Complex Events in the Evolution of the Human Pseudoautosomal Region 2 (PAR2)

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The 320-kb human pseudoautosomal region 2 (PAR2) at the tips of the long arms of the X and Y chromosomes is thought to have been duplicated onto the Y chromosome recently in primate evolution. The four genes within PAR2 have been proposed to constitute two zones with different base ratios and transcription, one of which was added recently to the X chromosome. To test this hypothesis, we cloned and mapped PAR2 genes in other species, the lemur, the cat, and a marsupial, the tammar wallaby. None of the human PAR2 genes colocalized with human PAR1 genes in the marsupial genome, confirming that the human PAR1 and PAR2 evolved independently. Of the four PAR2 genes, only *SYBL1* was located on the X chromosome in all species, including marsupials, so it was part of the ancient X. *HSPRY3* localized to the X in all the eutherians, but not marsupial, so it must have been added to the X 80–130 million years ago. *CXYorf1* was present on the X in primates and also in mouse, but autosomal in wallaby, suggesting a later addition 70–130 million years ago, and *IL9R* was on the X only in primate, suggesting addition 60–70 million years ago. The results therefore demonstrate that at least two independent additions were necessary for PAR2 evolution. The present gene order on the human X also requires two inversions. The complicated evolutionary pathway supports the hypothesis that terminal interchromosomal rearrangements are common in regions unpaired at meiosis.

[The sequence data from this study have been submitted to GenBank under accession nos. AF544202, AF544203, AF544204, and AF544205.]

The human X and Y chromosomes differ in size, morphology, and gene content, the X being large and gene rich and the Y being small and heterochromatic. They do not pair at male meiosis except within small, homologous, "pseudoautosomal" regions (PARs) (Vogt et al. 1997). PARs lie at either extremity of the human sex chromosomes (Cooke et al. 1985). The 2.6-Mb PAR region 1 (PAR1), at the tip of the short arm (Xp-YpPAR) contains 13 genes (Rappold 1993; Gianfrancesco et al. 2001b), and is required for pairing of the X and Y chromosomes at male meiosis. The 320-kb PAR region 2 (PAR2) at the end of the long arms (Xq-YqPAR) (Freije et al. 1992) shows a much lower frequency of pairing and recombination than PAR1 and is not necessary for fertility (Kvaloy et al. 1994; Li and Hamer 1995; Kuhl et al. 2001).

The first two genes to be mapped to the PAR2 were *SYBL1* (synaptobrevin-like protein 1) and *IL9R* (interleukin 9 receptor). Recently, the entire human PAR2 was sequenced and found to contain two other genes *HSPRY3* (homolog to *Drosophila sprouty* 3) and *CXYorf1*, as well as a number of fragmentary pseudogenes (Ciccodicola et al. 2000). The order of the genes from centromere to telomere is *HSPRY3*, *SYBL1*, *IL9R*, and *CXYorf1*. *HSPRY3* and *SYBL1* lie within the proximal 100 kb, while *IL9R* and *CXYorf1* are close together in the GC-rich distal 35 kb. *HSPRY3* and *SYBL1* both map to the X, but not the Y, in primate and the mouse. *IL9R* maps to the X in primate but is autosomal in mouse (Kermouni et al. 1995; D'Esposito et al. 1997; Vermeesch et al. 1997; Matarazzo et al. 1999; Ciccodicola et al. 2000) (Table 1). *HSPRY3* and *SYBL1* are both inactive on the Y and are subject to X inactivation in humans. In contrast, *IL9R* and *CXYorf1* are expressed from the Y and are not subject to X inactivation (Huber et al. 1999; Ciccodicola et al. 2000).

The presence of these four genes on the X but not the Y in primate and mouse indicated that the region was transferred to the Y during the last few million years, perhaps via an illegitimate LINE sequence recombination between the X and Y (Kvaloy et al. 1994). The absence of *IL9R* from the mouse X and the dichotomy in expression patterns between proximal and distal pairs of PAR2 genes led to the hypothesis that the regions containing them were added independently to the X chromosome during eutherian evolution. Ciccodicola et al. (2000) suggested a division into Zone 1 (*HSPRY3* and *SYBL1*) and a later added Zone 2 (*IL9R* and *CXYorf1*) possibly obtained by three independent events. As a result, there

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Table 1. Map Position of PAR2 Genes in Different Mammalian Species

		Location in						Time added to X (MY)
		human	apes	lemur	mouse	cat	wallaby	
Divergence from human (MY)			15	60	70	80	130	
PAR2 Genes	*HSPRY3*	X/Y	X	X	X	X	3	80–130
	SYBL1	X/Y	X	X	X + A	?	X	>130
	IL9R	X/Y + A	X	X + A	11	A	1	60–70
	CXYorfl	X/Y + A	X + A		X		3	70–130

MY indicates million years
A indicates localization on an autosome(s)
? indicates no positive identified.

are differences in base composition, recombination, and transcription that define operationally the two PAR2 zones.

We tested this hypothesis by comparing the location of homologs of PAR2 genes in two eutherian mammals that diverged from humans 60–70 million years ago (Mya) and in a distantly related marsupial mammal, which diverged independently from the eutherian lineage 130 Mya (Kumar and Hedges 1998). Comparative mapping of human X-borne genes in distantly related mammals can distinguish genes that were a part of the ancient mammalian X, and have been important in establishing the origin of the human PAR1. Mapping human X-borne genes in marsupial and monotreme mammals, which diverged from the eutherian lineage 130 and 170 Mya, respectively, have defined a conserved region (XCR) shared by the X chromosome in all three extant mammals, and a region (XAR) recently added to the eutherian X, but still autosomal in marsupials and monotremes (Graves 1995; Graves et al. 1998). The eutherian Y is also composed of a conserved (YCR) and an added region (YAR) that contains most of the ubiquitously expressed genes (Waters et al. 2001). The demonstration that cloned marsupial homologs of human PAR1 genes colocalize with other genes on human Xp (Toder and Graves 1998) implied that PAR1 is part of the large region added to the eutherian X and Y after the divergence of marsupials (130 Mya) but before the eutherian radiation (80 Mya).

To examine the origin of PAR2, we therefore cloned and mapped all four human PAR2 genes in a model marsupial species, *Macropus eugenii* (the tammar wallaby). We also cloned and mapped two PAR2 genes in *Felis cattus* (the domestic cat) and *Lemur catta* (the lemur). If the human PAR2 region originated as part of the conserved region present on the X in all mammals, we would expect the human PAR2 genes to map to the X also in marsupials. If PAR2 represents part of the same addition as PAR1, we would expect PAR2 genes to map with PAR1 genes on tammar 5p, and if PAR2 represents an independent addition, they will map on other autosomes. Our results further clarify PAR2 evolution, implying that most of PAR2 was independently added to the eutherian X and rearranged in at least four separate events and before it was transposed to the Y.

RESULTS

We cloned and characterized the wallaby homologs of all four PAR2 genes, the lemur and cat homologs of the human PAR2 genes, *HSPRY3*, *SYBL1*, and *IL9R*, and mapped their position in the tammar, lemur, and cat genomes.

Isolation and Mapping of PAR2 Gene Homologs in Tammar Wallaby

We screened a size-selected *M. eugenii* λ genomic library of more than 360,000 recombinant phage with cDNA probes for human *HSPRY3*, *SYBL1*, *IL9R*, and *CXYorf1*. Clones were isolated for each gene. Hybridization of *SalI*/*EcoRI*-digested positive λ clones with human *HSPRY3*, *IL9R*, and *CXYorf1* cDNA yielded clear and distinct bands (data not shown) that were subcloned into plasmid vector and partially sequenced to confirm clone identity. The marsupial clones displayed >80% homology to their human homologs within coding regions and were therefore confirmed as marsupial homologs of the human PAR2 genes. *HSPRY3* clones were of two types, one of which was highly homologous to human *HSPRY3*, and the other was identical to the related SPRY1 gene, which has been previously cloned and mapped (Charchar et al. 2000). A single *SYBL1* positive clone was sequenced after subcloning with TOPO Shotgun cloning kit (Invitrogen).

The tammar clones were each mapped to tammar chromosomes by fluorescence in situ hybridization (FISH). *HSPRY3* and *CXYorf1* hybridized to the long arm of chromosome 3 in a medial position (Fig. 1A,B). Tammar *IL9R* hybridized to the tip of the long arm of chromosome 1 and *SYBL1* mapped to the long arm of the X chromosome (Fig. 1D,E). FISH of *HSPRY3* followed by hybridization with tammar wallaby chromosome 3 paint was used to check if *HSPRY3* and *CXYorf1* mapped to chromosome 3 or 4, which are not distinguishable by size and morphology only (Fig. 1C).

Simultaneous hybridization of *CXYorf1* and *HSPRY3* confirmed that these genes are located very close together at 3q (Fig. 1F–1H).

Mapping of Human PAR2 Genes in Lemur and Cat

We screened a size-selected male bacterial artificial chromosome (BAC) library from lemur and cat and isolated single clones for *HSPRY3*, *SYBL1*, and *IL9R* in the lemur. We also isolated single clones for *HSPRY3* and *IL9R* in the cat. There was no positive signal for *SYBLI* in the cat. This represents a homologous gene from each PAR2 zone. The cat and lemur clones were each mapped to cat and lemur chromosomes respectively by FISH.

IL9R hybridized to autosome E3 in the cat (Fig. 1I) and to the X chromosome and telomeres of an autosome of the lemur (Table 1). *SYBL1* mapped to the long arm of the X chromosome in the lemur. *HSPRY3* hybridized to the X chromosome in both cat (Table 1) and lemur (Fig. 1J).

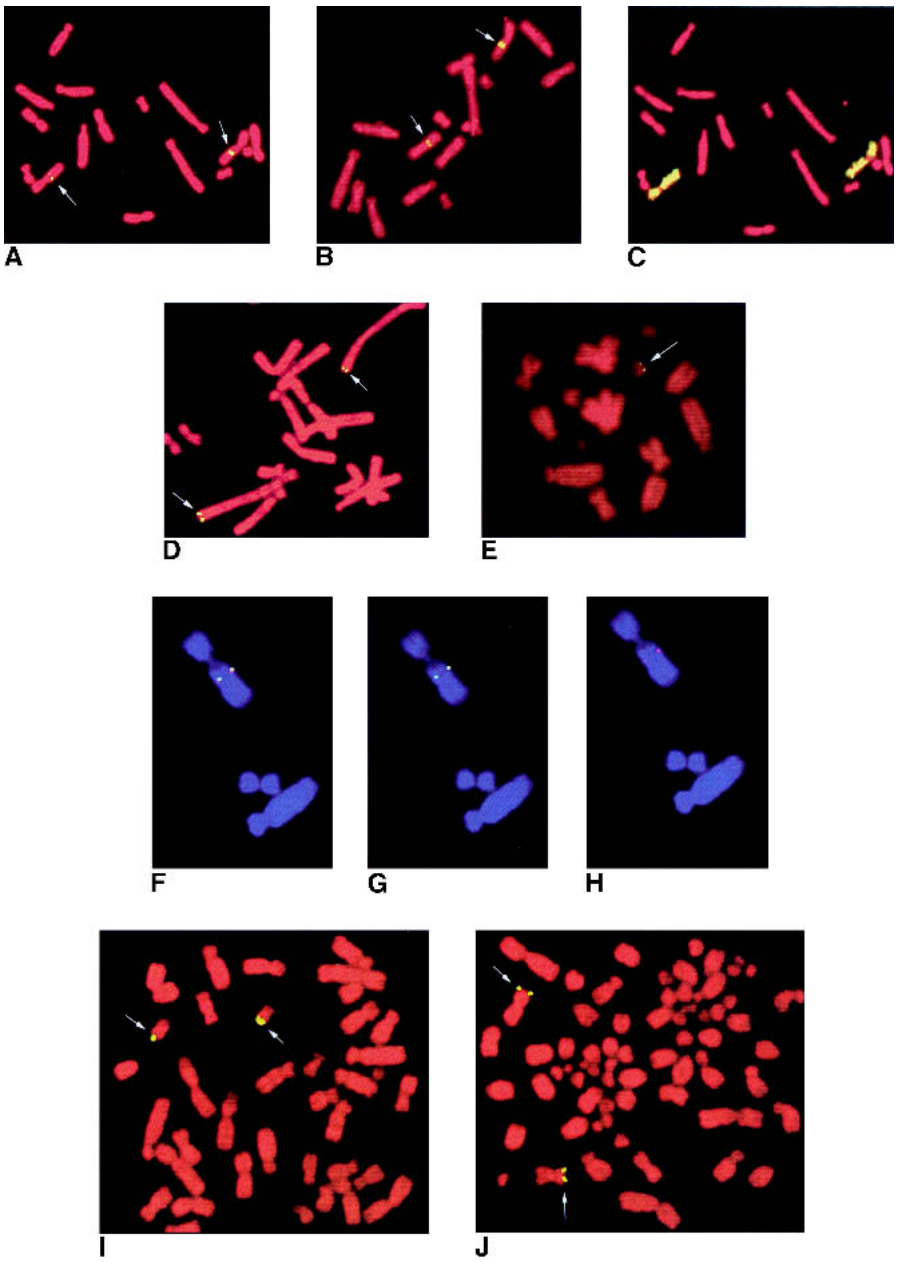

Figure 1 Chromosomal localization of the marsupial, cat and lemur orthologs as determined by fluorescence in situ hybridization analysis. (A) and (B) Localization of the *HSPRY3* and *CXYorf1* genes on chromosome 3. (C) The position is confirmed after sequential hybridization with *HSPRY3* and tammar chromosome 3 paint. (D) and (E) *IL9R* and *SYBL1* hybridized, respectively, to chromosome 1 and X. (F) Simultaneous hybridization of *CXYorf1* and *HSPRY3* to chromosome 3 where *CXYorf1* is yellow (G) and *HSPRY3* signal is red (H). (I) and (J) Chromosomal localization of the cat and lemur orthologs as determined by fluorescence in situ hybridization analysis. (I) Localization of *IL9R* to chromosome E3 in the cat, and (J) *HSPRY3* to the X chromosome in the Lemur.

DISCUSSION

Evolution of PAR 2 Region—Are There Two Evolutionary Zones?

The aims of this study were to test alternative theories for the origin of the human PAR2 region; either as part of the conserved region XCR present on the X in all mammals, or as part of an added region, either with PAR1, or as an independent addition. The first hypothesis requires that PAR2 genes lie on the X in other eutherians and marsupials as well as primates, and the second that they are autosomal in marsupials; on tammar chromosome 5p, if it was part of the same addition as PAR1, or elsewhere if it represents an independent addition. Our findings are hard to reconcile with any of these simple hypotheses.

The hypothesis that *HSPRY3* and *SYBL1* (zone 1) were part of the original eutherian X, but *IL9R* and *CXYorf1* (zone 2) were transferred later would be supported by evidence that zone 1 genes map together on the X in all mammal species whereas zone 2 genes map together on the X in species closely related to human but not on an autosome in distantly related species.

HSPRY3 and *SYBL1* lie on the X in all eutherian species, whereas *IL9R* is on the X in all primates, but autosomal in mouse and cat. This suggests that *HSPRY3* and *SYBL1* were part of the original eutherian X, but *IL9R* was added to the primate X after the divergence of the cat and the rodent lineage 70 and 80 Mya.

However, *CXYorf1,* the other Zone 2 gene, is located on the mouse X, implying that it was added earlier than *IL9R*. Our results from the wallaby further complicate the picture, since *SYBL1* was also on the X chromosome, whereas the other Zone 1 gene *HSPRY3* was autosomal. The two Zone 2 genes *IL9R* and *CXYorf1* were both autosomal.

These results imply that *SYBL1* was part of an original therian X in a common ancestor of eutherians and marsupials 130 Mya, and supports the results from cat and mouse. However, *HSPRY3,* being on the X in all eutherians but autosomal in wallaby, must have been added some time between the divergence of eutheria and marsupials 130 Mya and the eutherian radiation about 80 Mya. The two zone 2 genes *IL9R* and *CXYorf1* were also added to the X in independent events, since *CXYorf1* was present on the X in mouse, whereas *IL9R* was autosomal in mouse and cat and was therefore added only about 60–70 Mya. This independence is also favored by the different map positions of *IL9R* and *CXYorf1* in the wallaby, which suggests that the genes were added independently from different original sites. Thus

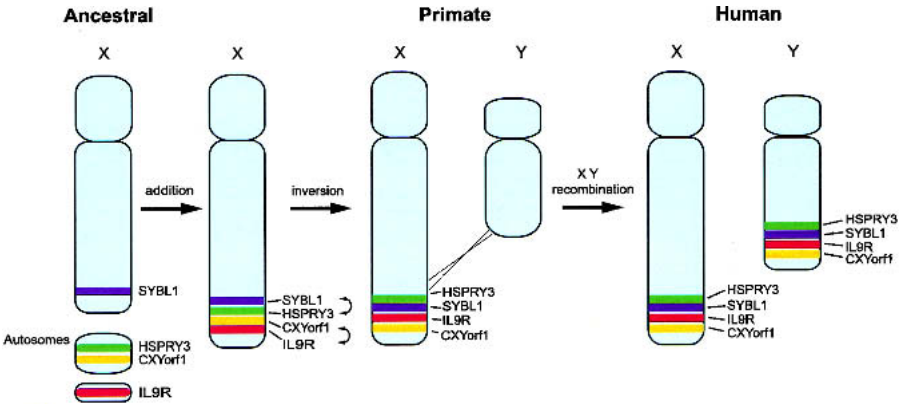

Figure 2 Schematic representation of the evolution of the PAR2 on the human X chromosome. SYBL1 lies at a telomeric position on the original therian X. HSPRY3 and CXYorf1 were added first followed by an inversion to make HSPRY3 proximal to SYBL1. IL9R was added later, and then a second inversion occurred to make CXYorf1 telomeric.

the results do not support the reality of either Zone 1 or Zone 2. The subdivision of the PAR2 into two zones is based on the different molecular and transcriptional status, rather than their evolutionary history. The differences in base composition and transcription between the pairs of genes on the human X and Y are therefore more likely to represent subsequent adaptations, as for example their position with respect to Y heterochromatin.

Independent Events in the Evolution of PAR2

Our results are more compatible with the hypothesis that three of the four genes within the human PAR2 were added to the X chromosome in at least two separate steps. The additions could be dated by the separation times of the species in which they were X linked or autosomal.

Our localization of *SYBL1* on the X in the lemur and tammar is consistent with its X location in other primates and mouse, as well as an early report that it lies on the X in a related marsupial, the potoroo, *Potorous tridactylus* (Ciccodicola et al. 2000). However, signal was detected on the short arm of the compound potoroo X, which is known from other studies to represent an added autosome (equivalent to tammar chromosome 4) in an XY_1Y_2 system (Toder et al. 1997; Rens et al. 1999). Presumably there was a rearrangement in long-term culture of the venerable PtK2 line. We therefore conclude that *SYBL1* is part of the ancient X chromosome that is conserved in all therian mammals and is therefore at least 130 million years old.

The other three PAR2 genes all proved to be autosomal in the wallaby, implying that they were translocated to the human sex chromosomes after the divergence of marsupials and eutherians about 130 Mya. None mapped to tammar chromosome 5p, the location of PAR1 genes. Thus the addition of PAR2 was independent of PAR1, which was a part of a large region added to both sex chromosomes 80–130 Mya. The stages in the genesis of the PAR2 region can be deduced from the positions of PAR2 homologs in primate, mouse, cat, and marsupial (summarized in Table 1). *HSPRY3* is on the X in all eutherians, but autosomal in marsupials, so must have been added 80–130 Mya, independently of PAR1. *CXYorf1* is on the X and autosomes in primate and mouse (Gianfrancesco et al. 2001a), but autosomal in the wallaby, so must have been added before the divergence of the marsupial lineage from eutherians ~130 Mya and most likely added with *HSPRY3*. *IL9R* is on the X in all primates, but autosomal in mouse, cat, and wallaby; it was therefore added to the primate X later (60–70 Mya). The most likely scenario to explain the current position of these genes in the PAR2 of the human X chromosome (Fig. 2) is therefore that the original therian X contained SYBL1 at a telomeric site (D'Esposito et al. 1997) and other genes were added in the order *HSPRY3*, ± *CXYorf1*, and *IL9R*. Addition of *HSPRY3* and *CXYorf1* was followed by inversion to make *HSPRY3* proximal to *SYBL1*. Similarly, addition of IL9R (the last gene) must have been followed by an inversion with the previously added *CXYorf1*. Because there are multiple copies of *IL9R* and *CXYorf1* on the autosomes, gene duplication may play a role in the evolution of *IL9R* and *CXYorf1*.

An alternative that we cannot rule out is that PAR2 genes were originally on the ancestral mammalian X, but were independently transferred from the X to autosomes. However, this would require independent loss of *HSPRY3*, *CXYorf1*, and *IL9R* in eutherian and marsupial, so it is considered unlikely. Mapping of PAR2 genes in monotremes should help to further clarify the origin of PAR2.

Thus the genesis of the PAR2 region has been complex, requiring three independent addition and two inversion events within a tiny region. This contrasts with the extreme stability of the X chromosome, which is almost invariant in eutherian mammals, and which includes a very large region that has been conserved for more than 170 million years. Remarkably, instability seems to be a special feature of both pseudoautosomal regions. Many genes such as *CSF2RA* and *IL3RA* within and near to the human PAR1 are autosomal in mouse, and some (e.g., *KAL*) appear to have been lost completely from the mouse genome (Toder and Graves 1998).

The Cause of PAR Instability

An explanation for this instability of PAR1 and PAR2 may be provided by the hypothesis that at meiosis unpaired chromosome ends tend to associate with nonhomologous regions with which they may recombine, producing terminal translocations (T. Ashley and J. Graves, unpubl.). This hypothesis is based on the observation that unpaired ends of a heteromorphic bivalent (sex chromosomes or rearranged autosomes) tend to associate nonhomologously (Ashley et al. 1981). This could explain the terminal locations and multiple copies of the transferred *Csf2ra* and *Il3ra* in mouse as well as explaining the multiple transfers and inversions in the genesis of the PAR2 region.

METHODS

Animal Tissue

Tammar wallaby (*M. eugenii*) material was obtained from The Melbourne Royal Zoological Gardens under the La Trobe University Animal Ethics Committee permit number RP96/4/V6. Ear tissue was used to establish cell cultures, and liver was

used for the isolation of genomic DNA. Cat and lemur material was obtained from cultured fibroblasts.

Isolation of Genomic Clones

A size-selected λ genomic library was previously constructed from tammar wallaby male liver (Delbridge et al. 1997). Briefly, the genomic DNA was partially digested in the 15–20 kb size range with *Sau3*A and packaged in λ EMBL 3 *Bam*HI arms. The library was titered and plated to a density of 120,000 plaque-forming units (pfu) on four 22 × 22 cm Nunc plates. The library plates were lifted twice onto nylon membrane (Hybond-N+, Amersham).

To isolate tammar wallaby genomic *IL9R*, *HSPRY3*, *SYBL1*, and *CXYorf1* clones, >360,000 recombinant phage were screened with human cDNA probes for each of the genes. Small hybridizing fragments of wallaby genomic clones were subcloned into pBluescript. The plasmid primers T3 and T7 were used to obtain sequence from either end of the cloned fragment with the AmpliCycle Sequencing Kit (Promega) according to the manufacturer's instructions. For *SYBL1*, small hybridizing fragments subcloned into the pCR4Blunt-TOPO vector of the TOPO Shotgun Subcloning Kits (Invitrogen) were sequenced with T3 and T7 primers.

To isolate cat and lemur genomic *IL9R*, *HSPRY3*, and *SYBL1*, specific primers for human PAR2 genes were selected and used to prepare probes for library screening. These probes were used to screen high-density filters of the entire RPCI-86 Feline Male BAC Library and LBNL-2 Lemur (*Lemur catta*) (BACPAC Resources). Hybridization of high-density filters were performed following standard protocol reported at http://www.chori.org/bacpac/. Clones used for FISH were confirmed by PCR.

Fluorescence In Situ Hybridization

Chromosome preparations were obtained from ear fibroblasts of a male tammar wallaby cultured in DME with 10% of fetal bovine serum. After harvesting, the cell pellet was dropped onto clean wet slides, which were stored at −20°C until use. For in situ hybridization, we followed the protocol detailed in Svartman and Vianna-Morgante (1999), with minor modifications. The probes were labeled with biotin-14-dCTP by nick translation (BioNick; Life Technologies) and, after precipitation with suppressor DNA (tammar wallaby genomic DNA sheared to 500 bp) in a proportion of 1:250, 200 ng of each probe were applied to the hybridization areas. Hybridization was carried out at 42°C overnight (*CXYorf1*, *HSPRY3*, and *SYBL1*) or at 37°C for 3 d (*IL9R*). Posthybridization washes were performed at 42°C, one in 50% formamide/2xSSS and one in 2xSSC, both for 3 min. Immunodetection was performed with polyclonal antibiotin raised in sheep (3:500, Vector), followed by antisheep IgG conjugated with FITC (1:100, Vector). Counterstaining was performed with propidium iodide (0.6 ng/μL) and the preparations were mounted with DAPI (0.8 ng/μL) in Vectashield Mounting Medium (Vector). Double hybridization was performed with digoxigenin-labeled *HSPRY3* and biotin-labeled *CXYorf1* in the same conditions described above, and for immunodetection we used antibiotin conjugated with FITC and antidigoxigenin conjugated with rhodamine (Oncor). For sequential FISH, after hybridization and analysis of *HSPRY3*, the chromosome preparation was left overnight in PBS at 4°C and hybridization with tammar wallaby chromosome 3 paint (Toder et al. 1997) was then performed as described above for single sequences. Analyses were performed in a Zeiss Axioplan microscope and images were collected with a liquid charge-coupled device (CCD) camera (Photometrics).

For the cat and lemur, chromosome preparation was obtained following standard protocols. Slides were hybridized in situ basically as described by Lichter et al. (1990), with minor modifications. We used 300 ng of BAC probe in each experiment; hybridization was performed at 37°C in 2XSSC, 50% (v/v) formamide, 10% (v/v) dextran sulfate, 5 mg human Cot1 DNA (Gibco-BRL), and 3 mg sonicated salmon sperm DNA, in a volume of 10 mL. Posthybridization washings were at 0.1XSSC at 60°C, three times each. Digital images were obtained using a Leica DMRXA epifluorescence microscope equipped with a cooled CCD camera (Princeton Instruments). DAPI was used to counterstain lemur and feline chromosomes to recognize them on the basis of the DAPI banding pattern. Cy3 and DAPI fluorescence signals, detected with specific filters, were recorded separately as gray-scale images. Pseudocoloring and merging of images were performed using Adobe Photoshop software.

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