

Giovanna Floridaia · Giorgio Gimelli
Orsetta Zuffardi · William C. Earnshaw
Peter E. Warburton · Chris Tyler-Smith

A neocentromere in the *DAZ* region of the human Y chromosome

Received: 14 December 1999 / Accepted: 24 March 2000 / Published online: 29 June 2000
© Springer-Verlag 2000

Abstract We describe a novel rearranged human Y chromosome consisting of an inverted duplication of the long arm heterochromatin and a small amount of euchromatin: *rea*(Y)(qter–q11.2::q11.2–qter). The normal centromere has been deleted and a neocentromere containing CENP-A, -C, -E and Mad2 but not CENP-B has formed close to the breakpoint. A 2.7 Mb yeast artificial chromosome contig spanning the breakpoint was constructed and the breakpoint was localised to a region of <120 kb close to the *DAZ* gene cluster. Combined immunofluorescence and fluorescence in situ hybridisation showed that the centromeric protein-binding domain of the neocentromere was located near the breakpoint and within the *DAZ* cluster.

Introduction

The centromere is an essential structure for proper segregation of the chromosome during meiosis and mitosis. It is the location where the kinetochore is assembled and proper

attachment to the spindle is monitored (Amon 1999). Normal human centromeres lie in a heterochromatic region containing an abundance of highly repetitive sequences, including an array of tandemly repeated alphoid satellite DNA from 130 kb to >5 Mb in size, and several specific proteins. Despite its importance, the molecular constituents of the centromere are incompletely known, and the role of the centromeric DNA is still a matter of debate.

Several proteins present at human centromeres have been identified. Among them, CENP-A (Palmer et al. 1987) is a histone-like protein that is bound to alphoid DNA at active centromeres in vivo (Vafa and Sullivan 1997; Warburton et al. 1997), supporting the possibility that alphoid DNA forms the natural centromere. CENP-C (Saitoh et al. 1992) and CENP-E (Yen et al. 1992) are also associated with active centromeres (Earnshaw et al. 1989; Sullivan and Schwartz 1995). In contrast, CENP-B is present at the normal autosomal and X-chromosomal centromeres, but has not been detected at the Y-chromosomal centromere (Pluta et al. 1990), and remains associated with inactive centromeres in dicentric chromosomes (Earnshaw et al. 1989), so seems not to be directly involved in centromeric function.

Several lines of evidence suggest that the alphoid DNA forms the functional centromere. Analysis of rearranged Y chromosomes (Tyler-Smith et al. 1993) or dissection of the Y centromere with telomeres (Brown et al. 1994) indicates that 150–200 kb of alphoid DNA plus about 300 kb of adjacent sequences are sufficient for centromeric function. The introduction of synthetic arrays of alphoid DNA or alphoid yeast artificial chromosomes (YACs) into human cells can lead to the formation of de novo centromeres and minichromosomes (Harrington et al. 1997; Ikeno et al. 1998). Nevertheless, there have been several reported cases of human marker chromosomes with functioning centromeres but no detectable alphoid DNA (reviewed by Choo 1997). These centromeres are called neocentromeres and all those tested bind the centromeric proteins CENP-A, -C and -E, normally associated with active centromeres, but not CENP-B.

Edited by: S. Henikoff

G. Floridaia · C. Tyler-Smith (✉)
CRC Chromosome Molecular Biology Group,
Department of Biochemistry, University of Oxford,
South Parks Road, Oxford OX1 3QU, UK
e-mail: chris@bioch.ox.ac.uk

G. Gimelli
Laboratorio di Citogenetica, Istituto G. Gaslini,
Largo G. Gaslini 5, 16148 Genoa, Italy

O. Zuffardi
Biologia e Genetica Medica, Via Forlanini 14, 27100 Pavia, Italy

W.C. Earnshaw · P.E. Warburton
Institute of Cell and Molecular Biology, Michael Swann Building,
King's Buildings, University of Edinburgh,
Mayfield Road, Edinburgh EH9 3JR, UK

Present addresses:

G. Floridaia, Lab. di Ultrastrutture, Istituto Superiore di Sanità,
Viale Regina Elena 299, 00161 Rome, Italy

P.E. Warburton, Dept. of Human Genetics,
Mount Sinai School of Medicine, New York, NY 10029, USA

The most common mechanism for the formation of marker chromosomes carrying neocentromeres is a de novo inverted duplication of the distal portion of a chromosome. In most of these inverted chromosomes, the neocentromere is located in one of the two arms, while in some of them it is found close to or at the breakpoint (Depinet et al. 1997). Alternative mechanisms for the formation of neocentromere-containing marker chromosomes include the re-joining of the two distal regions of a chromosome following deletion of the centomere (Voullaire et al. 1993) or involve no gross structural rearrangement (Tyler-Smith et al. 1999). In most of the markers, the neocentromere has been formed in euchromatic sequences specific to the chromosome. In the only case that has been studied at the molecular level (du Sart et al. 1997), the neocentromeric DNA of a mar del (10) chromosome is unrearranged in comparison with the corresponding region of the normal chromosome 10, and no sequence addition or removal could be detected (Barry et al. 1999). Thus it seems that both alphoid and non-alphoid DNA can be a site of centromeric activity. It has therefore been suggested that an epigenetic modification (a heritable change that does not involve a change in primary DNA sequence) is required to form an active centromere (Karpen and Allshire 1997). A candidate event would be the binding of CENP-A to a region where it is not normally present. However, the mechanism of activation of neocentromeres is unknown, and we do not understand the influence of DNA sequence organisation on the process. Furthermore, chromosomes vary in their segregation efficiency (Burns et al. 1999) and marker chromosomes with neocentromeres often show mosaicism, which may arise because the neocentromere, once activated, is still less efficient than a normal centromere. This in turn raises the possibility that the centromeric DNA has an important role in determining centromere efficiency. It is thus important to investigate a series of neocentromeres in order to understand the range of DNA sequences that can form them, and how these relate to segregation efficiency. To this end, we present here the analysis of a novel neocentromere that has formed in the euchromatic region of the human Y chromosome in a rearranged chromosome showing the inverted duplicated structure most commonly associated with neocentromeres.

Materials and methods

Case report

L.C., a 30 year old woman, underwent in vitro fertilisation embryo transfer (FIVET) in 1992 because of severe oligoasthenospermia in her husband. A dichorial diamniotic twin pregnancy was detected by ultrasonography at the sixth week of gestation. Both fetuses had regular cardiac activity. Fetal growth was normal at the 10th week of gestation, but 2 weeks later the patient was referred for observation because of metrorrhagia. At this time, one fetus appeared normal by level II ultrasonography, but the twin presented a voluminous septated cystic hygroma of the neck extending to the thorax and the abdominal wall, a hydrops and a bilateral hydrothorax. Chorionic villi were obtained by transabdominal sampling from both placentas. Cytotrophoblast cells showed a normal

female karyotype in the placenta from the normal fetus but an aneuploid cell line carrying three rearranged Y chromosomes in the placenta of the abnormal fetus.

At the 16th week of gestation a second cytogenetic analysis was performed on amniocytes and cultured hygroma cells. On this occasion an ultrasound examination showed poor growth of the female fetus with cystic hygroma (<5th percentile) and regular growth of the normal twin. The abnormal fetus died in utero at the 23rd week of gestation while the development of the normal twin and the pregnancy proceeded normally. A normal female weighing 3300 g and a fetus papyraceous were born at the 39th week of gestation by Caesarean section. Biopsies of both placentas were performed to confirm the cytogenetic analysis.

Cells and cell culture

A lymphoblastoid cell line, LB189, derived from the father was grown in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum (GlobePharm), 2 mM glutamine (Sigma), 20 U/ml penicillin and 20 µg/ml streptomycin (Gibco-BRL). Cytotrophoblast cells, cystic hygroma cells and amniocytes were cultured as described previously (Simoni et al. 1985; Welborn and Lewis 1990). Long-term growth of the amniocyte line SC27 was carried out in the above medium containing 20% serum.

Cytogenetic analysis

Chromosome preparations from the 13 week old chorionic villi were obtained by the short incubation method and by long-term villus culture (Simoni et al. 1985). QFQ, CBG and DA-DAPI (4',6-diamidino-2-phenylindole) banding were performed according to standard methods. Fluorescence in situ hybridisation (FISH) with the Y alphoid centromeric probe *DYZ3* (Oncor ONK5016) or the all-centromere probe (Oncor ONP5095) was carried out as suggested by the supplier. The DAZ region YAC probe yOX58 (Foote et al. 1992) was used as a FISH probe in the way described previously (Rossi et al. 1994). CREST immunostaining was performed as described (Haaf and Schmid 1989). Combined immunofluorescence/FISH on metaphase spreads was also as described (Warburton et al. 1997; Alonso et al. 2000), using methanol:acetic acid (3:1) fixation for CENP-B and CENP-C, and aqueous cyto-spin spreads for CENP-E and Mad2; Mad2 antibodies (Li and Benezra 1996) were generously provided by Y. Li and R. Benezra. Combined CREST/FISH staining on nuclei was carried out in the following way. Trypsinised cells were harvested, washed in PBS and resuspended in different hypotonic solutions [amniocytes in 0.8% sodium citrate, 0.075 M KCl, distilled H₂O (1:1:1) for 15 min at room temperature; lymphoblasts in 0.075 M KCl at room temperature for 10 min] at a concentration of 1×10⁵/ml. Aliquots (0.3–0.5 ml) of suspension were centrifuged at 1000 rpm for 10 min onto a glass slide in a cytocentrifuge (Shandon). Cells were fixed in absolute methanol at –20°C for 30 min and soaked in cold acetone for 30 s. Air-dried slides were washed in PBS for 15 min and then incubated with CREST antiserum (Antibodies Inc.; see Larin et al. 1994) diluted 1:3 in PBS for 1–2 h at 37°C in a humidified chamber. After a 15 min wash in PBS, slides were incubated with anti-human IgG-fluorescein isothiocyanate (Vector), diluted 1:75 in PBS. After one 15 min wash in PBS at room temperature, slides were fixed in 7.5% or 10% formaldehyde in 10 mM TRIS-HCl, 150 mM NaCl, 0.1% BSA for 10 min, washed in distilled H₂O for 10 min and fixed in 3:1 methanol:acetic acid for 15 min. Then FISH was performed as usual, except that denaturation in 70% formamide, 2×SSC at 80°C was carried out for 10 min. Slide screening was performed using an Olympus BH2 microscope and results were documented on 35 mm Fujichrome 400 slide film, or using an Olympus BX50 microscope equipped with a CCD camera (Princeton Instruments, RTE/CCD-1300-Y). Colour images were processed using Adobe Photoshop and an error in the alignment of the CENP-E layers was corrected at this stage.

Yeast artificial chromosome analysis

Yeast artificial chromosome clones were selected from the OXEN Y-chromosomal YAC contig (Foote et al. 1992) and the CEPH YAC contig (Cohen et al. 1993; Jones et al. 1994) on the basis of published sequence-tagged site content and our screening with the 50f2 and 49a probes. The YACs analysed were yOX58, yOX103, yOX190, yOX197 and yOX198 from the OXEN contig, and 786C10, 803B6, 913B1 and 933A6 from the CEPH contig. Clones were colony purified, and YACs similar in size to the largest major YAC band in the original mixed culture were chosen for further analysis. Restriction site maps were constructed by partial digestion with BsiWI, BssHII, MluI, SacII or SfiI followed by pulsed field gel electrophoresis (PFGE) and indirect end labelling (Cooper et al. 1992) using λ cI875 DNA concatemers as size standards. Maps were refined by hybridisation to complete digests made with the same enzymes.

Molecular methods

Preparation of DNA in agarose plugs, PFGE, DNA transfer, hybridisation and posthybridisation washes were performed as described (Cooper et al. 1992). The DAA (del Yq) and m38 (internal deletion of 50f2/C and adjacent sequences) DNA preparations were those described previously (Tyler-Smith et al. 1993; Jobling et al. 1996). For quantitation, hybridised filters were analysed using either a PhosphorImager (Storm 860, Molecular Dynamics) and ImageQuant software, or an InstantImager (Packard). A deletion map of the rearranged chromosome was constructed as described (Tyler-Smith et al. 1993). A cosmid library from the YAC 786C10 was prepared in the vector SuperCos 1 following the Stratagene protocol and using Gigapack III packaging extract. YAC-derived clones were selected by colony hybridisation to human DNA, and EcoRI fragments of these lacking highly repeated sequences were identified after digestion, agarose gel electrophoresis and filter hybridisation to human DNA. Gel-purified fragments were used directly as probes against digests of YAC and human DNA.

Results

Characterisation of a rearranged Y chromosome lacking alphoid DNA

Metaphase spreads prepared from chorionic villi associated with an abnormal fetus at 13 weeks of gestation showed three copies of a rearranged Y chromosome in 57/57 metaphases. When cytogenetic analysis was carried out on amniocytes at 16 weeks of gestation, mosaicism was seen, with 18/26 (~70%) colonies carrying one copy of the rearranged Y chromosome and the remaining 8/26 (~30%) being 45,X. Mosaicism was also seen in cultured hygroma cells analysed at the same time: 35/44 (~80%) metaphases carried a rearranged Y and 9/44 (~20%) were 45,X.

The rearranged chromosome consisted of two blocks of brilliant Q-positive heterochromatin separated by a tiny region of Q-negative euchromatin (Fig. 1b). No centromeric C-band was detected by CBG-banding (results not shown), and no alphoid DNA could be detected cytogenetically using either a Y alphoid probe or an all-centromere probe (Fig. 1c). The absence of Y alphoid DNA was confirmed by quantitative molecular hybridisation to EcoRI digests of DNA from LB189, SC27 and

an unrelated female. Hybridisation to a control α -globin probe followed by InstantImager quantitation revealed 16,429, 15,726 and 15,577 counts, respectively, in each track, showing that the loading ratios were 1.05:1.01:1. Re-hybridisation to a Y alphoid probe produced 60,045, 3021 and 2992 counts per track, which after normalisation for loading give alphoid hybridisation intensities of 57,186, 2991 and 2992 counts, respectively. The figure of 2992 in the female sample represents cross-hybridisation to non-Y sequences and was subtracted from each measurement to give 54,191, -1 and 0 Y alphoid counts from each sample. From the size of the Y alphoid array on a pulsed-field gel, it is known that LB189 has about 50 copies of the 5.7 kb Y alphoid unit, so one unit would contribute 1084 counts to the InstantImager quantitation. We therefore conclude from the quantitative hybridisation experiment that SC27 has less than one copy of the Y alphoid unit per cell and, from the combined molecular and cytogenetic results, that the rearranged Y chromosome lacks alphoid sequences. The father's karyotype (Fig. 1a), including the Y chromosome and the Y alphoid array, was normal, and derivation of the rea(Y) from the paternal Y chromosome was confirmed by examination of the hypervariable Y fingerprint pattern generated by hybridisation of a *DYZ1* probe to a PFGE-fractionated PvuII digest (Mathias et al. 1994). The Y chromosome was therefore interpreted as a de novo rearrangement in the karyotype 45,X/46,X rea(Y)(qter-q11.2::q11.2-qter).

A neocentromere in the rearranged Y chromosome

Despite mosaicism of the amniocyte karyotype, the rearranged Y chromosome was retained more efficiently than would be expected for an acentric structure. We therefore investigated whether it carried a centromere that bound detectable amounts of known centromeric proteins. Immunofluorescence with a CREST antiserum showed staining close to the centre of the chromosome (Fig. 1d), although the signal was weaker than that of most other chromosomes in the spread. A CENP-A-specific antiserum has already been shown to bind to this chromosome (Warburton et al. 1997); the presence of CENP-B could not be detected (Fig. 2a), but CENP-C (Fig. 2b), CENP-E (Fig. 2c) and Mad2 (Fig. 2d) were present at a similar location to the CREST signal. CENP-B is not expected to be detected at a Y chromosome centromere, but is also absent from other neocentromeres (Voullaire et al. 1993, 1999), so its lack is not surprising and may account for the low CREST signal. Thus this rearranged Y chromosome contains a functional neocentromere located near the breakpoint in Yq11.2 that carries an active kinetochore, as shown by the presence of the kinetochore proteins CENP-A, -C and -E. In addition, the presence of Mad2 at the neocentromere (in these Colcemid-treated cells) suggests that it is also competent to activate the spindle assembly checkpoint.

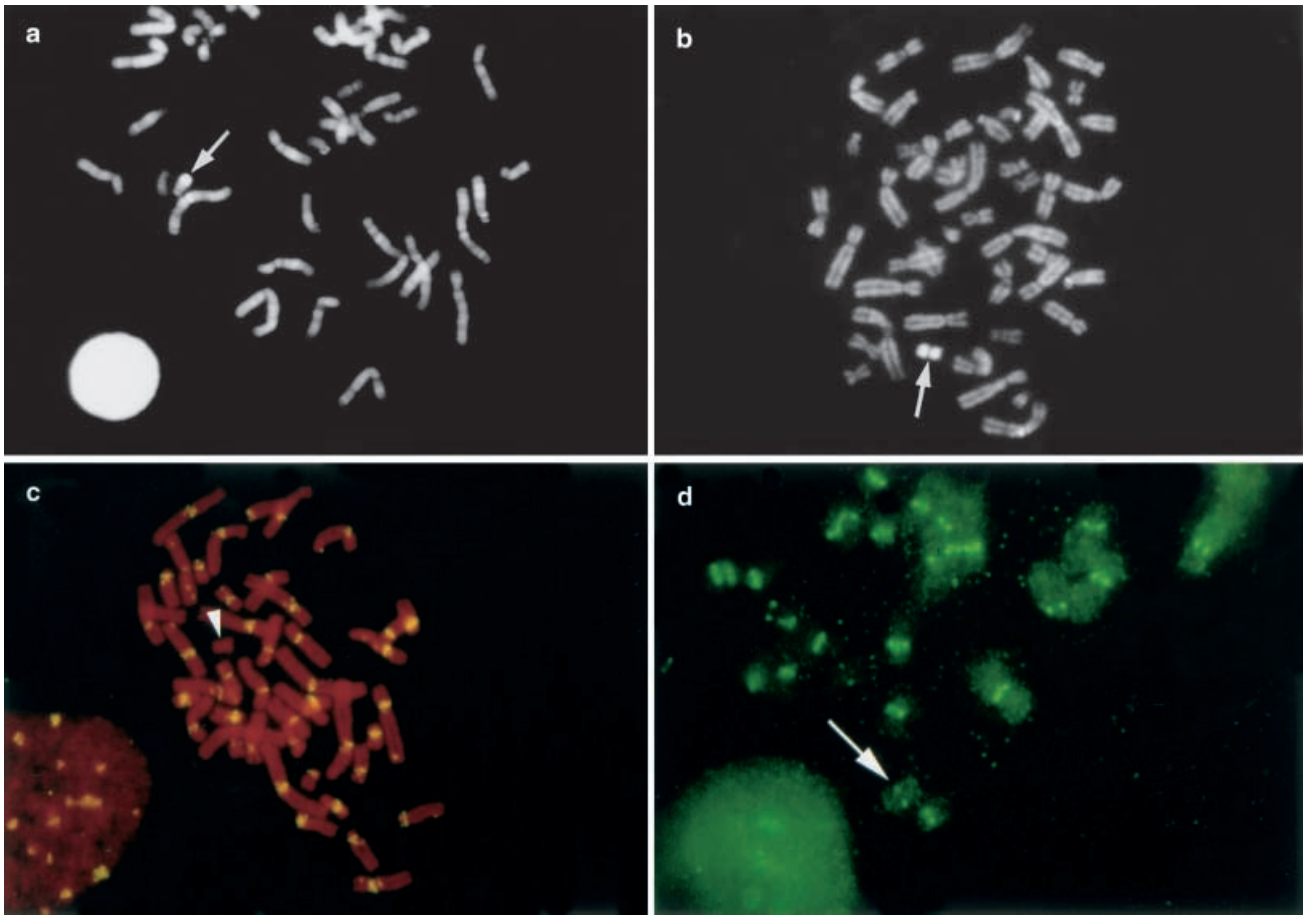


Fig. 1a–d Cytogenetic analysis of the paternal (LB189) and rearranged (SC27) Y chromosomes. **a** LB189, Q-banding; **b** SC27, Q-banding; **c** SC27, fluorescence in situ hybridisation (FISH) with all-centromere probe; **d** SC27, CREST immunostaining. The *arrow* indicates the Y chromosome

Deletion map of the rearranged Y chromosome

We wished to identify the DNA sequences forming the neocentromere. As a first step towards this, we set out to map the position of the nearby breakpoint. Since the Y is haploid, we could construct a deletion map by simply testing for the presence or absence of Y sequences from known locations on the chromosome in SC27 genomic DNA. Analysis of 32 sequences in this way showed that all loci tested from Y_p, Y_{cen} and proximal Y_q (intervals 1–18) were absent, while sequences from the more distal Y_q euchromatin and heterochromatin (intervals 21–23) were present (Fig. 3). Sequences mapping to deletion interval 19 were present but reduced in intensity, while one locus from deletion interval 20 was absent. This is likely to reflect the ambiguity in assigning dispersed repeated sequences to deletion intervals, rather than a complex rearrangement. The precise location of the breakpoint on the deletion map could potentially be complicated by the polymorphism of some sequences in this area of the chromosome. However, 50f2/C, which can be present in zero, one or two copies in normal Y chromosomes as a

result of natural polymorphisms (Jobling et al. 1996), was found to be present in one copy in LB189 and absent from SC27. 2D29/D was present on both chromosomes. The breakpoint defined by molecular markers therefore lies between these two loci in the distal Y_q euchromatin, in a position consistent with the cytogenetic analysis.

Construction of a restriction site map of the region spanning the breakpoint in Yq11.2

Restriction site maps of seven YACs containing 50f2/C or 2D29/D, and an eighth YAC containing other sequences from this region, were constructed. The maps of all eight YACs could be assembled into a single contig covering 2.7 Mb of DNA (Fig. 4a). The exceptionally large YAC 933A6 extended the contig by at least 2 Mb in the proximal direction, but this additional DNA lies outside the region of interest to us and was not included in the map. 50f2/C and 2D29/D were localised on the map by hybridisation to complete digests of the YACs and were found to lie about 1.6 Mb apart (Fig. 4b).

The breakpoint was localised to a smaller region by examination of additional loci. 92R7 detects multiple bands of Y DNA, including a duplicated ~9.5 kb HindIII fragment. Both of these copies are present in the YAC 786C10 (Fig. 4b), but only one is retained in SC27 ge-

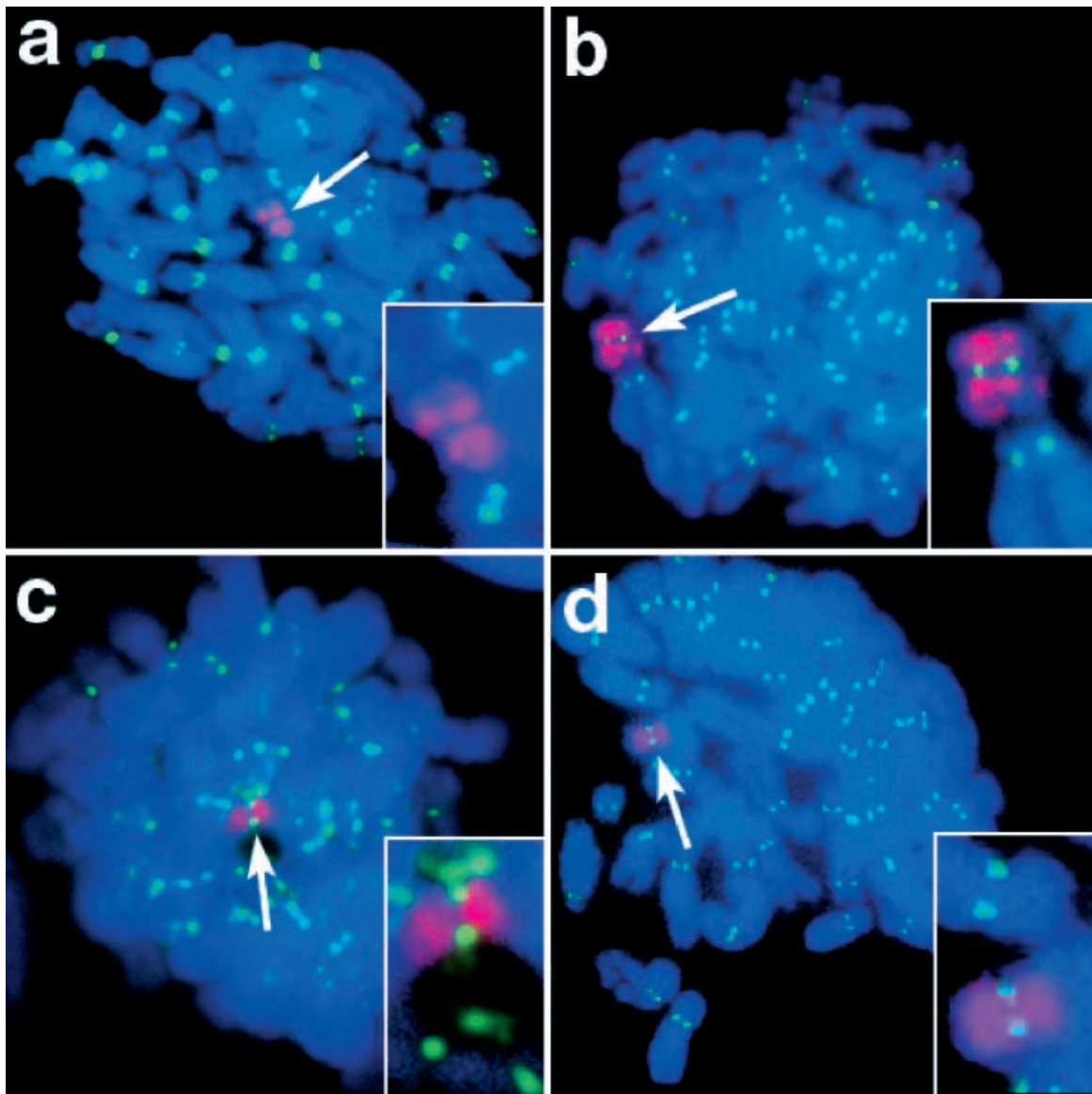


Fig. 2a–d Identification of centromeric and kinetochore proteins present at the SC27 neocentromere. Immunostaining with **a** CENP-B, **b** CENP-C, **c** CENP-E, **d** Mad2. Protein staining is shown in *green* and the Y chromosome (*white arrow*) is identified by FISH using a *DYZ1* probe (*red*); in each case an enlargement of the chromosome is included in the *inset*

nomeric DNA, so the breakpoint must lie within 786C10. A cosmid library was constructed from 786C10 DNA and additional probes were isolated and used to elucidate the structure of the region. The organisation of the DNA is quite complicated and most sequences are repeated, either within this YAC or elsewhere on the Y (Fig. 4c). In addition, the *Sfi*I sites marked with an asterisk were not cleaved to completion in YAC DNA or genomic DNA under any conditions used (Fig. 4d, e). Nevertheless, the probe 4G6, lying in the ~430 kb *Sfi*I fragment but detecting both ~430 kb and ~650 kb *Sfi*I fragments because of the incomplete *Sfi*I cleavage, revealed that these large *Sfi*I fragments were present and unaltered in size in SC27 (Fig. 4e), demonstrating that the breakpoint must

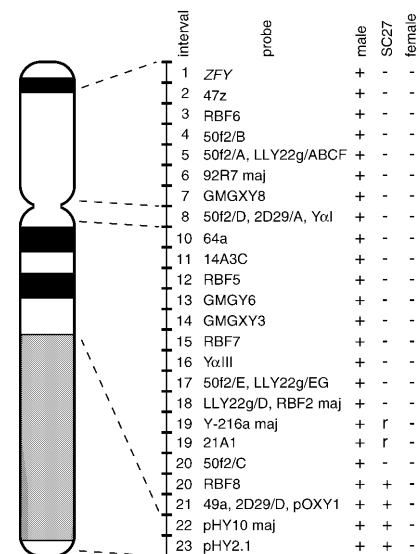
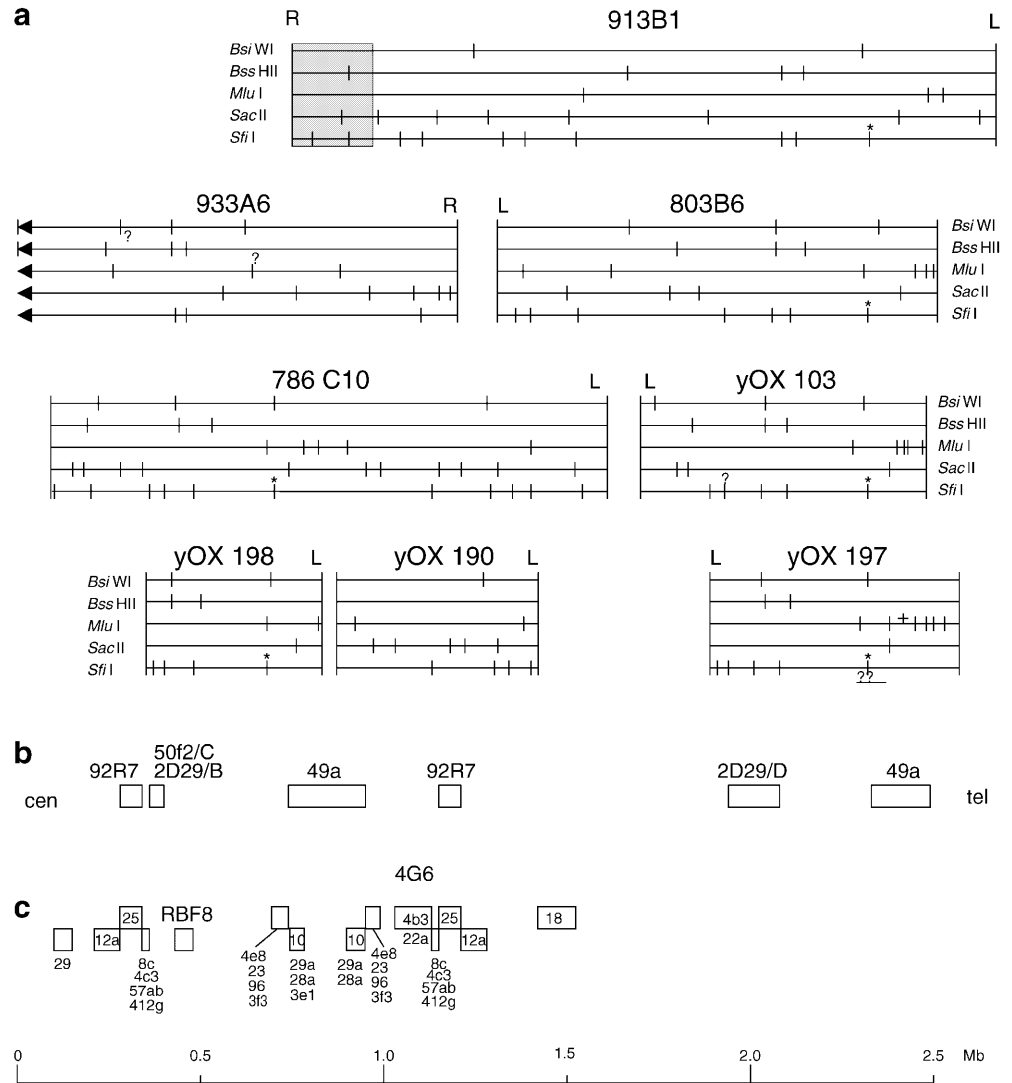


Fig. 4a–e Molecular analysis of the breakpoint region. **a** Restriction site maps of yeast artificial chromosomes (YACs) from contig spanning the breakpoint. Grey area co-cloned region. Asterisk SfiI site showing incomplete cleavage. **b** Location of probes on the contig. **c** Location of probes within YAC 786C10. **d** SfiI digests of human DNA fractionated by pulsed-field gel electrophoresis and probed with 49a. **e** The same filter probed with 4G6. Closed arrows DAZ gene-containing SfiI fragments located in the proximal part of **a** and hybridising to both probes. Open arrows SfiI fragments located in the distal part of **a** and hybridising to 49a alone. A pair of fragments is seen in each case because of partial cleavage at the site indicated in **a**



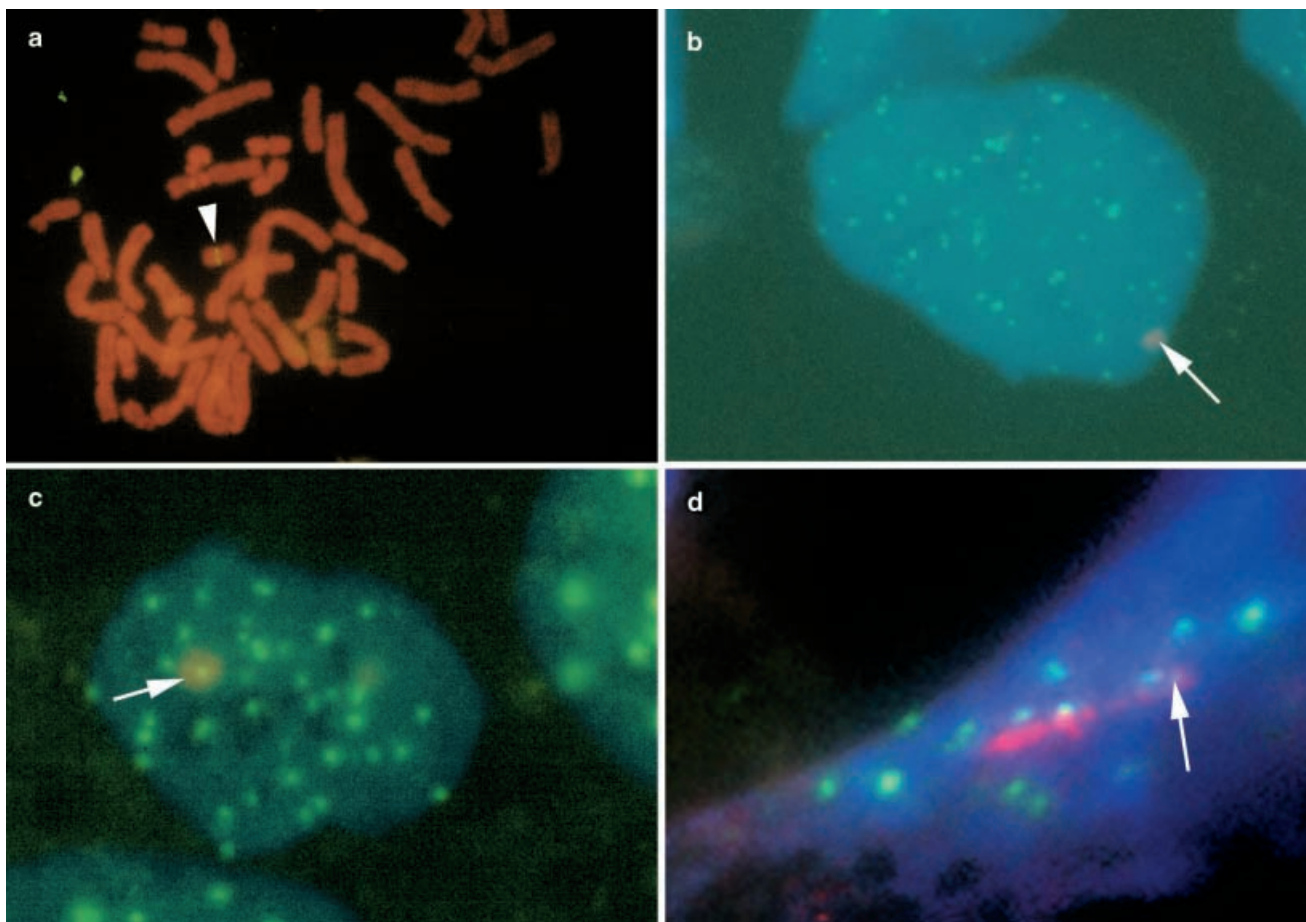


Fig. 5a–d Cytogenetic analysis of neocentromeric sequences. **a** SC27 metaphase chromosomes, FISH with yOX58 probe. **b** LB189 nucleus. **c** SC27 nucleus. **d** Extended SC27 nucleus. In **b–d**, the CREST immunostaining is shown in *green* and the YAC 786C10 FISH signal in *red*

lie within the immediately adjacent ~80 kb fragment or the next ~40 kb fragment, which itself contains 50f2/C: a region of less than 120 kb.

About 2.5 Mb of the 2.7 Mb contig consists of a large-scale imperfect tandem duplication, seen as a repetition of the 2D29 and 49a hybridisation sites (Fig. 4b). The copy found in YAC 786C10 (and thus studied in most detail) itself consists of an interrupted and imperfect inverted duplication, seen from the duplicated locations of YAC-derived probes (Fig. 4c). Since the structure was so complex, we needed to determine whether one copy or more of the entire 2.5 Mb region was present in genomic DNA, and undertook quantitative hybridisation experiments to answer this question. We assumed that the Y copy of *poxY1* was a single-copy sequence on a normal Y chromosome, since it is present as a single hypervariable fragment (Oakey and Tyler-Smith 1990), and used this as our copy number standard. Assuming that there were two copies of *poxY1* per SC27 Y chromosome, because of the inverted duplicated structure, we determined that there were also two copies per *rea*(Y) chromosome of 2D29/D, but only one copy of 4G6. 50f2/C and 2D29/B were absent.

Thus, although the chromosome is symmetrical at the cytogenetic level, it is not perfectly symmetrical at the molecular level. One arm of the structure extends from Yqter to the breakpoint between 50f2/C and 4G6 (at about 0.5 Mb in Fig. 4a), while the other arm is shorter by about 1 Mb, extending from a breakpoint between 4G6 and 2D29/D (1.1–1.9 Mb in Fig. 4a) to Yqter.

Localisation of the neocentromere on extended chromatin

Fluorescence in situ hybridisation with YAC yOX58 on SC27 metaphase chromosomes showed a signal close to the middle of the *rea*(Y) chromosome (Fig. 5a), as expected from the deletion mapping (Fig. 3). At this level of resolution, the position of the DNA signal is not distinguishable from the location of the centromeric protein staining (Figs. 1d, 2). In order to investigate the localisation of the centromeric protein domain on the DNA sequences more precisely, we analysed interphase nuclei, where the chromatin is more extended than in metaphase chromosomes. Centromeric protein location was revealed by immunostaining with a CREST antiserum and DNA sequences lying near the neocentromere were visualised by FISH using the YAC 786C10. Because of the repetitive nature of the DNA sequences in this part of the chromosome, including the large-scale duplication, we

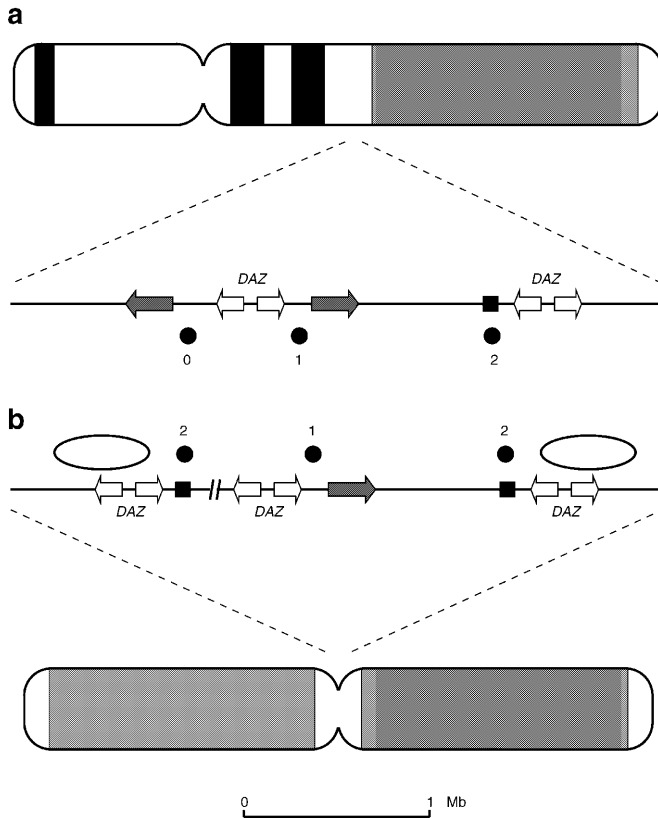


Fig. 6a, b Schematic summary of the chromosomal rearrangement and the two possible locations for the neocentromere. **a** Normal Y chromosome with the *DAZ* region shown below. Arrows indicate orientations of repeated sequences; 0, 1 and 2 show the copy number of three regions of the map in the rearranged chromosome. **b** Rearranged Y chromosome with the *DAZ* region shown above. Ovals indicate the two possible locations of the centromeric protein binding domain

Table 1 Association of YAC (neocentromere) and CREST (centromeric protein) signals in the normal paternal (LB189) and rearranged (SC27) Y chromosomes

	YAC + CREST signals associated	YAC + CREST signals not associated
LB189	6	54
SC27	31	43

expect this YAC probe to detect almost the entire region shown in Fig. 4a. In LB189, 10% (6/60) of the YAC signals were associated with a CREST signal and 90% were not (Fig. 5b; Table 1). We interpret these associations as due to chance events where, in the flattened nucleus, the YAC DNA sequences overlap with the centromere of another chromosome. In contrast, in SC27, 31/74 (42%) of the YAC signals were associated with a CREST signal (Fig. 5c; Table 1). This is less than the 100% that might be expected, but this is more likely to be due to the technical difficulty of detecting a CREST signal at all copies of the neocentromere than to a genuine lack of centromeric proteins from some copies of the *rea(Y)*. The

CREST signal is lower at the neocentromere than at other centromeres (Fig. 1d), and the conditions required for simultaneous visualisation of the CREST and FISH signals mean that neither is optimised. Nevertheless, the difference in association frequencies is highly significant ($P < 1\%$, Chi-squared test). In most nuclei, the FISH signal formed a larger region than the CREST, and the CREST signal usually lay towards the edge of the FISH region. In extended and distorted nuclei, found mainly around the edge of the slide, the relative localisation could be viewed in more detail. In the most highly extended nuclei, the CREST signal lay at one end of a single linear FISH signal (Fig. 5d). The centromere protein domain therefore lies within the *DAZ* gene cluster but close to its Yqter edge. The repetitive nature of these DNA sequences has hindered attempts to carry out a more precise examination of the sequences at the neocentromere.

Discussion

The unique rearranged Y chromosome and neocentromere described in this report throw some light on how neocentromeres originate and the range of sequences that can form a neocentromere. The father's Y chromosome was examined and was cytogenetically normal, so the *rea(Y)* represents a *de novo* rearrangement that must have taken place during spermatogenesis or early development of the embryo. The chromosome is an inverted duplication, grossly symmetrical but with one arm approximately 1 Mb longer than the other. It could have arisen from a single recombination event in slightly misaligned sister chromatids, between repeated sequences ~1 Mb apart and orientated in opposite directions (Fig. 6).

The father was severely oligospermic and the embryo arose from FIVET; infertile males may have deletions of the *DAZ* gene region on the Y chromosome (Reijo et al. 1995), and both the rearrangement and the neocentromere have arisen in the *DAZ* region. Are these coincidences, or is there a causal link? *DAZ* deletions are found at highest frequency in azoospermic males, where they may be present in over 10% of cases (Reijo et al. 1995), but are also known in severely oligospermic males (Vogt et al. 1996); however, the father did not have a *DAZ* deletion and the cause of his oligospermia is unknown. Thus, while the coincidence seems remarkable, and it remains possible that the general organisation of the *DAZ* region or the specific structure found in the father predisposes simultaneously to infertility, rearrangement and neocentromere formation, we have no evidence for a causal link. In vitro fertilisation is not generally associated with an increased risk of chromosomal abnormalities (Tarlitzis and Grimbizis 1999). The loss of most of the euchromatic portion of the Y chromosome would be expected to result in Turner Syndrome, which is usually lethal in utero, and would explain the phenotype observed.

Our map of the *DAZ* region differs from that previously published (Yen 1998), despite being based on many of the same YAC clones, in that it is about twice the size and does not postulate a high frequency of internal YAC deletions. Inspection of the mapping data reveals that individual YAC maps agree well, and that the difference is due to interpretation as a single genomic region with rearrangements in the YACs (Yen 1998), or a duplicated genomic region corresponding to the YAC structures (Fig. 4). Figure 4d, e provides evidence for two genomic copies of the large SfiI fragment carrying the *DAZ* gene, one associated with 4G6 and one not, and so we favour the structure shown in Fig. 4a. This is consistent with the generally complex organisation of Yq11 (Kirsch et al. 1996). The sequence complexity of the region has prevented us from localising the neocentromere precisely, and we consider that further work would be better directed towards simpler neocentromeric DNA sequences.

The detection of 48,Xrea(Y)rea(Y)rea(Y) cells in the placenta and a 45,X cell line in the amniocytes and hygrogram cells suggests that the rea(Y) chromosome is segregating less accurately than a normal Y chromosome. These differences between cell lineages could result simply from random segregation errors, although we cannot rule out the possibility of a higher error rate immediately after the formation of the neocentromere, or differential selection acting on cells carrying different numbers of the rea(Y). If one copy of the rea(Y) was present in the fertilised egg, chromosome loss was occurring at the same rate throughout development, the growth rates of the 46,Xrea(Y) and 45,X cells were the same, and 16 week old amniocytes had gone through 35–40 cell divisions, the observed 30% loss would correspond to a loss rate of about 1% per cell division. This is about twice as high as the maximum rate seen in transformed aneuploid HeLa cells (Burns et al. 1999), and is much higher than that expected for a normal Y chromosome in an embryo. The presence of Mad2 at the neocentromere (Figure 2d) suggests that a failure to activate the spindle assembly checkpoint cannot account for this inefficiency.

In conclusion, our work describes a novel Y chromosome lacking detectable alphoid DNA. Although the chromosome has undergone rearrangement, the position of the neocentromere can be distinguished from that of the breakpoint, and no evidence was found for DNA rearrangement at the site of the neocentromere. Centromeric proteins, however, have bound, but their composition or amount, or the underlying *DAZ* sequence results in a neocentromere that is less efficient than a natural centromere.

Acknowledgements G.F. was supported by an EMBO short-term fellowship and by the CNR (Consiglio Nazionale delle Ricerche), O.Z. by cofin98-MURST (Ministero dell'Università e della Ricerca Scientifica e Tecnologica) and IRCCS Policlinico San Matteo, Pavia, and C.T.S by the CRC.

References

- Alonso AL, Li S, Warburton PE (2000) Analysis of the structure and function of the human centromere/kinetochore complex using combined immunofluorescence and FISH to normal and variant centromeres. In: Speel E-J, Hopman A (eds) Chromosome analysis protocols. Humana Press, in press
- Amon A (1999) The spindle checkpoint. *Curr Opin Genet Dev* 9:69–75
- Barry AE, Howman EV, Cancilla MR, Saffery R, Choo KH (1999) Sequence analysis of an 80 kb human neocentromere. *Hum Mol Genet* 8:217–227
- Brown KE, Barnett MA, Burgdorf C, Shaw P, Buckle VJ, Brown WR (1994) Dissecting the centromere of the human Y chromosome with cloned telomeric DNA. *Hum Mol Genet* 3:1227–1237
- Burns EM, Christopoulou L, Corish P, Tyler-Smith C (1999) Quantitative measurement of mammalian chromosome mitotic loss rates using the green fluorescent protein. *J Cell Sci* 112:2705–2714
- Choo KH (1997) Centromere DNA dynamics: latent centromeres and neocentromere formation. *Am J Hum Genet* 61:1225–1233
- Cohen D, Chumakov I, Weissenbach J (1993) A first-generation physical map of the human genome. *Nature* 366:698–701
- Cooper KF, Fisher RB, Tyler-Smith C (1992) Structure of the pericentric long arm region of the human Y chromosome. *J Mol Biol* 228:421–432
- Depinet TW, Zackowski JL, Earnshaw WC, Kaffe S, Sekhon GS, Stallard R, Sullivan BA, Vance GH, Van Dyke DL, Willard HF, Zinn AB, Schwartz S (1997) Characterization of neo-centromeres in marker chromosomes lacking detectable alpha-satellite DNA. *Hum Mol Genet* 6:1195–1204
- du Sart D, Cancilla MR, Earle E, Mao JI, Saffery R, Tainton KM, Kalitsis P, Martyn J, Barry AE, Choo KH (1997) A functional neo-centromere formed through activation of a latent human centromere and consisting of non-alpha-satellite DNA. *Nat Genet* 16:144–153
- Earnshaw WC, Ratrie H, Stetten G (1989) Visualization of centromere proteins CENP-B and CENP-C on a stable dicentric chromosome in cytological spreads. *Chromosoma* 98:1–12
- Foote S, Vollrath D, Hilton A, Page DC (1992) The human Y chromosome: overlapping DNA clones spanning the euchromatic region. *Science* 258:60–66
- Haaf T, Schmid M (1989) Centromeric association and non-random distribution of centromeres in human tumour cells. *Hum Genet* 81:137–143
- Harrington JJ, Van Bokkelen G, Mays RW, Gustashaw K, Willard HF (1997) Formation of de novo centromeres and construction of first-generation human artificial microchromosomes [see comments]. *Nat Genet* 15:345–355
- Ikeno M, Grimes B, Okazaki T, Nakano M, Saitoh K, Hoshino H, McGill NI, Cooke H, Masumoto H (1998) Construction of YAC-based mammalian artificial chromosomes. *Nat Biotechnol* 16:431–439
- Jobling MA, Samara V, Pandya A, Fretwell N, Bernasconi B, Mitchell RJ, Gerelsaikhan T, Dashnyam B, Sajantila A, Salo PJ, Nakahori Y, Disteche CM, Thangaraj K, Singh L, Crawford MH, Tyler-Smith C (1996) Recurrent duplication and deletion polymorphisms on the long arm of the Y chromosome in normal males. *Hum Mol Genet* 5:1767–1775
- Jones MH, Khwaja OS, Briggs H, Lambson B, Davey PM, Chalmers J, Zhou CY, Walker EM, Zhang Y, Todd C, Ferguson-Smith MA, Affara NA (1994) A set of ninety-seven overlapping yeast artificial chromosome clones spanning the human Y chromosome euchromatin. *Genomics* 24:266–275
- Karpen GH, Allshire RC (1997) The case for epigenetic effects on centromere identity and function. *Trends Genet* 13:489–496
- Kirsch S, Keil R, Edelmann A, Henegariu O, Hirschmann P, LePaslier D, Vogt PH (1996) Molecular analysis of the genomic structure of the human Y chromosome in the euchromatic part of its long arm (Yq11). *Cytogenet Cell Genet* 75:197–206

- Larin Z, Taylor SS, Tyler-Smith C (1994) De novo formation of several features of a centromere following introduction of a Y aliphoid YAC into mammalian cells. *Hum Mol Genet* 3:689–695
- Li Y, Benezra R (1996) Identification of a human mitotic checkpoint gene: hSMAD2. *Science* 274:246–248
- Mathias N, Bayés M, Tyler-Smith C (1994) Highly informative compound haplotypes for the human Y chromosome. *Hum Mol Genet* 3:115–123
- Oakey R, Tyler-Smith C (1990) Y chromosome DNA haplotyping suggests that most European and Asian men are descended from one of two males. *Genomics* 7:325–330
- Palmer DK, O'Day K, Wener MH, Andrews BS, Margolis RL (1987) A 17-kD centromere protein (CENP-A) copurifies with nucleosome core particles and with histones. *J Cell Biol* 104:805–815
- Pluta AF, Cooke CA, Earnshaw WC (1990) Structure of the human centromere at metaphase. *Trends Biochem Sci* 15:181–185
- Reijo R, Lee TY, Salo P, Alagappan R, Brown LG, Rosenberg M, Rozen S, Jaffe T, Straus D, Hovatta O, de la Chapelle A, Silber S, Page DC (1995) Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. *Nat Genet* 10:383–393
- Rossi E, Faiella A, Zeviani M, Labeit S, Florida G, Brunelli S, Cammarata M, Boncinelli E, Zuffardi O (1994) Order of six loci at 2q24–q31 and orientation of the HOXD locus. *Genomics* 24:34–40
- Saitoh H, Tomkiel J, Cooke CA, Ratrie H, Maurer M, Rothfield NF, Earnshaw WC (1992) CENP-C, an autoantigen in scleroderma, is a component of the human inner kinetochore plate. *Cell* 70:115–125
- Simoni G, Fraccaro M, Terzoli G, Romitti L, Rosella F, Gimelli G, Cuoco C, Dalprà L, Nocera GF, Tibiletti MG (1985) Cytogenetics of chorionic villi sampling: technical developments and diagnostic applications. In: Fraccaro M, Simoni G, Brambati B (eds) *First trimester fetal diagnosis*. Springer-Verlag, Berlin Heidelberg New York, pp 99–108
- Sullivan BA, Schwartz S (1995) Identification of centromeric antigens in dicentric Robertsonian translocations: CENP-C and CENP-E are necessary components of functional centromeres. *Hum Mol Genet* 4:2189–2197
- Tarlatzis BC, Grimbizis G (1999) Pregnancy and child outcome after assisted reproduction techniques. *Hum Reprod* 14 (Suppl 1): 231–242
- Tyler-Smith C, Oakey RJ, Larin Z, Fisher RB, Crocker M, Affara NA, Ferguson-Smith MA, Muenke M, Zuffardi O, Jobling MA (1993) Localization of DNA sequences required for human centromere function through an analysis of rearranged Y chromosomes. *Nat Genet* 5:368–375
- Tyler-Smith C, Gimelli G, Giglio S, Florida G, Pandya A, Terzoli G, Warburton PE, Earnshaw WC, Zuffardi O (1999) Transmission of a fully functional human neocentromere through three generations. *Am J Hum Genet* 64:1440–1444
- Vafa O, Sullivan KF (1997) Chromatin containing CENP-A and alpha-satellite DNA is a major component of the inner kinetochore plate. *Curr Biol* 7:897–900
- Vogt PH, Edelmann A, Kirsch S, Henegariu O, Hirschmann P, Kiesewetter F, Kohn FM, Schill WB, Farah S, Ramos C, Hartmann M, Hartschuh W, Meschede D, Behre HM, Castel A, Nieschlag E, Weidner W, Grone HJ, Jung A, Engel W, Haidl G (1996) Human Y chromosome azoospermia factors (AZF) mapped to different subregions in Yq11. *Hum Mol Genet* 5:933–943
- Voullaire LE, Slater HR, Petrovic V, Choo KH (1993) A functional marker centromere with no detectable alpha-satellite, satellite III, or CENP-B protein: activation of a latent centromere? *Am J Hum Genet* 52:1153–1163
- Voullaire L, Saffery R, Davies J, Earle E, Kalitsis P, Slater H, Irvine DV, Choo KH (1999) Trisomy 20p resulting from inverted duplication and neocentromere formation. *Am J Med Genet* 85:403–408
- Warburton PE, Cooke CA, Bourassa S, Vafa O, Sullivan BA, Stetten G, Gimelli G, Warburton D, Tyler-Smith C, Sullivan KF, Poirier GG, Earnshaw WC (1997) Immunolocalization of CENP-A suggests a distinct nucleosome structure at the inner kinetochore plate of active centromeres. *Curr Biol* 7:901–904
- Welborn JL, Lewis JP (1990) Analysis of mosaic states in amniotic fluid using the in-situ colony technique. *Clin Genet* 38: 14–20
- Yen PH (1998) A long-range restriction map of deletion interval 6 of the human Y chromosome: a region frequently deleted in azoospermic males. *Genomics* 54:5–12
- Yen TJ, Li G, Schaar BT, Szilak I, Cleveland DW (1992) CENP-E is a putative kinetochore motor that accumulates just before mitosis. *Nature* 359:536–539