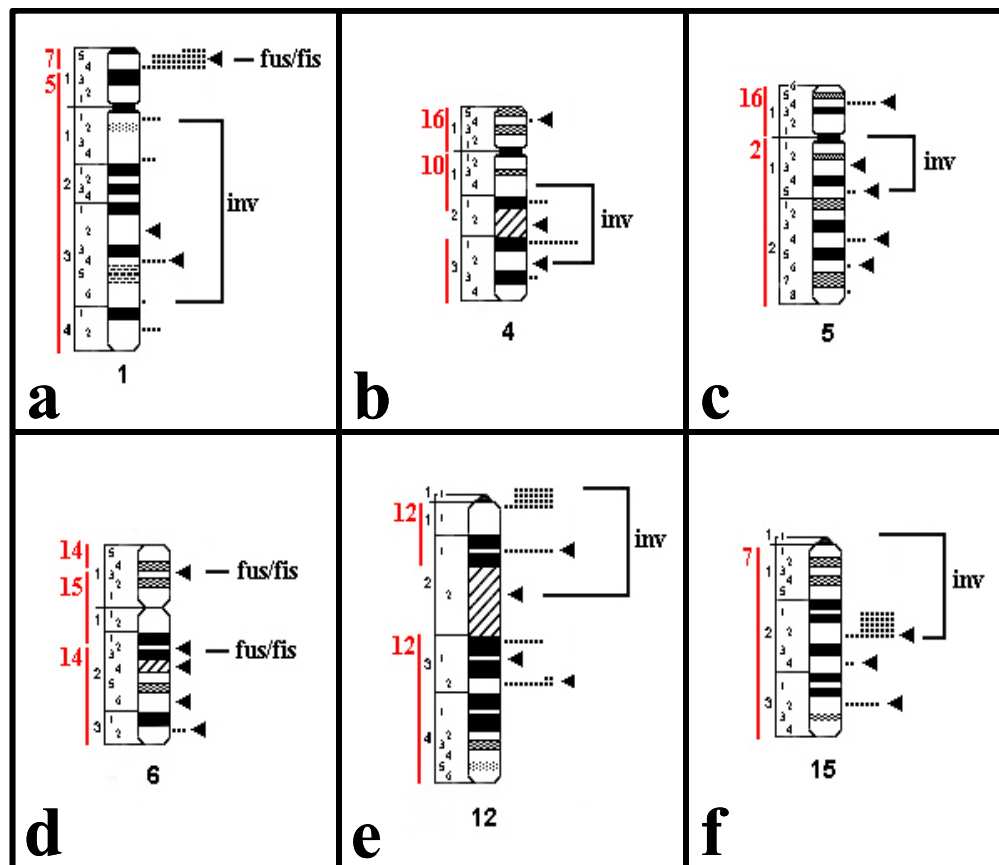
Figure 4: Idiograms showing some examples of evolutionary chromosome rearrangements localized in chromosome bands that express fragile sites and/or contain ITSs (intrachromosomal telomeric sequences) in Cebus apella chromosomes. Each dot shows a hybridization signal in both chromatids and arrowheads indicate the location of fragile sites. Homology with human chromosomes is shown on the left. (a) The chromosome band 1p14 implicated in the fusion expresses a fragile site and contains ITSs. Regarding the chromosome bands implicated in the inversion (1q11; 1q36), both contain ITSs. (b) C. apella chromosome 4 is homologous to human chromosomes 10 and 16 by a centromeric fusion and a paracentric inversion. One of the inversion points (4q32) expresses a fragile site. (c) The chromosome band implicated in the inversion (5q15) expresses a fragile site and contains ITSs. (d) C. apella chromosome 6 is homologous to human chromosomes 14 and 15. At the fusion/fission points fragile sites are expressed. (e) The chromosome band implicated in the inversion (12q22) expresses a fragile site and corresponds to a heterochromatic region. (f) The chromosome band implicated in the inversion (15q22) expresses a fragile site and contains ITSs.

In this sense, we have found two interesting examples in the *C. apella* karyotype that could support the hypothesis that consider ITSs the result of evolutionary reorganizations and/or unstable loci. *C. apella* chromosomes 1 and 11 contain associated fragments of HSA5/HSA7 and HSA3/HSA21, respectively (Figure 2a). In the ancestral Primate karyotype the two chromosomes homologous to HSA5 and HSA7 were not fusioned, whereas in the putative ancestral Platyrrhini karyotype the association of these two chromosomes is described (Neusser et al., 2001). In the

limit of the conserved chromosome segments in *C. apella*, chromosome band CAP1p14, a fragile site is expressed and an ITS has been detected very frequently, suggesting that the telomeric sequences are landmarks of the fusion events, and that these sequences have been retained after the reorganization. On the other hand, the HSA3/HSA21 association has been considered an ancestral trait, even present in the ancestral Primate karyotype (Richard and Dutrillaux, 1998; Chowdhary et al., 1998; Müller et al., 1999). The fact that in the limit of the conserved chromosome segments in *C. apella* another very frequent ITS has been detected suggests that in this locus the presence of telomeric repeats could provide alternative sites for telomere formation that would stabilize chromosomal reorganizations.
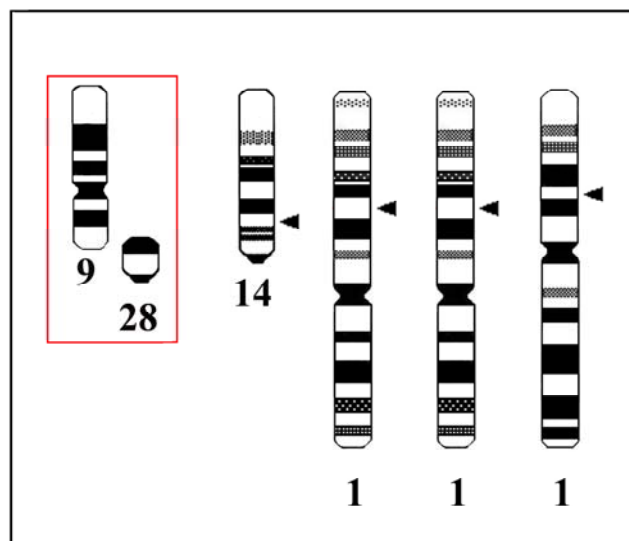
Figure 5: Ideograms showing the conservation of human (HSA) fragile site HSA1p22 located in the same chromosome band in the homologous *M. fascicularis* (MFA), *M. sphinx* (MSP) and *C. apella* (CAP) chromosomes. This chromosome band corresponds to the fission/fusion point of two chromosomes of *L. lagothricha* (LLA) (Ruiz-Herrera et al., 200b). Arrowheads indicate the location of fragile sites.

Regarding *C. apella* chromosomes 4, 5 and 7, where the junction of segments homologous to human chromosomes are located at the centromere level, telomeric sequences have not been detected. Chromosome CAP4 shows the HSA10/HSA16 association, whereas chromosomes CAP5 and CAP7 show the HSA2/HSA16 and HSA8/HSA18 associations, respectively (Figure 2a). These three associations are present in the putative ancestral Platyrrhini karyotype derived from centromeric fusions from the putative ancestral Primate karyotype (García et al., 2000; Neusser et al., 2001). This cytogenetic evidence allows us to consider that, when the chromosome

associations are ancestral and the limit is at the centromeric level, ITSs are not present. In the case of *M. fascicularis*, chromosomes MFA2 and MFA13 are the result of the fusion of two human chromosomes, 7/21 and 20/22, respectively. These are examples where the telomeric sequences have been retained after the fusion of ancestral chromosomes (Ruiz-Herrera et al., 2002b). These findings support the hypothesis that interstitial telomeric sequences should be considered as remnants of ancestral chromosomal rearrangements during karyotype evolution.

Focusing on intrachromosomal reorganizations, as described in the *M. fascicularis* karyotype (Ruiz-Herrera et al., 2002b), ITSs can be considered unstable loci where chromosome breaks for inversions can occur. This is the case of ITSs CAP8q14, CAP10q12 and CAP15q22, all of which are located in inversion points when *C. apella* is compared with other Primate species (Table 2, Figure 4).

Inter-individual differences of telomeric hybridization signals in *C. apella*.

The results obtained from the analysis of the distribution of intrachromosomal telomeric repeats in the three specimens of *C. apella* studied have revealed an interesting finding. The ITS located at the 11q11 locus is present in the three specimens, but the hybridization signals have different intensities. In all of the 81 metaphases analyzed from Specimen 2, a large telomeric signal at 11q11 is observed (Figure 3e), suggesting an array mechanism of telomeric repeat amplification. Mondello et al. (2000) described a variability in the length of four interstitial telomeric sequences in a human population. A molecular analysis of these sequences revealed the presence of a different number of TTAGGG repeats in these loci. In the case of the *C. apella* karyotype, the difference in FISH signal intensity is probably a consequence of the number of telomeric repeats localized in this locus, although it remains to be confirmed by molecular analysis. In any case, interstitial telomeric sequences, like other microsatellites, show variations in the length of telomeric repeats that can be due to polymerase template slippage or recombination mechanisms (Mondello et al., 2000).

Heterochromatin regions on *Cebus* chromosomes, fragile sites and ITSs.

An important characteristic of the genus *Cebus* is the high proportion of constitutive heterochromatin (10%-15% of the haploid genome) located in the centromeric, terminal as well as interstitial regions of certain chromosomes (Ponsà et al., 1995; García et al., 1999). Extensive cytogenetic studies have reported a wide chromosomal variability of heterochromatin polymorphisms in *Cebus* species (Garcia et al., 1978; Mudry de Pargament et al., 1985; Ponsà et al., 1995; García et al., 1999; 2003). In particular, variations in size of interstitial heterochromatic bands in chromosomes 4, 6, 11, 12, 13, 17, 18, 19 and 20 have been described in *C. apella*, as well

as paracentric inversions involving these regions (Ponsà et al., 1995; García et al., 1999). In the case of *C. apella* and *C. nigrivittatus*, all interstitial heterochromatic regions interrupt chromosomal regions that are homologous to one single human chromosome.

The fact that the 4q22, 6q24, 12q22, 13q24, 17q21 and 18q15 interstitial heterochromatin regions, as well as the 11q15 terminal heterochromatin block express fragile sites in *C. apella* and *C. nigrivittatus* karyotypes (Figures 2a and 2b), suggests an implication of these fragile sites in the high heterochromatin variability described in these species. The 11q15 fragile site is one of the most affected by breaks and/or other chromosomal abnormalities both in *C. apella* and *C. nigrivittatus* karyotypes (24% of total abnormalities). This fragile site is always located in the same position in the heterochromatic block in relation to the centromere (11q15 proximal) and corresponds to a heterochromatin region affected by a high frequency of gaps/breaks in cultures treated by aphidicolin, as well as in control cultures, in all specimens studied. Molecular studies of cloned, human common fragile sites have revealed that the DNA sequence at these loci adopts structures with high flexibility and low stability (Mishmar et al., 1998; Mangelsdorf et al., 2000); these features would be implicated in chromosome instability observed in these regions.

In contrast with what happens in other mammalian species (Meyne et al., 1990; Garagna et al., 1997; Hirai, 2001), interstitial heterochromatin regions as well as the large heterochromatin block from chromosome 11 in the *C. apella* and *C. nigrivittatus* karyotypes do not hybridize with the telomeric probe. In these species, the constitutive heterochromatin regions do not contain the telomeric sequence (TTAGGG)n.

Chromosome bands significantly affected by X-irradiation and ITSs vs. fragile sites.

In order to support previous suggestions from cytogenetic studies showing a relationship between the location of ITS and fragile sites (Farr et al., 1991; Musio et al., 1996; Ruiz-Herrera et al., 2002b; Zou et al., 2002), the distribution of *C. apella* ITSs was compared with the location of aphidicolin-induced fragile sites. Out of the 55 *C. apella* chromosome bands that contain ITSs described in this work, 27 (49.09%) express fragile sites (Figure 2a, Table 3). These results support the previous study in the M. fascicularis (Ruiz-Herrera et al., 2002a), where 51.14% of chromosome bands where ITSs are located express aphidicolin-induced fragile sites. This association between fragile sites and telomeric sequences is also supported by recent results in the Chinese hamster, which suggest the insertion of intrachromosomal telomeric sequences within unstable regions (Faravelli et al., 2002). So, the presence of ITSs in certain chromosome bands can be considered as a "marker" of instability (Azzalin et al., 2001).

Table 3: Correspondence between intrachromosomal telomere-like sequences (ITS) in human (HSA), *M. fascicularis* (MFA) and *C. apella* (CAP) chromosomes.

ITS-CAP	ITS-HSA	ITS-MFA	ITS-CAP	ITS-HSA	ITS-MFA
1p14 [a,b]	-	-	12q21 [b]	-	12p13
1q11	-	-	12q31	-	-
1q14	-	-	12q32 [a,b]	12q13	12q13 [b]
1q34 [a,b]	-	-	-	12q21 [b]	12q21
1q36	5q31.1 [a,b]	-	-	12q23	12q23
1q42	-	-	-	2q23	15q13
2p12 [b]	-	-	-	2q33 [a,b]	15q21 [a,b]
-	4p15 [b]	4p24 [b]	-	2q35	15q23 [b]
-	4q25	4q21 [b]	-	6p21	6q23 [a,b]
2q24 [b]	-	4q23 [b]	-	6cen	6q21
2q26 [b]	-	-	-	6q21 [b]	6q12
2q32 [b]	-	-	-	5q23	5q23
2q43 [b]	-		-	1p35	1q34
3p13	6p22.2	-	14q24	1p32 [a,b]	1q32 [b]
3q21 [b]	-	-	14q11	1q21 [a,b]	1q12
3q22 [b]	-	-	22q11	-	-
3q33	-	-	-	1p22 [a,b]	1q22 [b]
3q36	6q25	6p13	-	1q23	1q14 [b]
4p14 [b]	16p13.1	-	-	1q25 [b]	1p22
4q21	-	-	22q16 [b]	-	-
4q31	10q21	10q21	-	1q32	1p31
-	10q22 [b]	10q14 [b]	-	11q13 [a,b]	11p12 [b]
4q33	10q25 [b]	-	-	11q23 [b]	11q23
5p14 [b]	-	20q15 [b]	-	7p15	2q14 [b]
5q15 [b]	2p13 [b]	-	15q22 [b]	-	-
5q24 [b]	-	-	15q24 [b]	-	-
5q26 [b]	-	-	-	7q21 [b]	2q21
5q28	-	-	15q32 [b]	7q32.3 [a,b]	2q24 [b]
6q32 [b]	-	-	-	13q32 [b]	16p13 [b]
7p14	8p21	8p13	17q22 [b]	13q21.1 [b]	16q13 [b]
7q12	-	-	18q13	-	-
7q24 [a,b]	18q21 [b]	-	18q25	-	-
8p14	8q13	8q14	-	9p21	14q23
8p12	-	-	-	9q11	14q12
8q12 [a]	-	-	-	9q22 [b]	14q14
8q14 [b]	8q22 [b]	-	-	9q31	14q21
8q16 [b]	8q24.1 [a,b]	-	21q11	17q12	-
10q12 [b]	-	-	21q14	-	-
11q11	-	-	26q11	-	-
11q14	-	-	Xp22 [b]	-	-
12q11	-	-	-	Xq21	Xq21

[a] Chromosome bands with a significantly higher number of breaks after X-irradiation (Barrios *et al.*,1989; Borrell *et al.*, 1998a; Borrell *et al.*, 1998b).
[b] Chromosome bands that express aphidicolin-induced fragile sites (Human Gene Mapping, 1991; Ruiz-Herrera et al., 2002b; this report).

Out of the 26 chromosome bands significantly affected by X-irradiation in the *C. apella* karyotype (Borrell et al., 1998a), 12 (46.15%) are co-localized within fragile sites. This situation is quite different from that of *M. fascicularis* karyotype, where only 26.32% of chromosome bands significantly affected by X-irradiation are located in fragile sites (Borrell et al., 1998b; Ruiz-Herrera et al., 2002a). It is difficult to understand the relationship between fragile sites and breakpoints induced by X-ray when we compare this to the situation with the human karyotype, where a significant number of X-ray induced breaks (65%) (Barrios et al., 1989) coincide (p<0.005) with the location of fragile sites.

Overview of evolutionary breakpoints, fragile sites and ITSs.

Since fragile sites have been considered a universal mammalian genome feature, we have demonstrated that approximately 40% of *C. apella*, *C. nigrivittatus* and *M. sphinx* aphidicolin-induced fragile sites are conserved in the homologous human chromosomes. This could lead to the conclusion that these fragile sites were probably present in the putative ancestral Primate karyotype. The fact that a high number of chromosomal reorganizations among different Platyrrhini and Catarrhini species are located at chromosome bands which express fragile sites and/or contain ITSs suggests a relationship among these phenomena: evolutionary breakpoints, fragile sites and telomeric sequences. In the comparative study presented here, some ITSs correspond to inversion and fusion/fission points and some cytogenetic evidence of this relationship is represented in Table 2 and Figure 4. *C. apella* chromosome 1 is homologous to human chromosomes 5 and 7 by a paracentric inversion and a fusion/fission (Figure 4a). The chromosome bands involved in the inversion contain ITSs and the fusion/fission point contains ITSs and also expresses an aphidicolin-induced fragile site. This is the same situation as *C. apella* chromosome 15 that is homologous to human chromosome 7 by a pericentric inversion (Figure 4f). The inversion point contains ITSs and expresses an aphidicolin-induced fragile site.

It seems that evolutionary breakpoints in Primates are "concentrated" in some particular chromosomal regions or loci (those where their particular characteristics induce chromosomal breakage), since at least 40% of *C. apella* chromosome bands that express fragile sites are evolutionary breakpoints. A comparison with other Primate species located in different branches of the evolutionary tree will allow for the support or rejection of this conclusion.

ACKNOWLEDGMENTS

REFERENCES

Azzalin C.M., Mucciolo E., Bertoni L., Giulotto E. 1997. Fluorescence in situ hybridization with a synthetic (T2AG3)n polynucleotide detects several intrachromosomal telomere-like repeats on human chromosomes. Cytogenet Cell Genet 78:112--115.

Azzalin C.M., Nergadze S.G., Giulotto E. 2001. Human intrachromosomal telomeric-like repeats: sequence organization and mechanisms of origin. Chromosoma 110:75--82.

Barrios L., Miró R., Caballín M.R., Fuster C., Guedea F., Subias A., Egozcue J. 1989. Cytogenetic effects of radiotherapy. Breakpoint distribution in induced chromosome aberrations. Cancer Genet Cytogenet 41:61--70.

Böhm U., Dahm P.F., McAllister B.F., Greebaum I.F. 1995. Identifying chromosomal fragile sites from individuals: a multinomial statistical model. Hum Genet 95:249--256.

Borrell A., Ponsà M., Egozcue J., Rubio A., Garcia M. 1998a. Chromosome abnormalities in peripheral blood lymphocytes from *Cebus apella* (Cebidae, Platyrrhini) after X-irradiation. Mutat Res 401: 65--76.

Borrell A., Ponsà M., Egozcue J., Rubio A., Garcia M. 1998b. Chromosome abnormalities in peripheral blood lymphocytes from *Macaca fascicularis* and *Erythrocebus patas* (Cercopithecidae, Catarrhini) after X-irradiation. Mutat Res 403: 185--198.

Brown C.J., Dunbar V.G., Shafer D.A. 1986. A comparison of the karyotypes of six species of the genus *Macaca* and the species of the genus *Cercocebus*. Folia Primatol 46: 164--172.

Chowdhary B.P., Raudsepp T., Frönicke L., Scherthan H. 1998. Emerging patterns of comparative genome organization in some mammalian species as revealed by Zoo-FISH. Genome Res 8: 577--589.

Clemente I.C., Ponsà M., Garcia M., Egozcue J. 1990. Chromosome evolution in the Cercopithecidae and its relationship to human fragile sites and neoplasia. Int J Primatol 11: 377--398.

Djalali M., Adolph S., Steinbach P., Winking H., Hameister H. 1987. A comparative mapping study of fragile sites in the human and murine genomes. Hum Genet 77:157--162.

Dutrillaux B., Biemont M.C., Viegas-Pequignot E., Laurent C. 1979. Comparison of the karyotypes of four Cercopithecoidea: *Papio papio*, *P. anubis*, *Macaca mulatta*, and *M. fascicularis*. Cytogenet Cell Genet 23: 77--83.

Dutrillaux B. and Couturier J. 1981. The ancestral karyotype of platyrrhine monkeys. Cytogenet Cell Genet 30: 232--242.

Fagundes V. and Yonenaga-Yassuda Y. 1998. Evolutionary conservation of whole homeologous chromosome arms in the Akodont rodents *Bolomys* and *Akodon* (Muridae, Sigmodontinae): maintenance of interstitial telomeric segments (ITBs) in recent event of centric fusion. Chromosome Res 6: 643--648.

Faravelli M., Azzalin C.M., Bertoni L., Chernova O., Attilini C., Mondello C., Giulotto E. 2002. Molecular organization of internal telomeric sequences in Chinese hamster chromosomes. Gene 283: 11--16.

Farr C., Fantes J., Goodfellow P., Cooke H. 1991. Functional reintroduction of human telomeres into mammalian species. Proc Natl Acad Sci USA 88: 7006--7010.

Finato A.O., Varella-Garcia M., Tajara E.H., Taddei V.A., Morielle-Versute E. 2000. Intrachromosomal distribution of telomeric repeats in *Eumops glaucinus* and *Euntops perotis* (Molossidae, Chiroptera). Chromosome Res 8: 563--569.

Finelli P., Stanyon R., Plesker R., Ferguson-Smith M.A., O'Brien P.C.M., Wienberg J. 1999. Reciprocal chromosome painting shows that the great difference in diploid number between human and African green monkey is mostly due to non-Robertsonian fissions. Mamm Genome 10:713--718.

Fundia A., Gorostiaga M., Delprat A., Mudry M. 1991. Fragile sites analysis and definition of chromosome landmarks, regions and bands in *Alouatta caraya* (ACA). Primatology Today. Elsevier Science Publishers B.V.(Biomedical Division) pp. 617--618.

Fundia A., Gorostiaga M., Mudry M. 2000. Expression of common fragile sites in two Ceboidea species: *Saimiri boliviensis* and *Alouatta caraya* (Primates: Platyrrhini). Genet Sel Evol 32: 87--97.

Garagna S., Ronchetti E., Mascheretti S., Crovell S., Formenti D., Rumpler Y., Manfredi Romanini M.G. 1997. Non-telomeric chromosome localization of (TTAGGG)n repeats in the genus *Eulemur*. Chromosome Res 5: 487--491.

Garcia M., Miro R., Freitas L., Egozcue J. 1978. Banding patterns of the chromosomes of *Cebus apella*: unstable chromosomes and pericentric inversion. Folia Primatol 29: 196--205.

García F., Nogués C., Garcia M., Egozcue J., Ponsà M. 1999. Characterization of constitutive heterochromatin in *Cebus apella* (Cebidae, Primates) and *Pan troglodytes* (Hominidae, Primates): comparison to human chromosomes. Am J Primatol 49: 205--21.

García F., Nogués C., Ponsà M., Ruiz-Herrera A., Egozcue J., Garcia Caldés M. 2000. Chromosomal homologies between humans and *Cebus apella* (Primates) revealed by ZOO-FISH. Mamm Genome 11: 399--401.

García F., Ruiz-Herrera A., Egozcue J., Ponsà M., Garcia M. 2002. Chromosome homologies between *Cebus* and *Ateles* (Primates) based on ZOO-FISH and G-banding comparisons. Am J Primatol 57: 177--188.

García F., Garcia M., Mora L., Alarcón L., Egozcue J., Ponsà M. 2003. Qualitative analysis of constitutive heterochromatin and Primate evolution. Biol J Linn Soc (in press).

Glover T.W. and Stein C.K. 1988. Chromosome breakage and recombination at fragile sites. Am J Hum Genet 43: 265--273.

Go Y., Rakotoarisoa G., Kawamoto Y., Randrianjafy A., Koyama N., Hirai H. 2000. PRINS analysis of the telomeric sequence in seven lemurs. Chromosome Res 8: 57--65.

Handt O., Sutherland G.R., Richards R.I. 2000. Fragile sites and minisatellite repeats instability. Mol Genet Metab 70:99--105.

Hirai H. 2001. Relationship of telomere sequence and constitutive heterochromatin in the humans and apes as detected by PRINS. Methods Cell Science 23: 29--35.

Human Gene Mapping 11. 1991. Cytogenet Cell Genet **58:**1--2200.

Ijdo J.W., Baldini A., Ward D.C., Reeders S.T., Wells R.A. 1991. Origin of human chromosome 2: an ancestral telomere-telomere fusion. Proc Natl Acad Sci USA 88: 9051--9055.

Lee C., Sasi R., Lin C.C. 1993. Interstitial localization of telomeric DNA sequences in the Indian muntjak chromosomes; further evidence for tandem chromosome fusions in the karyotype evolution of the asian muntjaks. Cytogenet Cell Genet 63: 156--159.

Mangelsdorf M., Ried K., Woollatt E., Dayan S. Eyre H., Finnis M., Hobson L., Nancarrow J., Venter D., Baker E. et al. 2000. Chromosomal fragile site FRA16D and DNA instability in cancer. Cancer Res 60: 1683--1689.

McAllister B. and Greenbaum I.F. 1997. How common are common fragile sites: variation of aphidicolin-induced chromosomal fragile sites in a population of the deer mouse (*Peromyscus maniculatus*). Hum Genet 100: 182--188.

Metcalfe C.J., Eldridge M.D., McQuade L.R., Johnston P.G. 1997. Mapping the distribution of the telomeric sequence (T2AG3)n in rock-wallabies, *Petrogale* (Marsupialia: Macropodidae), by fluorescence in situ hybridization. I. The penicillata complex. Cytogenet Cell Genet 78: 74--80.

Metcalfe C.J., Eldridge M.D., Toder R., Johnston P.G. 1998. Mapping the distribution of the telomeric sequence (T2AG3)n in the Macropodoidea (Marsupialia), by fluorescence in situ hybridization. I. The swamp wallaby, *Wallabia bicolor*. Chromosome Res 6: 603--610.

Meyne J., Ratliff R.L., Moyzis R.K. 1989. Conservation of the human telomere sequence (TTAGGG)n among vertebrates. Proc Natl Acad Sci USA 86: 7049--7053.

Meyne J., Baker R.J., Hobart H.H., Hsu T.C., Ryder O.A., Ward O.G., Wiley J.E., Wurster-Hill D.H., Yates T.L., Moyzis R.K. 1990. Distribution of non-telomeric sites of the (TTAGGG)n telomeric sequence in vertebrate chromosomes. Chromosoma 99: 3--10.

Miró R., Clemente I.C., Fuster C., Egozcue J. 1987. Fragile sites, chromosome evolution, and human neoplasia. Hum Genet 75: 345--349.

Mishmar D., Rahat A., Scherer S.W., Nyakatura G., Hinzmann B., Kohwi Y., Mandel-Gutfroind Y., Lee J.R., Descher B. et al. 1998. Molecular characterization of a common fragile site (FRA7H) on human chromosome 7 by the cloning of a simian virus 40 integration site. Proc Natl Acad Sci USA 95: 8141--8146.

Mondello C., Pirzio L., Azzalin C.M., Giulotto E. 2000. Instability of interstitial telomeric sequences in the human genome. Genomics 68: 111--117.

Mudry de Pargament M.D., Labal de Vinuesa M.L., Brieux de Salum S. 1985. Quantitative estimation of heteromorphism in C-bands of *Cebus apella*. J Hum Evol 14: 693--698.

Müller S., Stanyon R., O'Brien P.C.M., Ferguson-Smith M.A., Plesker R., Wienberg J. 1999. Defining the ancestral karyotype of all Primate by multidirectional chromosome painting between tree shrew, lemurs and humans. Chromosoma 108: 393--400.

Müller S., Stanyon R., Finelli P., Archidiacono N., Wienberg J. 2000. Molecular cytogenetic dissection of human chromosomes 3 and 21 evolution. Proc Natl Acad Sci USA 97: 206--211.

Musio A., Rainaldi G., Sbrana I. 1996. Spontaneous and aphidicolin-sensitive fragile site 3cen co-localizes with the (TTAGGG)n telomeric sequence in Chinese hamster cells. Cytogenet Cell Genet 75: 159--163.

Nanda I., Schrama D., Feichtinger W., Haaf T., Schartl M., Schmid M. 2002. Distribution of telomeric (TTAGGG)(n) sequences in avian chromosomes. Chromosoma 111: 215--227.

Napier J.R. and Napier P.H. 1985. The natural history of the Primates. British Museum (Natural History) Cambridge University Press.

Neusser M., Stanyon R., Bigoni F., Wienberg J., Müller S. 2001. Molecular cytotaxonomy of New World monkeys (Platyrrhini) − comparative analysis of five species by multi-color chromosome painting gives evidence for a classification of *Callimico goeldii* within the family of Callitrichidae. Cytogenet Cell Genet 94: 206--215.

Pellegrino K.C., Rodrigues M.T., Yonenaga-Yassuda Y. 1999. Chromosomal evolution in the Brazilian lizards of genus *Leposoma* (Squamata, Gymnophthalmidae) from Amazon and Atlantic rain forests: banding patterns and FISH of telomeric sequences. Hereditas 131: 15--21.

Ponsà M., Estop A., Egozcue J., Garcia M., Miró R. 1986. Comparative chromosome banding studies in the family Cercopithecidae. Hum Evol 1: 525--533.

Ponsà M., Garcia M., Borrell A., García F., Egozcue J., Gorostiaga M.A., Delprat A., Mudry M. 1995. Heterochromatin and cytogenetic polymorphisms in *Cebus apella* (Cebidae, Platyrhhini). Am J Primatol 37: 325--331.

Richard F., Lombard M., Dutrillaux B. 1996. ZOO-FISH suggests a complete homology between human and capuchin monkey (Platyrrhini) euchromatin. Genomics 36: 417--423.

Richard F. and Dutrillaux B. 1998. Origin of human chromosome 21 and its consequences: a 50-million-year-old story. Chromosome Res 6: 263-268.

Rubio-Goday A., Caballin M.R., Garcia-Caldés M., Egozcue J. 1976. Comparative study of the banding patterns of the chromosomes of Cercopithecidae. I. Subfamily Papinae: *Macaca fascicularis* and *Papio sphinx*. Folia Primatol 26: 306-309.

Ruiz-Herrera A., Ponsà M., García F., Egozcue J., Garcia M. 2002a. Fragile sites in human and *Macaca fascicularis* chromosomes are breakpoints in chromosome evolution. Chromosome Res 10: 33--44.

Ruiz-Herrera A., García F., Azzalin C.M., Giulotto E., Egozcue J., Ponsà M., Garcia M. 2002b. Distribution of intrachromosomal telomeric sequences (ITS) on *Macaca fascicularis* (Primates) chromosomes and their implication for chromosome evolution. Hum Genet 110: 578--586.

Schmid M., Ott G., Haaf T., Scheres J.M. 1985. Evolutionary conservation of fragile sites induced by 5-azacytidine and 5-azadeoxycytidine in man, gorilla, and chimpanzee. Hum Genet 71: 342--350.

Smeets D.F. and Van de Klundert F.A. 1990. Common fragile sites in man and three closely related Primate species. Cytogenet Cell Genet 53: 8--14.

Stanyon R., Fantini C., Camperio Ciani A., Chiarelli B., Ardito G. 1988. Banded karyotypes of 20 Papionini species reveal no necessary correlation with speciation. Am J Primatol 16: 3--17.

Stanyon R., Consigliere S., Bigoni F., Ferguson-Smith M., O'Brien P.C., Wienberg J. 2001. Reciprocal chromosome painting between a New World Primate, the woolly monkey, and humans. Chromosome Res 9: 97-106.

Sutherland G.R. 1979. Heritable fragile sites on human chromosomes I. Factors affecting expression in lymphocyte culture. Am J Hum Genet 31:125-35.

Svetlova E.Y., Razin S.V., Debatisse M. 2001. Mammalian recombination hot spot in a DNA loop anchorage region: a model for the study of common fragile sites. J Cell Biochem 81: 170--178.

Thomsen P.D., Hoyheim B., Christensen K. 1996. Recent fusion events during evolution of pig chromosomes 3 and 6 identified by comparison with the babirusa karyotype. Cytogenet Cell Genet 73: 203--208.

Vermeesch J.R., De Meurichy W., Van Den Berghe H., Marynen P., Petit P. 1996. Differences in the distribution and nature of the interstitial telomeric (TTAGGG)n sequences in the chromosomes of the Giraffidae, okapai (*Okapia johnstoni*), and giraffe (*Giraffa camelopardalis*): evidence for ancestral telomeres at the okapi polymorphic rob(5;26) fusion site. Cytogenet Cell Genet 72: 310-315.

Wienberg J., Stanyon R., Jauch A., Cremer T. 1992. Homologies in human and *Macaca fuscata* chromosomes revealed by in situ suppression hybridization with human chromosome specific DNA libraries. Chromosoma 101: 265-270.

Yunis J.J. and Prakash O. 1982. The origin of man: A chromosomal pictorial legacy. Science 215: 1525-1530.

Zou Y., Yi X., Wright W.E., Shay J.W. 2002. Human telomerase can immortalize Indian muntjac cells. Exp Cell Res 281: 63-76.

Trabajo 5

Molecular cytogenetic conservation of aphidicolin-induced fragile sites in Papionini species and man

Ruiz-Herrera A, García F, Fröenicke L, Ponsà M, Egozcue J, Garcia M, Stanyon R

(en preparación)

Molecular cytogenetic conservation of aphidicolin-induced fragile sites in Papionini (Primates) species and man.

Aurora Ruiz-Herrera, Francisca García, Lutz Fröenicke, Montserrat Ponsà, Josep Egozcue, Montserrat Garcia, Roscoe Stanyon

ABSTRACT

Fragile sites are considered structural features of mammalian chromosomes and they are conserved during evolution. In the present report, the homology at the molecular level, of relevant chromosome regions which express aphidicolin-induced fragile sites in human, *Macaca fascicularis*, *Mandrillus sphinx* and *Macaca arctoides* chromosomes by FISH of BAC/YAC clones is reported. The results show that fragile sites studied are present in homologous chromosome bands in humans and Papionini chromosomes and we are able to conclude from the hybridization data that banding is a good guide to compare FS location between humans and Papionini. In addition, the FISH with BAC clones has allowed us to corroborate chromosome rearrangements that have been taking place among the species studied and to establish the breakpoints involved.

INTRODUCTION

Fragile sites (FS) are specific chromosomal *loci* that present non-random gaps and breaks under specific culture conditions (Sutherland, 1979). Although FS were described for the first time in 1965 (Dekaban, 1965), their biological significance is still speculative because most are not directly associated with pathological processes and underlying molecular mechanisms are poorly understood. Out of 117 FS described in the human genome, more than 80 are classified as common fragile sites (cFS). Most cFS can be induced by aphidicolin and are expressed in almost all individuals of the population.

The FS expression in non-human Primates has been studied in Catarrhini species (Schimd et al. 1985, Smeets and Van de Klundert, 1990; Ruiz-Herrera et al., 2002a; Ruiz-Herrera et al., submitted) as well as in Platyrrhini species (Mudry, 1990; Fundia et al., 1991; 2000; Ruiz-Herrera et al., submitted). Authors reporting on Old World Primates propose that most FS are evolutionarily conserved and therefore homologous to human FS. They also propose a relationship between the location of cFS and chromosome rearrangements in evolution because FS often appear

to lie at or near hypothesized chromosomal breakpoints (Miró et al., 1987; Clemente et al., 1990; Fundia et al., 1991; 2000; Ruiz-Herrera et al., 2002a; Ruiz-Herrera et al., submitted).

Here we aimed to test whether the hypothesis of FS conservation would be sustained in a wider range of Primate species and confirmed by molecular methods. Previously Ruiz-Herrera et al. (2002a) observed that 38 of the human common fragile sites described in the literature on the basis of banding comparisons mapped to the equivalent common FS in the long-tailed macaque, *Macaca fascicularis* (MFA). A research goal was to test whether molecular cytogenetic tools such as FISH could demonstrate the conservation of FS in evolution. The hypothesis of FS conservation and its relationship to chromosomal rearrangements is based on G-banding comparisons. Over the last few years comparative molecular cytogenetics has developed the tools necessary to test these hypotheses at the DNA level. Different sources of DNA probes specific for chromosome sub-regions, like bacterial artificial chromosomes (BAC), yeast artificial chromosomes (YAC) or P1-derived artificial chromosomes (PAC) libraries, are currently available for comparative molecular cytogenetic studies (Nickerson and Nelson, 1998; Vallente-Samonte et al. 2000). These sub-chromosomal probes allow a refined and precise mapping of homology at the band-to-band level between species.

In this paper, the homology, at the molecular level, of relevant chromosome regions which express aphidicolin FS in human, *Macaca fascicularis* (MFA), *Mandrillus sphinx* (MSP) and *Macaca arctoides* (MAR) chromosomes by FISH of BAC/YAC clones is reported, and its implication for chromosomal evolution is discussed. For this purpose, fourteen BAC/YAC clones, which are mapped to human chromosome bands which contain common FS, were chosen from the National Cancer Institute (NCI) FISH mapped BAC database and provided by Dr. Glower (University of Michigan). These BAC/YAC clones were hybridized to Primate metaphases expressing aphidicolin induced FS to confirm, at the molecular level, that the bands which contain various human, macaque and mandrill FS are indeed homologous. In order to confirm the chromosome homologies between human and macaque chromosomes previously described (Wienberg et al., 1992; Ruiz-Herrera et al., 2002a), another set of seven BAC clones from the same database were used.

MATERIALS AND METHODS

Blood samples, cell lines and metaphase spread preparations. For the BAC/YAC hybridizations, peripheral blood samples from one female mandrill (*M. sphinx*, MSP; Parc Zoològic de Barcelona; Spain) and established cell lines were also used: a lymphoblastoid cell line from a

stump-tailed macaque (*M. arctoides*, MAR, obtained from Coriell Institute for Medical Research, Repository no. GM03443) and an additional fibroblast cell line from a long-tailed macaque (*M. fascicularis*, MFA, obtained from the Laboratory of Genomic Diversity, NCI-Frederick). Cultures from peripheral blood samples and lymphoblastoid cell lines were processed under standard conditions in order to obtain chromosome preparations.

BAC probes preparation. Twelve BAC clones and two YAC clones, which are mapped to human chromosome bands, which contain common fragile sites, were chosen by reference to the FISH mapped BAC NCI database (http://cgap.nci.nih.gov). DNA from BACs (purchased from Children's Hospital Oakland Research Institute, Research Genetics) and YACs (provided by Dr. Glover, University of Michigan) was extracted according to standard protocols. The BAC/YAC clones and their position in the human, MFA, MSP and MAR chromosomes are listed in Table 1. In addition, seven BAC clones from the human chromosome 1 (PR11-161A11, RP11-4J2, RP11-45F21), 5 (RP11-62D9), 7 (GS1-6E1) and 12 (RP11-666F17 and RP11-1022B3) obtained from the same database (http://cgap.nci.nih.gov), were chosen in order to confirm the chromosome homologies among human and Papionini species chromosomes 1, 5, 7 and 12 (Figure 1a).

Fluorescence *in situ* hybridization (FISH). Degenerate oligonucleotide primer PCR (DOP-PCR) was performed as previously described (Stanyon *et al*. 2001) for the direct labelling of BAC/YAC DNA with Rodamine110 (Applied Biosystems), Cy-5 (Amersham) and TAMRA (Applied Biosystems). For three-colour FISH, probes were combined by mixing different BAC/YAC DNA probes and precipitating with competitor DNA (Cot-1 human DNA), Salmon sperm DNA and 3 M sodium acetate overnight at –20ºC. The precipitated probe mix was resuspended in 14µl hybridization buffer (50% deionised formamide, 10% dextran sulphate, 2xSSC and 0.5M phosphate), denatured at 80ºC for 10 minutes and preannealed by incubation at 37ºC for 1 hour. Chromosome preparations were denaturalised in 70% formamide/2xSSC at 65ºC for 1 minute and 30 seconds, and overnight hybridization was performed at 37ºC. Post-hybridization washes were performed in 50% formamide/2xSSC at 42ºC for 10 minutes followed by three washes in 2xSSC at 42ºC for 5 minutes each. Chromosomes were counterstained with DAPI and observed with a Zeiss Axioplan2 microscope equipped with a Photometrics Quantrix CCD camera. Digital images were taken by SmartCapture2 software coupled to a Macintosh G4 computer.

RESULTS

The BAC/YAC probes (21) used in this experiment were hybridized to human metaphase chromosomes first to determine the hybridization conditions and then to confirm chromosome location. At least 10 metaphases were analysed per experiment. The results of the hybridization obtained in human, MFA, MSP and MAR chromosomes are shown in Table 1, Figure 1 and Figure 2a. For the molecular analysis of fragile sites, metaphases with aphidicolin-induced breaks/gaps were hybridised with the BAC/YAC probes (Fig 2b, 2c, 2d). Results confirm, at molecular level, that the bands which contain human, macaque and mandrill FS are indeed homologous.

Table 1: Bacterial artificial chromosomes (BAC) clones hybridised to human, *Macaca fascicularis* (MFA), *Mandrillus sphinx* (MSP) and *Macaca arctoides* (MAR) chromosomes, their location in human chromosomes and their assignment to Papionini chromosome bands (*asterisk*: chromosome band that contains a FS).

| BAC/YAC clones	Location Human (FS denomination)		MFA	MSP	MAR
BAC RP11-161A11	1p42.2	-	1q36	1q36	1q36
BAC RP11-55M23	1p32*	(FRA1B)	1q32*	1q32	1q32
BAC RP11-125P20	1p22*	(FRA1D)	1q22*	1q22*	1q22
BAC RP11-4J2	1q25	-	1p31	1p31	1p31
BAC RP11-45F21	1q32	-	1p21	1p21	1p21
BAC RP11-155C15	1q42*	(FRA1H)	1p35*	1p35*	1p35
YAC 850a6	3p14.2*	(FRA3B)	3q22*	no data	no data
BAC 42C19	7p22*	(FRA7B)	2p12	no data	no data
BAC GS1-220D8	7p13*	(FRA7D)	2q13*	2q13	2q13
BAC GS1-6E1	7p12	-	2q12	2q12	2q12
BAC CTB-20D2	7q22*	(FRA7F)	2q22*	2q22*	2q22
BAC GS1-259E18	7q32*	(FRA7H)	2q24*	2q24*	2q24
BAC RP11-88L18	5p14*	(FRA5E)	5p13*	5p13*	5p13
BAC RP11-19F12	5p13*	(FRA5A)	5p12*	5p12	5p12
BAC RP11-62D9	5q13.3	-	5q14	5q14	5q14
BAC RP11-4E3	5q31*	(FRA5C)	5q31*	5q31	5q31
BAC RP11-666F17	12p11	-	12p12*	12p12	12p12
BAC RP11-1022B3	12q14	-	12q14	12q14	12q14
BAC RP11-90B5	18q12.2*	(FRA18A)	18q13*	18q13*	18q13*
BAC RP11-15C15	18q21.3*	(FRA18B)	18p12	18p12	18p12
YAC 29c11	Xp22*	(FRAXB)	Xp22*	no data	no data
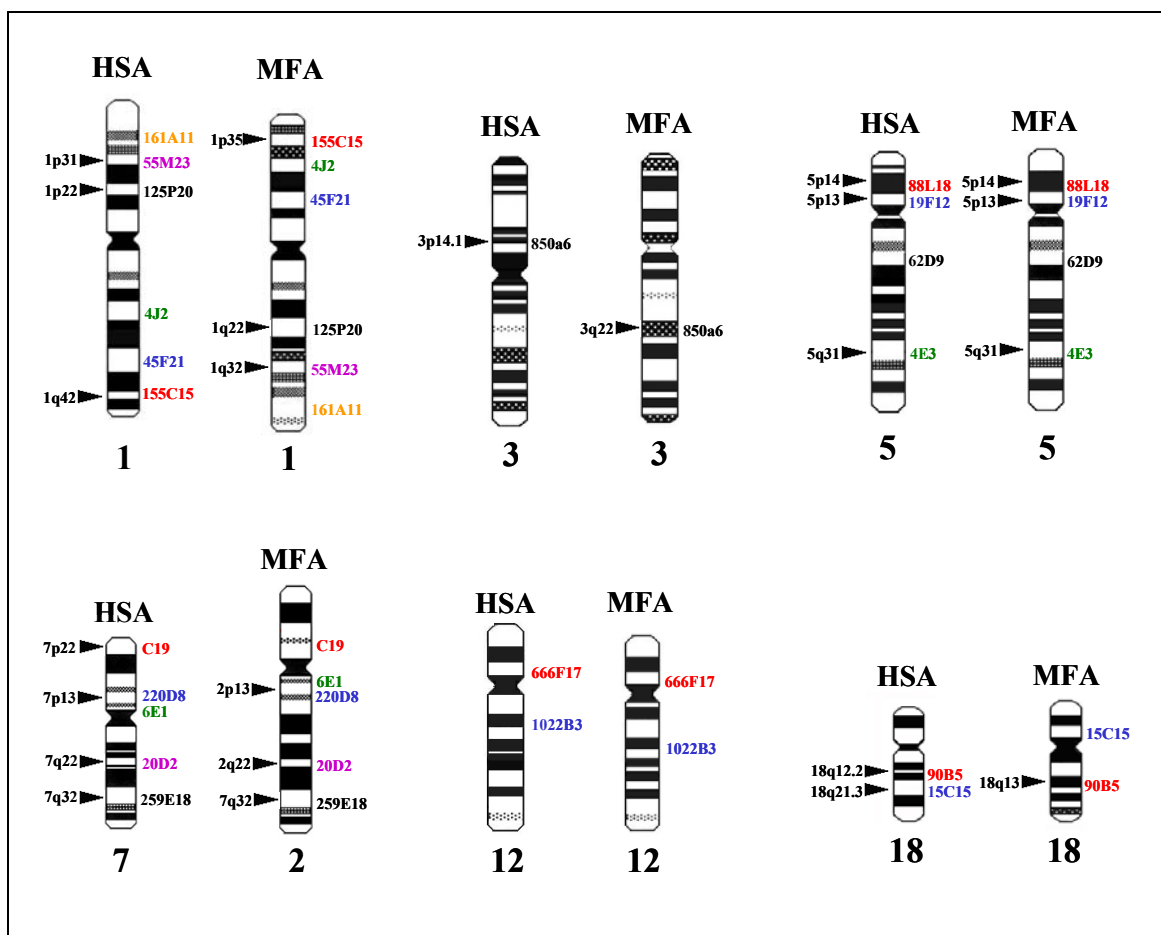
Figure 1: Ideograms from human chromosomes 1, 3, 5, 7, 12 and 18 and their homologous in Papionini species (represented by *M. fascicularis*). All the BAC/YAC signals positions are represented on the right. Arrowheads indicate chromosome bands that express fragile sites in each species.

From the fourteen human fragile sites conserved in MFA chromosomes previously reported, all of them have been confirmed by BAC/YAC hybridisation, except for two of them, FRA7B (HSA7p22) and FRA18B (HSA18q21.3). Although the G-banding assignment between the Papionini species chromosomes homologous to human chromosome 3 is difficult to establish due to the highly complex evolution of this chromosome, the hybridisation with the YAC clone 850a6 shows that human fragile sites FRA3B is conserved as a fragile sites in MFA (MFA3q22).

DISCUSSION

Up to now the hypothesis of FS homology between species has been supported only by comparing banding patterns. Studies carried out in great apes (Smeets and Van de Klundert, 1990) suggested an apparent evolutionary conservation of FS. Recently, banding analyses of FS in MFA

lead to the hypothesis that a number of aphidicolin-induced FS of humans were conserved in Old World monkeys (Ruiz-Herrera et al., 2002a; Ruiz-Herrera et al., submitted) and in New World monkeys (Ruiz-Herrera et al., submitted).

Intrachromosomal rearrangements between Papionini and human chromosomes revealed by FISH of BAC clones

FISH of BAC clones has allowed to corroborate chromosome rearrangements that have been taking place among these species and to establish the breakpoints involved. Figure 1 and 2a represents the hybridisation patterns of the BACs clones in human, MFA, MSP and MAR chromosomes and some intrachromosomal reorganizations became evident.

Chromosome 1: MFA, MSP and MAR chromosomes 1 are homologous to human chromosome 1 by one paracentric inversion and one pericentric inversion (Dutrillaux et al. 1979; Müller et al. 2001; Ruiz-Herrera et al., 2002b). Our results confirm the paracentric inversion in MFA, MSP and MAR in the region cover by the clones RP11-4J2 and PR11-45F21 corresponding to Papionini chromosome bands 1p31 and 1p21, respectively (Fig.2).

Chromosome 2: MFA, MSP and MAR chromosomes 2 are homologous to human chromosomes 7 and 21 by a chromosome fusion and a pericentric inversion (Ruiz-Herrera et al., 2002a). In MFA, MSP and MAR chromosome 2, the order of the clones GS1-6E1 and GS1-220D8 are inverted respecting to human chromosome 7. Cytogenetic comparative studies suggested that macaque chromosome 2 has a chromosomal form similar to the ancestral chromosome number 7 in Primates (O'Brien and Stanyon, 1999; Ruiz-Herrera et al., 2002b).

Chromosome 5: According to Dutrillaux et al. (1979) MFA chromosomes 5 is homologous to MSP and MAR chromosomes 5 by a paracentric inversion. Moreover, Papionini species chromosomes 5 are homologous to human chromosome 5. No further reorganizations among human, MFA, MSP and MAR homologous chromosomes have been detected since al the BAC clones hybridised on *Macaque* chromosomes do not reveal any rearrangement.

Chromosome 12: M. fascicularis, M. sphinx and *M. arctoides* chromosomes 12 are homologous to human chromosome 12 (Ruiz-Herrera et al., 2002a). No further reorganizations among human, MFA, MSP and MAR homologous chromosomes have been detected after the hybridisation with BAC clones.

Chromosome 18: *M. fascicularis, M. sphinx* and *M. arctoides* chromosomes 18 are homologous to human chromosome 18. Previous reports postulated inversions or centromere dislocation for the establishment of the homology (Müller et al., 2001; Ruiz-Herrera et al., 2002b).

Results from FISH with BACs clones show that the most likely reorganization that could explain this chromosome homology should be a centromeric shift, according to Müller et al. (2001).
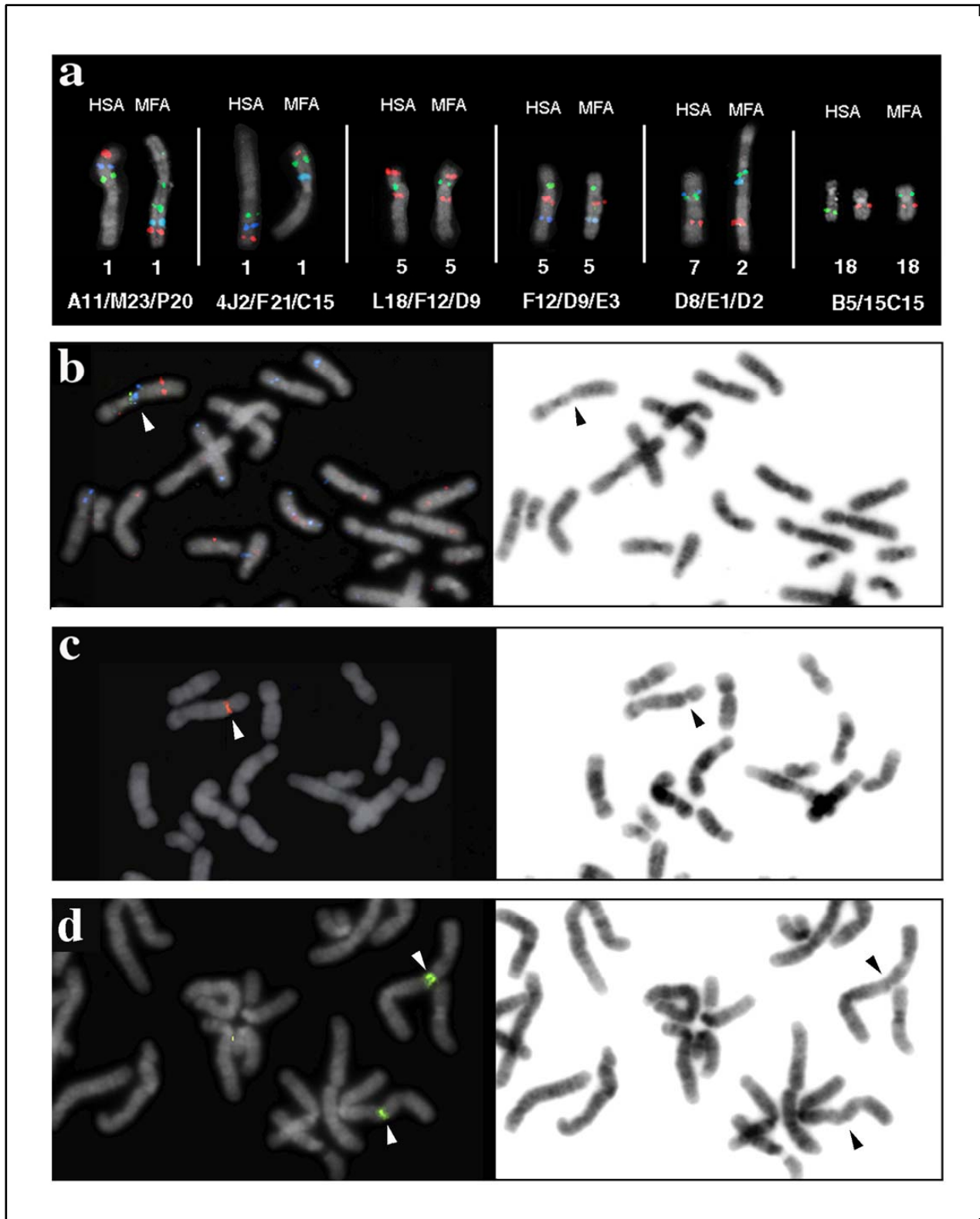
Figure 2: (a) Summary of three-colour FISH of BAC clones on human, *Macaca fascicularis* (MFA), *Mandrillus sphinx* (MSP) and *Macaca arctoides* (MAR) chromosomes. The results obtained in MFA, MSP and MAR are the same, so only the MFA chromosomes are represented. BAC clone labels: A11 (red), M23 (blue), P20 (green), 4J2 (green), F21 (blue), C15 (red), L18 (red), F12 (green), D9 (red), E3 (blue), D8 (blue), E1 (green), D2 (red), B5 (red), 14C15 (green). (b) Three-colour FISH with BAC clones D8 (blue), E1 (green) and D2 (red) on MFA chromosomes prepared after incubation with 0.2 μmol/L aphidicolin. Arrow indicates a chromatid break at fragile site MFA2q13. Note that BAC clone D8 (blue) spans break point 2q13. (c) One-colour FISH with the BAC clone D2 (red) on MSP chromosomes prepared after incubation with 0.2 μmol/L aphidicolin. Arrow indicates a chromatid break at fragile site MFA2q22. Note that BAC clone D2 spans break point 2q22. (d) One-colour FISH with the YAC clone 850a6 (green) on MFA chromosome prepared after incubation with 0.2 μmol/L aphidicolin. This YAC hybridisation is split by the chromatid breakage of fragile site MFA3q22.

Comparison of FS in Papionini and humans

In this paper, the homology, at the molecular level, of relevant chromosome regions known to harbor FS in human, MFA, MSP and MAR chromosomes by FISH of BAC/YAC clones is reported. The results show that FS studied are present in homologous chromosome bands in humans and Papionini chromosomes.

The chromosome band homology previously determined by G-banding comparisons (Ruiz-Herrera et al., 2002a) was confirmed in all cases by BAC/YAC hybridizations except for the human chromosome bands 18q21 and 7p22. The chromosome homology between human and macaque chromosome 18 has been interpreted by a pericentric inversion (Müller et al., 2001; Ruiz-Hererra et al., 2002b) or by a centromeric shift (Müller et al., 2001). In the Papionini species studied the BAC clone RP11-15C15 do not hybridises on 18q12 chromosome band, but on 18p12 chromosome band, showing that the most likely reorganization that could explain the chromosome 18 homology is a centromeric shift according to Müller et al. (2001). Regarding Papionini species homologous to human chromosome 7, although the BAC clone 42C19 do not hybridised on MFA 2q18 as previously described (Ruiz-Herrera et al., 2002a), but on 2p12 chromosome band, the pericentric inversion has been maintained (Figure 1, Figure 2a).

We are able to conclude from the hybridization data that banding is a good guide to compare FS location between humans and Papionini. The tribe Papionini (F. Cercopithecidae, Catarrhini) includes four genera (*Macaca*, *Mandrillus*, *Papio* and *Cercocebus*), which are highly chromosomally conservative, both in diploid number (2n=42) and G-banding patterns (Rubio-Goday et al., 1976; Dutrillaux et al., 1979; Brown et al., 1986; Stanyon et al., 1988;). The almost complete homology of the banding patterns in these species and the similarity with the human karyotype (Wienberg et al.,

1992; Ruiz-Herrera et al., 2002a), makes a comparison of the cFS location based on banding possible.

The G-banding pattern comparisons show that 40 FS are conserved between *M. sphinx* and *M. fascicularis* karyotypes (Ruiz-Herrera et al., submitted). Among these apparently conserved FS, the BAC/YAC hybridization results confirm the location of eight Papionini FS in the same chromosome band in the human karyotype: 1q22 corresponds to the human FS FRA1D (HSA1p22), 1p35 corresponds to the human FS FRA1H (HSA1q42), 3q22 corresponds to the human FS FRA3B (HSA3p14.2), 2q22 corresponds to the human FS FRA7F (HSA7q22), 2q24 corresponds to the human FS FRA7H (HSA7q32), 5p13 corresponds to the human FS FRA5E (HSA5p14), 18q13 corresponds to the human FS FRA18A (HSA18q12.2), Xp22 corresponds to the human FS FRAXB (HSAXp22).

Perspectives for an evolutionary contribution to FS research

Comparative evolutionary studies on FS may contribute to our understanding of the mechanisms of FS formation and their relationship to disease. The comparative study of FS expression performed in the present work has revealed that 8 human FS may have been conserved between MFA and MSP: HSA1p22 (FRA1D), HSA1q42 (FRA1H), HSA3p14.2 (FRA3B), HSA7q22 (FRA7F), HSA7q32 (FRA7H), HSA5p14 (FRA5E), HSA18q12.2 (FRA18A) and HSAXp22 (FRAXB).

The study of FS location conservation in Primates proceeds in different levels and phases: (1) the assignment by banding of Primate FS to a similar chromosomal location to human FS; (2) fluorescence *in situ* hybridization of BAC/YAC clones which demonstrate that the FS observed in various species are indeed located on homologous chromosome bands and (3) confirmation that FS located in both humans and Primates are spanned by the same BAC/YAC clone. The data in this report deal with level 3 of cytogenetic evidence for FS conservation.

Although the molecular mechanisms responsible for the genomic instability remain unclear, cFS are probably targets for chromosome breakpoints and this DNA instability could be a common trait of these *loci*. The phylogenetic depth of FS conservation may be considerable. Some human FS seen in great apes, and Papionini, HSA7q32, HSAXp22 and HSAXq22 may well be conserved in the karyotype of a New World monkey, *Cebus apella* (F. Cebidae, Platyrrhini) (Ruiz-Herrera et al., submitted).

A reasonable hypothesis is that many FS may even be conserved among species at the sequence level. The molecular characteristics of FS that are conserved during chromosome evolution

compared with FS that are not conserved or expressed may provide clues to the mechanisms underlying FS formation. Future comparative research on fragile sites in higher Primate species may therefore be one key to clarifying our understanding the role FS in both evolution and disease.

From the 14 BAC clones which are mapped to human chromosome bands which contain common fragile sites hybridized on Papionini chromosomes, 12 support the FS band homology, that would mean that little over 85% of human chromosome bands studied herein that contain FS are located in the homologous Papionini chromosome band previously reported by G-banding comparison.

ACKNOWLEDMENTS:

REFERENCES

Barrios L, Miró R, Caballín MR, Fuster C, Guedea F, Subias A, Egozcue J (1989) Cytogenetic effects of radiotherapy. Breakpoint distribution in induced chromosome aberrations. Cancer Genet Cytogenet 41:61-70.

Böhm U, Dahm PF, McAllister BF, Greebaum IF (1995) Identifying chromosomal fragile sites from individuals: a multinomial statistical model. Hum Genet 95:249-256.

Borrell A, Ponsà M, Egozcue J, Rubio A, Garcia M (1998) Chromosome abnormalities in peripheral blood lymphocytes from *Macaca fascicularis* and *Erythrocebus patas* (Cercopithecidae, Catarrhini) after X-ray irradiation. Mut Res 403:185-198.

Brown CJ, Dunbar VG, Shafer DA (1986) A comparison of the karyotypes of six species of the genus *Macaca* and the species of the genus *Cercocebus*. Folia Primatol 46:164-72.

Clemente IC, Ponsà M, Garcia M, Egozcue J (1990) Chromosome evolution in the Cercopithecidae and its relationship to human fragile sites and neoplasia. Int J Primatol 11:377-398.

Dekaban A (1965) Persisting clone of cells with an abnormal chromosome in a woman previously irradiated. J Nucl Med 6:740-6.

Djalali M, Adolph S, Steinbach P, Winking H, Hameister H (1987) A comparative mapping study of fragile sites in the human and murine genomes. Hum Genet 77:157-162.

Dutrillaux B, Biemont MC, Viegas-Pequignot E, Laurent C (1979) Comparison of the karyotypes of four Cercopithecoidae: *Papio papio*, *P. anubis*, *Macaca mulatta*, and *M. fascicularis*. Cytogenet Cell Genet 23:77-83.

Elder F and Robinson T (1989) Rodent common fragile sites: Are they conserved? Evidence in mouse and rat. Chromosoma 97:459-464.

Fang JM, Arlt MF, Burgess AC, Dagenais SL, Beer DG, Glover TW (2001) Translocation breakpoints in FHIT and FRA3B in both homologs of chromosome 3 in an esophageal adenocarcinoma. Genes Chromosomes Cancer 30:292-98.

Faravelli M, Azzalin CM, Bertoni L, Chernova O, Attilini C, Mondello C, Giulotto E (2002) Molecular organization of internal telomeric sequences in Chinese hamster chromosomes. Gene 283:11-16.

Fundia A, Gorostiaga M, Delprat A, Mudry M (1991) Fragile sites analysis and definition of chromosome landmarks, regions and bands in *Alouatta caraya* (ACA). Primatology Today, Elsevier Science Publishers B.V.(Biomedical Division) pp. 617-618.

Fundia A, Gorostiaga M, Mudry M (2000) Expression of common fragile sites in two Ceboidea species: *Saimiri boliviensis* and *Alouatta caraya* (Primates: Platyrrhini). Genet Sel Evol 32:87-97.

Huang H, Qian C, Jenkins RB, Smith DI (1998) Fish mapping of YAC clones at human chromosomal band 7q31.2: identification of YACs spanning FRA7G within the common region of LOH in breast and prostate cancer. Genes Chrom Cancer 21:152-159.

Human Gene Mapping 11 (1991) Cytogenet Cell Genet 58: 1-2200.

Krummel KA, Roberst LR, Kawakami M, Glover TW, Smith DI (2000) The characterization of the common fragile site FRA16D and its involvement in multiple myeloma translocations. Genomics 69:37-46.

Mangelsdorf M, Ried K, Woollatt E et al. (2000) Chromosomal fragile site FRA16D and DNA instability in cancer. Cancer Res 60:1683-1689.

McAllister B and Greenbaum IF (1997) How common are common fragile sites: variation of aphidicolin-induced chromosomal fragile sites in a population of the deer mouse (*Peromyscus maniculatus*). Hum Genet 100:182-188.

Miró R, Clemente IC, Fuster C, Egozcue J (1987) Fragile sites, chromosome evolution, and human neoplasia. Hum Genet 75:345-9.

Mishmar D, Rahat A, Scherer SW et al. (1998) Molecular characterization of a common fragile site (FRA7H) on human chromosome 7 by the cloning of a simian virus 40 integration site. Proc Natl Acad Sci USA 95:8141-8146.

Mudry MD (1990) Cytogenetic variability within and across populations of *Cebus apella* in Argentina. Folia Primatol 54:206-216.

National Cancer Institute (NCI) database (http://cgap.nci.nih.gov).

Nickerson E and Nelson DL (1998) Molecular definition of pericentric inversion breakpoints occurring during the evolution of humans and chimpanzees. Genomics 50:368-72.

Ohta M, Inoue H, Cotticelli MG et al. (1996) The FHIT gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma-associated t(3;8) breakpoint, is abnormal in digestive tract cancers. Cell 84:587-597.

Paige AJ, Taylor KJ, Stewart A et al. (2000). A 700-kb physical map of a region of 16q23.2 homozygously deleted in multiple cancers and spanning the common fragile site FRA16D. Cancer Res 60:1690-97.

Ponsà M, Estop A, Egozcue J, Garcia M, Miró R (1986) Comparative chromosome banding studies in the family Cercopithecidae. Hum Evol 1:525-533.

Richards RI (2001) Fragile and unstable chromosomes in cancer: causes and consequences. Trends Genet 17:339-345.

Ried K, Finnis M, Hobson L et al. (2000) Common chromosomal fragile site FRA16D sequence: identification of the FOR gene spanning FRA16D and the homozygous deletions and translocation breakpoint in cancer cells. Hum Mol Genet 9:1651-63.

Rubio-Goday A, Caballín MR, Garcia-Caldés M, Egozcue J (1976) Comparative study of the banding patterns of the chromosomes of Cercopithecidae. I. Subfamily Papinae: *Macaca fascicularis* and *Papio sphinx*. Folia Primatol 26:306-9.

Ruiz-Herrera A, Ponsà M, García F, Egozcue J, Garcia M (2002a) Fragile sites in human and *Macaca fascicularis* chromosomes are breakpoints in chromosome evolution. Chrom Res 10:33-44.

Ruiz-Herrera A, García F, Azzalin C, Giulotto E, Egozcue J, Ponsà M, Garcia M (2002b) Distribution of intrachromosomal telomeric sequences (ITS) on *Macaca fascicularis* (Primates) chromosomes and their implication for chromosome evolution. Hum Genet 110:578-86.

Ruiz-Herrera A, García F, Giulotto E, Attolini C, Egozcue J, Ponsà M, Garcia M. Implications of fragile sites and intrachromosomal telomeric (TTAGGG)n sequences in Primate chromosome evolution (submitted).

Schmid M, Ott G, Haaf T, Scheres JM (1985) Evolutionary conservation of fragile sites induced by 5-azacytidine and 5-azadeoxycytidine in man, gorilla, and chimpanzee. Hum Genet 71:342-50.

Smeets DF and Van de Klundert FA (1990) Common fragile sites in man and three closely related Primate species. Cytogenet Cell Genet 53:8-14.

Stanyon R, Fantini C, Camperio Ciani A, Chiarelli B, Ardito G (1988) Banded Karyotypes of 20 Papionini Species Reveal No Necessary Correlation with Speciation. Am J Primatol 16: 3-17.

Stanyon R, Consigliere S, Bigoni F, Ferguson-Smith M, O'Brien PCM, Wienberg J (2001) Reciprocal chromosome painting between a New World Primate, the woolly monkey, and humans. Chromosome Res 9:97-106.

Sutherland GR (1979) Heritable fragile sites on human chromosomes I. Factors affecting expression in lymphocyte culture. Am J Hum Genet 31:125-35.

Sutherland GR and Richards RI (1999) Fragile sites: cytogenetic similarity with molecular diversity. Am J Hum Genet 64:354-359.

Vallente-Samonte RU, Conte RA, Verma RS (2000) Syntenic homology of human unique DNA sequences within chromosome regions 5q31, 10q22, 13q32-33 and 19q13.1 in the great apes. Genet Mol Biol 23: 527-29.

Wienberg J, Stanyon R, Jauch A, Cremer T (1992) Homologies in human and *Macaca fuscata* chromosomes revealed by *in situ* suppression hybridization with human chromosome specific DNA libraries. Chromosoma 101:265-70.

Wilke CM, Hall BK, Hoge A, Paradee W, Smith DI, Glover TW (1996) FRA3B extends over a broad region and contains a spontaneous HPV 16 integration site: direct evidence for the coincidence of viral integration sites and fragile sites. Hum Mol Genet 5:187-195.

Yunis JJ and Soreng AL (1984) Constitutive fragile sites and cancer. Science 226:1199-1204