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Generation of a human X-derived minichromosome using telomere-associated chromosome fragmentation

Christine J.Farr[1], Rosemary A.L.Bayne[2], David Kipling[2], Walter Mills, Ricky Critcher and Howard J.Cooke[2]

Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH and [2]MRC Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK

[1]Corresponding author

A linear mammalian artificial chromosome vector will require at least three functional elements: a centromere, two telomeres and replication origins. One route to generate such a vector is by the fragmentation of an existing chromosome. We have previously described the use of cloned telomeric DNA to generate and stably rescue truncated derivatives of a human X chromosome in a somatic cell hybrid. Further rounds of telomere-associated chromosome fragmentation have now been used to engineer a human X-derived minichromosome. This minichromosome is estimated to be <10 Mb in size. *In situ* hybridization and molecular analysis reveal that the minichromosome has a linear structure, with two introduced telomere constructs flanking a 2.5 Mb α-satellite array. The highly truncated chromosome also retains some chromosome-specific DNA, originating from Xp11.21. There is no significant change in the mitotic stability of the minichromosome as compared with the X chromosome from which it was derived.

Keywords: α-satellite DNA/chromosome fragmentation/human X chromosome/mammalian artificial chromosome (MAC)/telomere

Introduction

The DNA of a mammalian artificial chromosome (MAC) would be a molecule of defined structure that carries all the functional elements known to be required for normal mammalian chromosome survival, replication and segregation. There are numerous reasons for wanting to generate MACs: they could be used to explore detailed chromosome function–structure relationships in mammalian cells; they would allow the functional analysis of large genes, gene clusters and genomic regions; and would provide an alternative strategy for somatic gene therapy (Huxley, 1994; Monaco and Larin, 1994).

There are two basic approaches to building a MAC. The first involves cutting and pasting together the various cloned functional elements, an approach used successfully to create artificial chromosomes in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Murray and Szostak, 1983; Hahnenberger *et al.*, 1989). An alternative strategy is the fragmentation of an existing chromosome (Farr *et al.*, 1991, 1992; Brown, 1992; Brown *et al.*, 1994).

Extrapolating from yeast it is assumed that a functional mammalian chromosome will need to have at least three components for stable replication and segregation: a centromere, two telomeres and replication origins (Tyler-Smith and Willard, 1993). In the mammalian system, the telomere is the only functional element to have been defined and for which a reliable assay is available. Origins of replication are thought to be abundant, with one located every 50–300 kb in the mammalian genome. Therefore, given the frequency with which they occur, it would seem reasonable to assume that DNA capable of acting as a replication origin will be present in most large (several hundred kilobase pairs) cloned pieces of DNA and will not need to be identified independently. Currently the major limitation to MAC-building strategies is defining a piece of DNA which will be capable of acting as a mitotically functional centromere (Huxley *et al.*, 1994).

In situ hybridization studies have shown that the centric heterochromatic regions of mammalian chromosomes contain a variety of satellite DNAs (Miklos, 1985). In the human genome one such family of tandemly organized repetitive DNA, α-satellite, is found in high copy number at the primary constriction of each human chromosome (Manuelidis, 1978; Mitchell *et al.*, 1985; Willard, 1985). Based on tandem repeat arrays of a diverged 171 bp motif, α-satellite DNA is arranged in a largely chromosome-specific manner, with the amount of α-satellite DNA at different centromeres varying widely (from 200–1200 kb on the human Y chromosome, to as much as 2500–9000 kb on human chromosome 7).

Several studies have indicated a major role for α-satellite DNA in centromere function. For example, transfection of α-satellite DNA into cells has shown that when it integrates into chromosomes it forms, or induces, a constriction and disrupts normal chromosome segregation (Haaf *et al.*, 1992; Larin *et al.*, 1994). Intriguingly, however, a number of linear marker chromosomes have been described in the literature which appear to lack α-satellite DNA (Callen *et al.*, 1992; Crolla *et al.*, 1992; Voullaire *et al.*, 1993; Blennow *et al.*, 1994; Ohashi *et al.*, 1994), suggesting that perhaps more than one DNA sequence can function to ensure correct chromosome segregation. Given the rapid turnover of most DNA sequences at the centromere [i.e. α-satellite in primates, the mouse minor satellite, the presence of (TTAGGG)*n* tracts at many other vertebrate centromeres] it is conceivable that the bulk of DNA sequences at centromeres may only be important to 'space' the various domains (the kinetochore, the central domain and the pairing domain) in the correct orientation. Thus, the critical aspects of DNA organization at mammalian centromeres remain poorly understood.

One approach to building a MAC would be to assemble the various components as a modified yeast artificial chromosome (YAC) and then introduce it into mammalian

cells and assay for mitotic function. However, in addition to the fact that a functional centromere has not yet been isolated, MAC-building strategies based in yeast are faced with the problem that highly repetitive regions of the human genome (such as the centromeric α-satellite DNA) are inherently unstable in *S.cerevisiae* (Neil *et al.*, 1990; Chartier *et al.*, 1992). In most current YAC libraries cloned stretches of α-satellite DNA are generally smaller than 300 kb. Another limiting feature of MAC manipulations in yeast is the fact that yeast telomerase will cap mammalian chromosome ends with DNA characteristic of yeast telomeres (Cross *et al.*, 1990). YAC or natural yeast telomeres have never been reported to function in mammalian cells. Recently Taylor and colleagues (Taylor *et al.*, 1994) retrofitted a YAC with cloned human telomeric DNA and tested how well the human telomere would function in mammalian cells after propagation of the YAC in yeast. They reported that the efficiency with which the modified human telomere functions when capped with *S.cerevisiae* repeats is dramatically reduced. Therefore, a MAC-generating strategy for which the starting point is a mitotically functional human chromosome would offer many advantages.

We and others have previously shown that short stretches (a few hundred base pairs) of (TTAGGG)*n* DNA can function to generate *de novo* telomeres at previously interstitial sites when introduced into mammalian cells (Farr *et al.*, 1991; Barnett *et al.*, 1993; Hanish *et al.*, 1994). Moreover, by combining this approach with the application of biochemical selections, it is possible to isolate fragmentation events involving specific chromosomes (Farr *et al.*, 1992). In some cases whole chromosome arms have been removed, suggesting a strategy for generating a minimal human chromosome. We have previously investigated whether the juxtaposition of a selectable marker and centromeric heterochromatin might result in down-regulation of gene expression through position effects. No such effects were observed, suggesting that the physical proximity of exogenous selectable markers and functional chromosomal elements need not be a limiting feature in this system (Bayne *et al.*, 1994).

In this report we describe the application of telomere-associated chromosome fragmentation (TACF) in the generation of a human minichromosome. Cell line HyTM1/36 is a Chinese hamster fibroblast cell line carrying a truncated version of a human X chromosome, from which the whole of the long arm has been removed by TACF. The chromosome was stabilized by the integration of a telomere construct, pHyTM1, into the α-satellite array and the seeding of a new telomere at this position (Farr *et al.*, 1992; Bayne *et al.*, 1994). This stabilizing integration resulted in the loss of 750 kb of α-satellite DNA, leaving an array of ~2.5 Mb with full mitotic function. This chromosome has now been subjected to a further round of fragmentation. The result is a small linear human chromosome in which the centromere is flanked by two introduced telomere constructs. This chromosome still retains the original hygromycin (Hyg)-associated telomere construct, juxtaposed with the α-satellite array. The second telomere construct (pHpTS1) has rescued a breakage event in Xp11.21. Using pulsed-field gel electrophoresis (PFGE) we estimate that this minichromosome is <10 Mb.

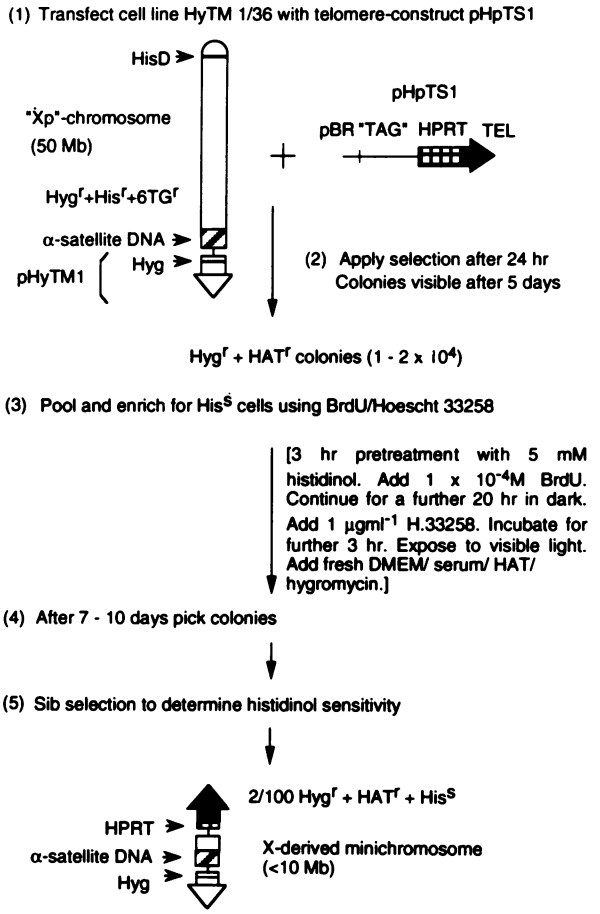

Fig. 1. Strategy for generating a X-derived minichromosome using TACF.

Results

Generation of a small human X chromosome

The strategy for generating an X-derived minichromosome is outlined in Figure 1. A telomere construct (pHpTS1), based on a plasmid carrying a human hypoxanthine phosphoribosyltransferase (HPRT) minigene (Reid *et al.*, 1990), was introduced into the hamster–human somatic cell hybrid HyTM1/36 by electroporation. Stable transfectants, in which the construct had randomly integrated, were selected in HAT-containing media. Preliminary screening of pools of HAT-resistant (HAT^r) transfectants using fluorescence *in situ* hybridization (FISH) failed to identify any in which the construct was integrated into the human X chromosome. To enrich for cells in which integration of the HPRT telomere construct had resulted in the loss of distal Xp sequences, a 5-bromodeoxyuridine (BrdU)-selection technique was used. The short arm of the HyTM1/36 human X chromosome has a bacterial histidinol dehydrogenase gene (*HisD*) targeted into the *MIC2* locus in Xp22.32. Following selection for uptake and stable integration of the linearized plasmid, 10 pools, each consisting of $1–2\times10^3$ Hyg^r+HAT^r colonies, were reseeded and the cells exposed to 5 mM histidinol for 24 h. Three hours after the initial exposure, 5-BrdU was added to the culture medium. Histidinol-resistant (His^r), and therefore actively growing, cells incorporate BrdU into their DNA with lethal consequences. Cells in which

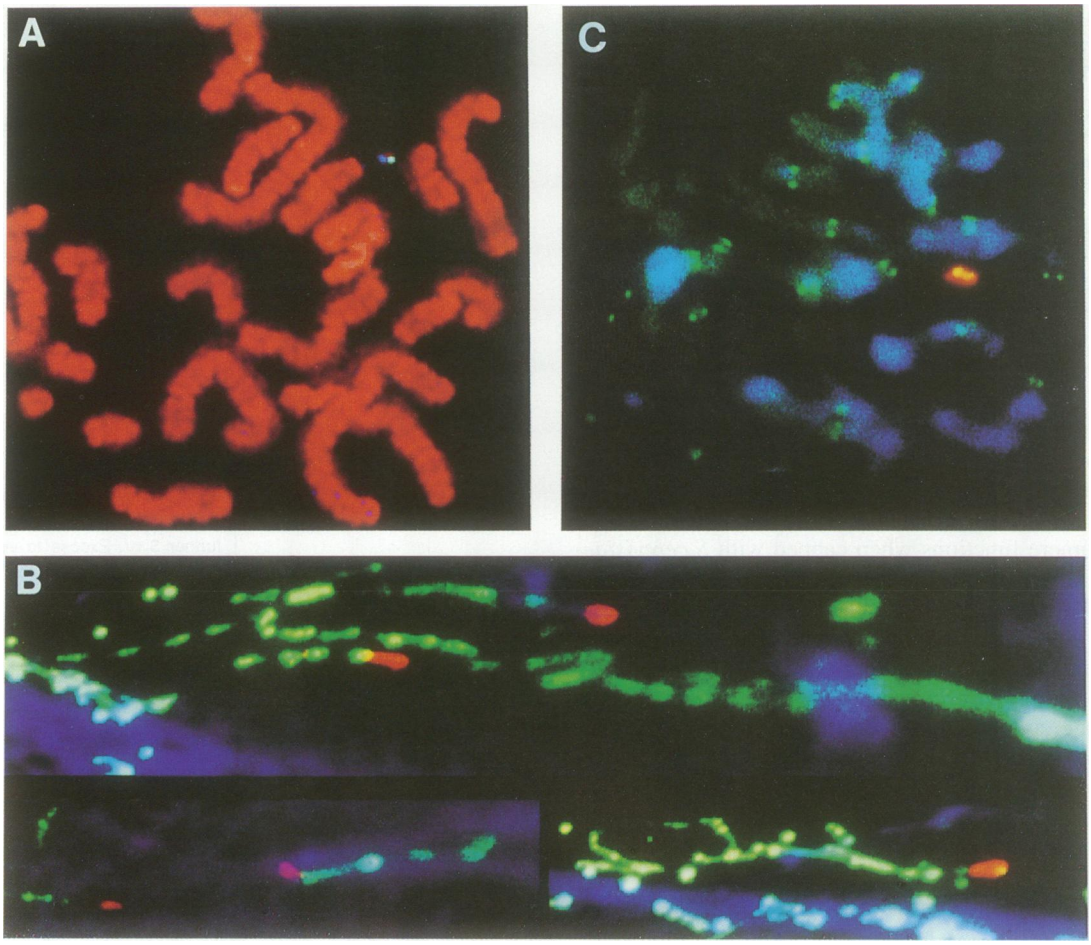

Fig. 2. *In situ* hybridization of the TACF-generated minichromosome. (**A**) Metaphase chromosomes from cell line 36HB7-7 probed simultaneously for both telomere constructs. The hygromycin-based construct, pHyTM1, was detected using Cy5.29-avidin and appears blue, while pHpTS1 was detected with FITC-labelled anti-digoxigenin antibodies and appears green. The chromosomal DNA was counterstained with propidium iodide (red). (**B**) Fluorescence *in situ* hybridization to extended DNA fibres prepared from agarose-embedded 36HB7-7 cells. Hybridization of DXZ1 (an X-specific α-satellite probe) detected with FITC-labelled anti-digoxigenin antibodies (green), and pHyTM1 detected with avidin-DCS-conjugated TRITC (red). (**C**) Metaphase spreads from 36HB7-7 showing colocalization of centromeric proteins (CENPs) and α-satellite DNA sequences. CENPs were visualized by indirect immunofluorescence with CREST patient serum and green FITC-conjugated goat anti-human IgE (Sigma). Human X-specific α-satellite was visualized by Texas Red fluorescence. The hamster chromosomes were counterstained with DAPI (blue).

the integration of the telomere construct has resulted in loss of distal Xp, including the *hisD* locus, are unable to grow in the presence of histidinol and, since they are not metabolizing, will not incorporate BrdU. As long as the exposure time to histidinol is kept relatively brief (to avoid killing the cells of interest) this strategy will enrich for histidinol-sensitive (His^s) cells in the predominantly His^r population. Colonies from the BrdU-selected flasks were picked and established as cell lines. Sib selections were undertaken to determine whether or not the cell lines were sensitive to histidinol.

Two lines out of 100 tested were His^s. These cell lines (referred to as 36HB7-2 and 36HB7-7), together with two His^r transfectants (36HB7-1 and 36HB7-10), were analysed further using FISH.

Cytogenetic analysis of the TACF-generated cell lines

The location of the pHpTS1 construct in these cell lines was determined by FISH. The HPRT telomere construct minus the (TTAGGG)*n* array (pHTAG3), and DXZ1 (specific for the human X α-satellite DNA) were used as probes. The control cell lines 36HB7-1 and 36HB7-10 both retained the whole of the short arm of the X chromosome present in HyTM1/36 and had single copy random integrations of the HPRT construct into the hamster genome: in 36HB7-10 the construct was located internally on a hamster chromosome, while in 36HB7-1 the construct appeared terminal. In both cell lines 36HB7-2 and 36HB7-7 the HPRT probe hybridized to a small independent chromosome, which is also labelled by DXZ1. These lines were picked from the same tissue culture flask and subsequent molecular analysis indicated that they had arisen from the same integration event. However, while 36HB7-7 is diploid, 36HB7-2, which has a noticeably longer doubling time, is triploid (but still retains only a single copy of the minichromosome). Cell line 36HB7-7 was also probed simultaneously for the original telomere construct pHyTM1 and for the more recently introduced pHpTS1. As shown in Figure 2A the two signals are co-detected on the small independent chromosome. Extended DNA fibre FISH provides direct visual evidence for the juxtaposition of the hygromycin-associated telomere construct with the α-satellite DNA (Figure 2B). The same

Table I. PCR analysis of TACF-generated cell lines

Xp	Introduced markers	Endogenous loci	TACF-generated cell lines				
			HyTM1/36	36HB7-1	36HB7-2	36HB7-7	36HB7-10
22.33	HisD		+	+	–	–	+
22.31		DXS1224	+	+	–	–	+
22.13		DXS1229	+	+	–	–	+
11.4		DXS993	+	+	–	–	+
11.3		DXS1055	+	+	–	–	+
11.3		DXS1003	+	+	–	–	+
11.23		DXS1039	+	+	–	–	+
11.22		DXS988	+	+	–	–	+
11.21		DXS741	+	+	–	–	+
11.21		DXS991	+	+	–	–	+
11.21		ZXDB	+	+	–	–	+
11.21		DXS14	+	+	–	–	+
11.21		ZXDA	+	+	+	+	+
cen		DXZ1	+	+	+	+	+
cen	Hyg		+	+	+	+	+
–	HPRT		–	+	+	+	+

Symbols are as follows: +, present; –, absent. The cell lines lack Xq DNA sequences.

technique reveals that the α-satellite DNA and pHpTS1 are not juxtaposed (data not shown). The combined application of *in situ* hybridization and protein immunofluorescence using CREST autoimmune sera reveals colocalization of centromere proteins (CENPs) with the human α-satellite DNA on the minichromosome (Figure 2C). Hybridization of metaphase spreads from cell line 36HB7-7 with a total hamster DNA probe, followed by a low stringency wash (2× SSC at 42°C), did not reveal any hybridization to the minichromosome (data not shown). This suggests that no hamster DNA has been integrated into the minichromosome, although the retention of very small amounts of hamster-derived sequences cannot be totally ruled out.

PCR analysis of the TACF generated cell lines

To determine how much of the human X chromosome had been retained, the cell lines were screened using a panel of PCR primers for Xp loci, for human X α-satellite DNA, and for the various selectable markers (Table I). Apart from the telomere constructs and α-satellite DNA, the only marker retained by cell lines 36HB7-2 and 36HB7-7 is the zinc finger protein gene ZXDA. The PCR primer pair used recognizes two highly similar zinc finger protein genes, ZXDA and ZXDB (Greig *et al.*, 1993), which lie within 400 kb of each other in Xp11.21. ZXDA maps proximal to ZXDB, and is estimated, by interphase FISH, to be within 500 kb of the α-satellite array (Miller *et al.*, 1995). PCR followed by diagnostic restriction enzyme digestion with *Ava*I or *Dde*I allows the two DNA products generated by this primer pair to be distinguished. This revealed that only the human ZXDA locus is present in cell line 36HB7-7 (Figure 3), data consistent with the fact that DXS14, which maps between the two zinc finger protein genes, is not retained.

Molecular analysis of the de novo telomeres

Southern blot analysis of *Bam*HI-cut genomic DNA probed with an HPRT DNA fragment indicated that in 36HB7-1, 36HB7-2 and 36HB7-7 the HPRT telomere construct had seeded a new telomere; heterogeneous smears were clearly visible. In 36HB7-10 a discrete band was seen indicating

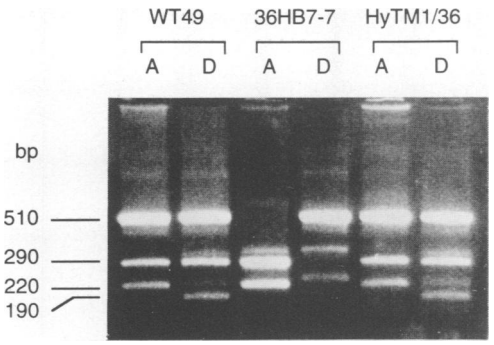

Fig. 3. PCR and diagnostic restriction enzyme analysis for ZXDA. PCR products from the highly similar zinc finger protein genes ZXDA and ZXDB were digested with either *Ava*I or *Dde*I. In the amplified product from the ZXDA locus there is one *Ava*I site and no *Dde*I sites, while in the equivalent product from ZXDB, two *Dde*I sites are present, but no *Ava*I sites. WT49 is a normal female lymphoblastoid cell line; HyTM1/36 is a hamster–human cell line which retains the short arm of the human X chromosome; 36HB7-7 is a hamster–human hybrid which retains an X-derived minichromosome. PCR amplified product was digested with either *Ava*I (lane A) or *Dde*I (lane D). The sizes of the relevant products are indicated. The PCR product corresponding to ZXDA is 510 bp in lane D (non-digested) and 290 bp and 220 bp in lane A. If present, the product corresponding to ZXDB is 510 bp in lane A (non-digested) and 290 and 190 bp in lane D.

that the telomere construct had integrated without seeding a new chromosome end (data not shown). These results are fully consistent with the cytogenetic and PCR analyses. The terminal location of both telomere constructs in cell line 36HB7-7 was confirmed by their sensitivity to *Bal*31 exonuclease digestion (Figure 4B).

DNA adjacent to both the telomere constructs stabilizing the small X chromosome was recovered by plasmid rescue following digestion of 36HB7-7 genomic DNA with *Bam*HI (Figure 4A). Sequence generated using primers to the pBR322 vector DNA was searched against the EMBL database. DNA adjacent to the hygromycin-based construct (referred to as pEC36) matched human X α-satellite DNA, confirming that on this side of the minichromosome the construct and α-satellite DNA are juxtaposed. Sequence generated from the Xp-derived end clone (referred to as

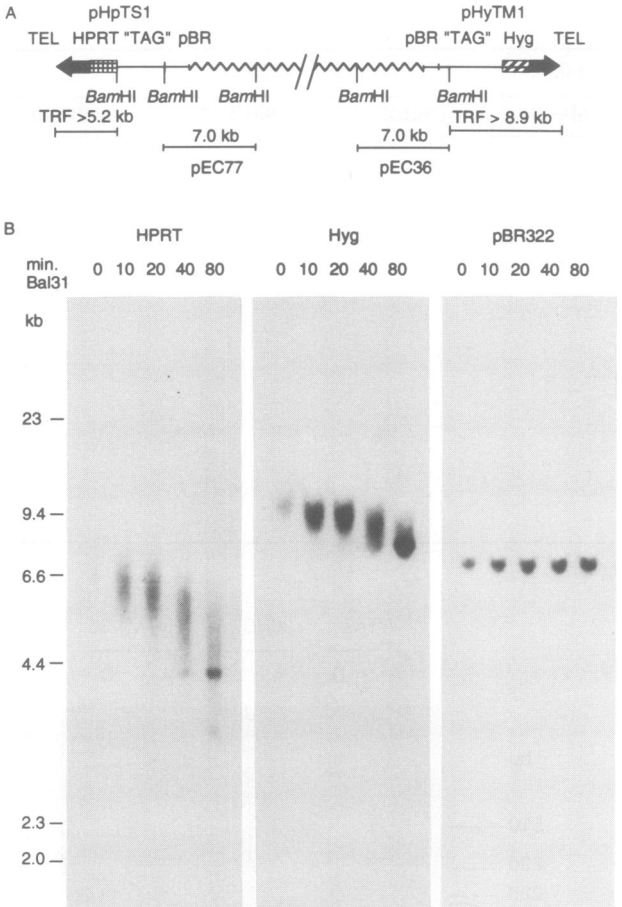

Fig. 4. (**A**) A schematic representation of the X-derived minichromosome showing the *Bam*HI terminal restriction fragments (TRF) and rescued end clones (pEC). (**B**) *Bal*31 nuclease digestions showing that pHpTS1 and pHyTM1 have both seeded new telomeres in cell line 36HB7-7. DNA was extracted, exposed to *Bal*31 for increasing periods of time and then digested with *Bam*HI. Digests were analysed by filter hybridization with a probe specific for each construct, and with a pBR322 control probe.

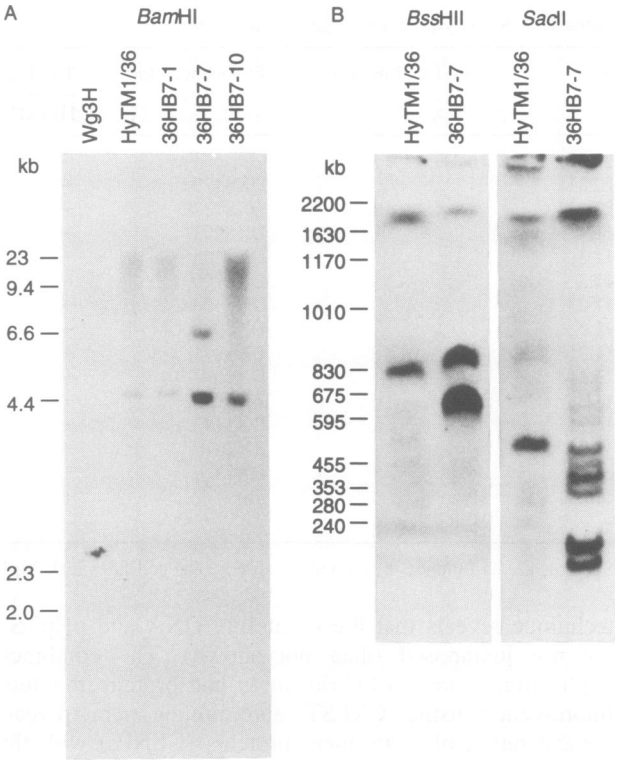

Fig. 5. Southern blot analysis of the X-derived minichromosome in cell line 36HB7-7 using the rescued end clone pEC77 as the probe. (**A**) Genomic DNA was digested with *Bam*HI and separated on a 0.8% agarose gel. DNAs are as follows: Wg3H is the Chinese hamster parent cell line; HyTM1/36 is the TACF-generated hamster–human hybrid which retains the whole of human Xp; cell lines 36HB7-1, 36HB7-7 and 36HB7-10 were generated by the present fragmentation experiment. (**B**) PFGE of high molecular weight DNA from cell lines HyTM1/36 and 36HB7-7 digested with *Bss*HII and *Sac*II. A 1% agarose gel was electrophoresed at 200 V for 25 h with pulse parameters of 60–100 s.

pEC77) included an Alu repeat, but no other database matches were identified. A *Pst*I subclone from pEC77 (which excludes the Alu repeat) was used in a Southern blot analysis. The probe detects a 5 kb fragment in the hamster–human somatic cell hybrid DNAs, but not in the parent hamster cell line Wg3H, confirming that the integration is into the human X chromosome and not into the hamster genome. In cell line 36HB7-7, from which the end clone was rescued, the probe detects a new 7 kb fragment (the same size as the rescued plasmid), but still detects the parental band. In addition the signal from the parental band is more intense, suggesting a 2- to 5-fold amplification of the terminal DNA sequences in this cell line (Figure 5A). The same result was obtained when probing DNA from cell line 36HB7-2 (data not shown).

Pulsed-field gel analysis of the minichromosome

The minichromosome was characterized further using PFGE. In order to determine the overall size of the minichromosome, uncut DNA from agarose blocks was electrophoretically separated (without any prior strand-cleaving treatment), using conditions that allow the resolution of DNA molecules from 3 to 10 Mb. The DNA was then transferred to nylon membrane and probed using DXZ1. In Figure 6 no signal is detectable beyond the wells for HyTM1/36, the starting cell line which retains the whole of the short arm of the X chromosome. In cell line 36HB7-7, which carries the minichromosome, a band of hybridization is detected migrating just above the 5.7 Mb *S.pombe* chromosome I. This signal runs ahead of the limit mobility (LM) DNA and was reproducibly detectable, using several different DNA preparations. No equivalent band was seen in cell line HyTM1/36, or in any other control cell lines which retain large fragments of the human X chromosome. DNA degradation induced experimentally (by γ irradiation of HyTM1/36 high molecular weight DNA) does not produce a signal at the position seen in 36HB7-7, but instead gives rise to a signal largely at the position of LM. This excludes the possibility that the hybridization signal detected in 36HB7-7 is an artefact arising from an accumulation of sheared DNA at the LM position. This suggests that cell line 36HB7-7 contains a separable linear chromosomal entity of ~7–8 Mb. We would expect that only a few percent of these large DNA molecules would migrate beyond the well, which contains the vast majority of nuclear DNA. Non-specific trapping of DNA in the agarose plug is a common technical limitation with very high molecular weight DNA in PFGE.

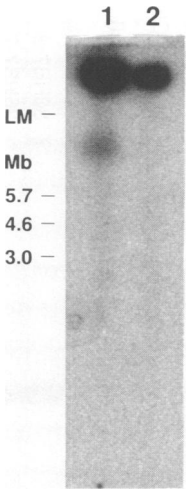

Fig. 6. PFGE of the intact X-derived minichromosome. High molecular weight DNA from cell lines 36HB7-7 (lane 1) and HyTM1/36 (lane 2) was electrophoresed though a 0.7% agarose gel under the following conditions: 0.25× TBE at 11°C, 5000–1000 s switch (logarithmically ramped), 110–100° linearly ramped electrode angle, at 50–45 V (ramped linearly), for 120 h. Markers are *S.pombe* chromosomes. The gel was neutral blotted and probed with DXZ1 (human X-specific α-satellite DNA). The final wash was in 0.5× SSC at 65°C. The image is from a Molecular Dynamics PhosphorImager. LM, limit mobility DNA.

Additional PFGE was carried out in order to elucidate the overall organization of the DNA in the minichromosome. A small internal deletion occurred within the α-satellite array during generation of the minichromosome. This resulted in the loss of an *Spe*I site (Figure 7A) and an estimated 50–100 kb of centromeric α-satellite DNA (see *Bgl*II digest in Figure 7A and B). Amplification of the retained Xp11.21 DNA in 36HB7-7 was confirmed using the rescued end clone to probe *Bss*HII- and *Sac*II-cut DNA (Figure 5B). With *Bss*HII a parental fragment of 850 kb is detected in addition to a new band of 650 kb. The hybridization signal from the new fragment is considerably more intense than from the parental band. With *Sac*II, in addition to the parental band (500 kb), several (four or five) new bands, ranging from 450 to 150 kb, hybridize to the end clone probe. This suggests that some of the Xp-specific DNA retained by the minichromosome has been amplified and is now present in multiple copies. Several *Sac*II sites are present within the amplified unit. *Bss*HII appears to cut less frequently and releases a larger fragment within which there are presumably several copies of the end clone DNA.

Mitotic stability of the human X minichromosome

The mitotic stability of the minichromosome in cell lines 36HB7-2 and 36HB7-7 was analysed and compared with the basal stability of the 50 Mb singly-truncated Xp chromosome retained in the control HPRT transfectants 36HB7-1 and 36HB7-10. The cells were initially grown under both hygromycin and HAT selection for maintenance of the derivative X chromosome. They were then transferred to non-selective medium for up to 2 months (60 doublings). Chromosome stability was estimated by the plating efficiency of each cell line at selected time points (i.e. relative colony numbers obtained when cells were plated in the presence and absence of hygromycin selection). The data presented in Table II show that the X-derived minichromosome in cell line 36HB7-7 is as stable as the 50 Mb Xp chromosome retained in the control HPRT transfectants 36HB7-1 and 36HB7-10. In 36HB7-2 the minichromosome shows significant instability, presumably due to the triploid nature of this cell line and its longer doubling time.

Plating efficiencies allow a distinction to be drawn between cells with or without a selectable marker-carrying human chromosome, but provide no information about the number of X chromosomes retained. Therefore the stability of the TACF-generated chromosomes was also investigated using *in situ* hybridization. The derivative human X chromosomes carried by cell lines 36HB7-7 and 36HB7-10 were visualized by FISH using an X-specific α-satellite probe. At least 100 metaphase spreads from each cell line at each time point were examined to ensure that no chromosome rearrangements had occurred. One thousand interphase nuclei per cell line that were positive with the probe, were analysed at each time point (Table III). One signal indicates a cell that has stably maintained the chromosome at mitosis, while multiple copies indicate that non-disjunction has occurred. (In this experiment we did not assess the number of cells with zero copies due to the complicating factor of variable probe penetration.) In cell line 36HB7-7 the proportion of cells containing one copy of the deleted X chromosome did not change significantly over the time course of the experiment. [In the control cell line 36HB7-10 the proportion of cells with a single X chromosome has apparently declined in cells maintained for 60 days on non-selective media. This variation does, however, fall within the range previously reported for X-chromosome hybrids (Bayne *et al.*, 1994).] Both the plating efficiencies and *in situ* hybridization data indicate that the minichromosome in 36HB7-7 is as stably maintained in the absence of selection as the 50 Mb Xp chromosome present in 36HB7-10 and 36HB7-1, and previous work has shown that the 50 Mb Xp chromosome is as stable as the whole X chromosome present in the hamster–human hybrids 2D7 and HTM18TC8 (Bayne *et al.*, 1994). The dramatic reduction in size of the X chromosome in 36HB7-7 (and the accompanying deletion of a further 50 kb of the α-satellite array) has not affected the stability of the chromosome in the Chinese hamster background over the time period studied.

Discussion

Previously, we and others have reported the use of cloned telomeric DNA to fragment mammalian chromosomes (Farr *et al.*, 1991, 1992; Itzhaki *et al.*, 1992; Barnett *et al.*, 1993; Hanish *et al.*, 1994). More recently a similar approach was used to produce long- and short-arm truncations of a human Y chromosome (Brown *et al.*, 1994). In the X chromosome experiments frequent breakage/telomere seeding was observed within or close to the centromere, suggesting a way of producing a minimal human chromosome, which could be used as the basis for a MAC vector. This strategy also offers a powerful approach for investigating the effect of decreasing size on the stability of human chromosomes and a way of functionally dissecting centromere domains (Brown *et al.*, 1994).

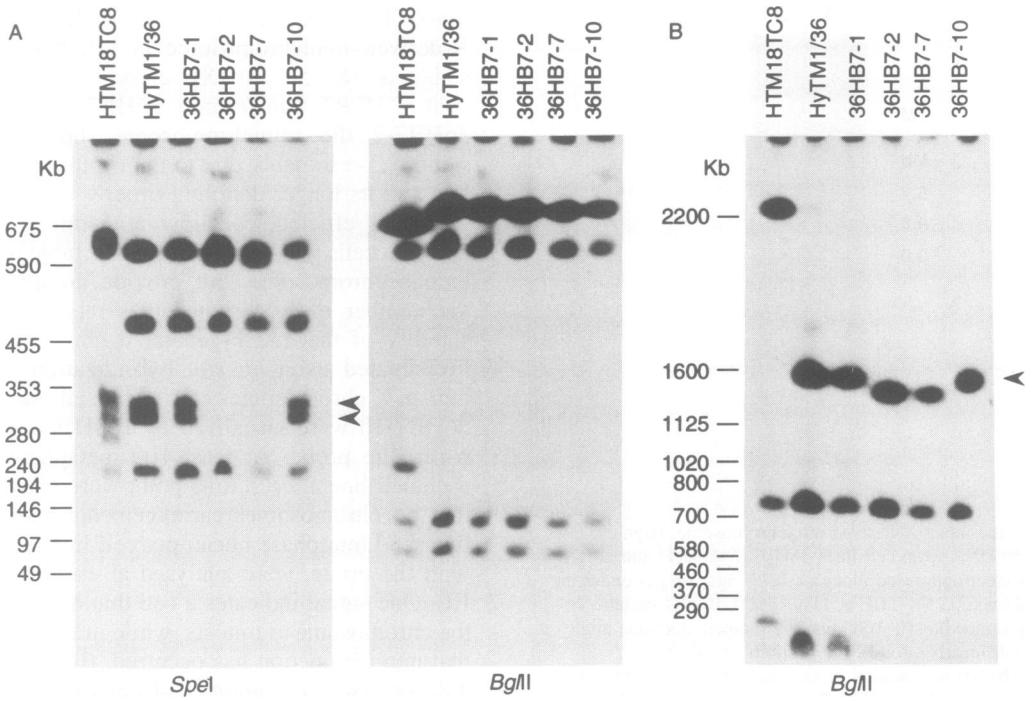

Fig. 7. Pulsed-field gel analysis of the α-satellite DNA in the X-derived minichromosome. Genomic DNA was digested with *Spe*I or *Bgl*II and the filters probed with the X-specific α-satellite DNA DXZ1. *Saccharomyces cerevisiae* chromosomes were used as markers. Cell line HTM18TC8 is a hamster–human hybrid which retains the whole X chromosome. Gel conditions were as follows: (**A**) 200 V, pulse parameters 20–60 s for 24 h; (**B**) 150 V, pulse parameters 120–200 s for 45 h. Separation of the *Bgl*II digests using two sets of PFGE conditions is shown, allowing resolution from 50 to 2000 kb. Arrows indicate hybridizing bands missing or shifted in 36HB7-2 and 36HB7-7 compared with HyTM1/36, 36HB7-1 or 36HB7-10.

This paper reports the generation and characterization of a small (<10 Mb), mammalian chromosome. Molecular and cytogenetic analyses show that it has a linear structure and is mitotically as stable as the human X chromosome from which it was derived. The minichromosome consists of ~2500 kb of α-satellite DNA, together with several Mb of rearranged DNA, which originates from Xp11.21. PCR analysis places the original Xp breakpoint between ZXDA and DXS14. Since the distance between these two markers is estimated to be not more than 400 kb and the distance separating the zinc finger protein gene from the α-satellite array is probably no more than 500 kb (Miller *et al.*, 1995), a straightforward breakage and healing event would have generated a minichromosome in the 3–4 Mb size range. However, PFGE places the size of this chromosome at ~7–8 Mb, considerably larger. This disparity can be accounted for by the presence of multiple copies of the DNA fragment detected using the rescued end clone. This amplification of the terminal DNA sequences has presumably arisen by a breakage–fusion–bridge cycle following chromosome breakage and before telomere healing, as has been discussed previously (Farr *et al.*, 1992).

The fact that a breakage event was rescued originating in this part of proximal Xp is of interest in light of evidence recently presented (Wolff *et al.*, 1994) for a chromosomal breakage hotspot in Xp11.21, which encompasses the duplicated zinc finger protein genes ZXDA and ZXDB. They describe four marker chromosomes with breaks between ZXDA and DXS423E. These authors suggest that this hotspot may result from misalignment and/or exchanges due to the presence of inverted repeat sequences, directly duplicated gene sequences, or to one or more inversion polymorphisms in the pericentric region. This region has been implicated in a number of X

Table II. Stability of the X-derived minichromosome as determined by plating efficiency

Cell line/days off selection	Colony number when plated on selection/colony number plated off selection (%)
36HB7-1 (Xp 50 Mb human chromosome/diploid)	
0	1913/2125 (90)
30	1754/1812 (97)
60	2408/2589 (93)
36HB7-2 (<10 Mb human minichromosome/triploid)	
0	2292/2510 (91)
30	1686/2245 (75)
60	1608/3063 (53)
36HB7-7 (<10 Mb human minichromosome/diploid)	
0	1422/1493 (95)
t30	1280/1288 (99)
t60	2010/2030 (99)
36HB7-10 (Xp 50 Mb human chromosome/diploid)	
0	1719/1738 (99)
30	1549/1671 (93)
60	1574/1589 (99)

Colonies were stained and counted 6 days after plating. Percentage stability is shown in parentheses.

Table III. Stability of the human X-derived minichromosome as determined by *in situ* hybridization

Cell line/days off selection	Number of signals with the human X α-satellite DNA probe					
	0	1	2	3	4	5
36HB7-7 (<10 Mb human minichromosome/diploid)						
0	nd (2)	864 (89)	125 (8)	11 (1)	0 (0)	0 (0)
30	nd (3)	791 (87)	191 (9)	18 (1)	0 (0)	0 (0)
60	nd (4)	874 (82)	111 (13)	13 (0)	2 (0)	0 (0)
36HB7-10 (50 Mb Xp chromosome/diploid)						
0	nd (4)	851 (83)	143 (11)	6 (2)	0 (0)	0 (0)
30	nd (2)	859 (84)	128 (13)	11 (0)	2 (1)	0 (0)
60	nd (0)	655 (61)	323 (39)	17 (0)	5 (0)	0 (0)

A total of 1000 interphase nuclei were scored for each cell line at each time point. The X-derived chromosomes were identified by *in situ* hybridization with DXZ1. Doublets of dots were counted as one signal. Data for 100 metaphases scored at each time point for the two cell lines are shown in brackets. nd = not determined.

chromosome abnormalities, such as isochromosomes and X;autosome translocations ascertained in patients with incontinentia pigmenti (de Grouchy *et al.*, 1985; Gilgenkrantz *et al.*, 1985; Kajii *et al.*, 1985; Gorski *et al.*, 1991).

Our work will now focus on reducing the overall size of the minichromosome still further. It is not known whether a minimum size is required for mammalian chromosomes to replicate, segregate and recombine efficiently. Normal human chromosomes range from 45 to 250 Mb, with ~20–30 microtubules attaching each kinetochore to the spindle pole during mitosis (Bloom, 1994). A number of naturally arising 'dot' chromosomes have been reported in mammalian cells (Olsen *et al.*, 1981; Law *et al.*, 1982; Hamkalo *et al.*, 1985). Although they appear mitotically stable, their analysis has often not been taken beyond the cytogenetic level, with no accurate information about size or structure (linear or ring) available. A somatic cell hybrid retaining a small stable derivative of human chromosome 1 has been described (Carine *et al.*, 1986, 1989). Based on slot blots probed with repetitive DNA the human content of the hybrid line was estimated to be <0.05%. This suggested that the minichromosome retained ~1000–2000 kb of chromosome-specific DNA together with a reduced centromeric domain. However, the possibility of a small ring chromosome was not ruled out.

Our knowledge of centromeric DNA and the way in which it is organized into a functional element is still rather superficial. The amount of α-satellite DNA at human centromeres varies widely, from just a few hundred kilobases to as much as several megabases. Indeed, naturally arising, mitotically stable variants that have undergone large deletions of α-satellite DNA have been described (Wevrick *et al.*, 1990). Moreover, the contributions of the other repetitive DNA families located in and around human centromeres are unclear. Most information available relates to the organization of DNA at the human Y centromere. This has come from two complementary, but very different, approaches: the analysis of naturally arising rearranged Y chromosomes, and the dissection of the Y centromere using cloned telomeres. These studies have shown that sequences necessary for Y centromere function can be localized to an interval containing ~200 kb of α-satellite and 300 kb of adjacent short arm sequences (Tyler-Smith *et al.*, 1993; Brown *et al.*, 1994).

To the extent that it is known, the X chromosome α-satellite DNA appears to be organized as an uninterrupted block of tandemly repetitive DNA surrounded by DNA of normal sequence composition (Mahtani and Willard, 1990). However, the possibility that this tandemly repetitive DNA is interspersed with short oligonucleotides that confer essential function cannot be ruled out. Functional assays, such as the one described in this report, are one way to address this possibility. The truncated X chromosome present in the starting cell line HyTM1/36 is as stable as the whole X chromosome from which it was derived (Bayne *et al.*, 1994). This suggests that the sequences on the long-arm side of the α-satellite array, which were removed during its generation, are not required for mitotic centromere function. Further manipulations of this functional array should also allow us to determine whether DNA sequences adjacent to the α-satellite DNA on the short arm-side of the X chromosome are essential for correct chromosome segregation.

A vital consideration in TACF-based MAC experiments is whether a stable chromosome can be generated that consists only of a functional centromere domain and replication origins flanked by introduced telomere constructs. The spatial concentration of the various chromosomal elements into such a small structure may result in functional interference. Indeed, it is conceivable that the limiting size of such a chromosome will reflect the length of the centromeric domain retained. The use of cloned telomeres systematically to manipulate the structure of human chromosomes in living cells will allow us to answer at least some of these questions.

Materials and methods

Recombinant DNA constructs

All molecular biology manipulations were carried out using standard methods (Sambrook *et al.*, 1989). The telomere construct used in this study (pHpTS1, Figure 1) is based on a pSV_2 plasmid backbone into which has been inserted a human HPRT minigene (Reid *et al.*, 1990). A 1.6 kb array of (TTAGGG)*n* (de Lange *et al.*, 1990) and flanking Bluescript polylinker was cloned into the unique *Nde*I site of the pBR322 sequence. In addition the construct contains a 4.5 kb 'tag' of genomic DNA from human chromosome 17 inserted into the *Bam*HI of the SV40 DNA. This 'tag' serves to increase the overall size of the plasmid,

allowing direct visualization of each integration site by FISH. A unique *Not*I site, which lies immediately distal to the (TTAGGG)*n* repeat array, was used to linearize the plasmid before transfection.

Cell culture and transfection

All cell lines are Chinese hamster fibroblasts containing a deletion derivative of the human X chromosome [originally derived from the somatic cell hybrid 2D (Goss and Harris, 1977)]. Cells were routinely grown in Dulbecco's modified Eagle's medium with penicillin and streptomycin, plus 10% fetal bovine serum and 500 μg/ml hygromycin B (Calbiochem). This basic medium was supplemented with additional selective agents as required. HPRT minigene transfectants were selected in the presence of 1× HAT (100 μM hypoxanthine/10 μM aminopterin/ 1.6 μM thymidine; Gibco BRL). Histidinol sensitivity was tested by the addition of 5 mM histidinol (Sigma).

Cells were transfected by electroporation using a Bio-Rad Gene Pulser. Actively growing cells were harvested by trypsinization, washed and resuspended in phosphate-buffered saline (without Ca^{2+} or Mg^{2+}). Cells (2×10^6) were electroporated in a volume of 800 μl with 10 μg of linearized plasmid DNA at 25 μF and 3 kV/cm. Cell viability under these conditions was routinely 20–50%. Selection was applied 24 h after transfection.

Cell line HyTM1/36 was transfected with the telomere construct pHpTS1 and 10 pools, each consisting of $1–2\times10^3$ Hyg^r + HAT^r colonies, were generated. The colonies were trypsinized and the cells from each pool seeded into 175 cm^2 tissue culture flasks. When confluent the bulk of the cells were frozen down, while 1×10^6 cells from each pool of HAT^r transfectants (passage 2) were seeded into 175 cm^2 flasks. To enrich for histidinol-sensitive transfectants a BrdU-killing approach was employed (Puck and Kao, 1967; Kaufman and Davidson, 1977). After attachment (2 h after plating) cells were pre-exposed to 5 mM histidinol for 3 h. 5-BrdU (Sigma) was added to the histidinol-containing culture medium at 1×10^{-4} M and the cells protected from light for 20 h. For the last 3 h of BrdU exposure the bisbenzimidazole dye, 33258 Hoechst (Sigma), was added at a final concentration of 1 μg/ml (2×10^{-6} M). After this time the flasks were exposed to visible light for 60 min at room temperature. 33258 Hoechst has been shown by others (Stetton *et al.*, 1976, 1977) to enhance the photosensitivity of BrdU-substituted cells. Fresh medium containing HAT and hygromycin was then added to the cells. As a control the untransfected parental cell line HyTM1/36 was taken through the same protocol. After 10 days growth 10 colonies were picked from each 175 cm^2 flask. Total colony numbers were in the range 50–200 per flask; no significant difference was apparent between the experimental and control flasks.

The plating efficiency of cells passaged on non-selective media for up to 2 months was determined by plating $1–2\times10^3$ cells per 75 cm^2 flask (in triplicate) into media both with and without hygromycin. Colonies were stained with Crystal violet and counted after 6 days.

DNA preparation, digestion and analysis

DNA from cell lines and plasmids was prepared by standard methods (Sambrook *et al.*, 1989). Restriction digests were carried out according to the manufacturer's recommendations (Gibco BRL). *Bal*31 nuclease (Gibco BRL) digestions were carried out as previously described (Farr *et al.*, 1991). Southern transfer from 0.5% agarose gels was onto nylon membranes (Hybond N+, Amersham). Filters were probed with DNA fragments labelled by the random-primer method (Feinberg and Vogelstein, 1984). Southern hybridizations were carried out in a Hybaid oven and washed at high stringency (0.1× SSPE/0.1% SDS) at 65°C unless otherwise stated.

Probes

Southern blot filters were probed with the following DNA fragments: a 2.1 kb *Eco*RI–*Nde*I from pBR322; DXZ1, an X-chromosome-specific α-satellite 2 kb *Bam*HI fragment from pSV_2X5 (Wolfe *et al.*, 1985); a 900 bp *Pst*I–*Xba*I 5′ fragment of human HPRT minigene (Reid *et al.*, 1990); a 550 bp *Eco*RI–*Sst*II fragment from the hygromycin B resistance gene (Kaster *et al.*, 1983); a 2.5 kb *Pst*I fragment from pEC77, the end clone rescued from the Xp side of the minichromosome. *In situ* hybridization was carried out using the human X-chromosome α-satellite probe and derivatives of the telomere constructs pHyTM1 and pHpTS1, which lack the (TTAGGG)*n* array (pHyTMΔ and pHTAG3).

Polymerase chain reaction

Polymerase chain reaction (PCR) was carried out as previously described (Farr *et al.*, 1992). Primers used to determine the retention or loss of the various constructs were as follows: HYG (bacterial hygromycin dehydrogenase) Forward: TGC AAG ACC TGC CTG AAA CC; Reverse: ACA TTG TTG GAG CCG AAA TCC. Conditions: 5 min 94°C [1 min 94°C/1 min 58°C/1 min 72°C]×30; product size 312 bp. HisD (bacterial histidinol dehydrogenase) Forward: GAG CTT CAA TAC CCT GAT TGA CT; Reverse: TCA GCA CCG TTG AGA AGA GCG. Conditions: 5 min 94°C [1 min 94°C/1 min 62°C/1 min 72°C]×30; product size 428 bp. HPRT minigene Forward: ACG TCT TGC TCG AGA TGT GAT; Reverse: CGC GGT ATT TTG CTT TTC CAG. Conditions: 5 min 94°C [1 min 94°C/1 min 56°C/2 min 72°C]×30; product size 500 bp. Sources of the primer pairs used to amplify endogenous human Xp loci are as follows: DXZ1(cen) (Farr *et al.*, 1992); ZXDAB (Greig *et al.*, 1993); DXS14 and DXS741, GDB; DXS991, DXS988, DXS1039, DXS1003, DXS1055, DXS993, DXS1229 and DXS1224 (Gyapay *et al.*, 1994).

End clone recovery and sequencing

Genomic DNA was digested with *Bam*HI and ligated at a concentration of 5 μg/ml with 1 U/ml T4 ligase (New England Biolabs) in the manufacturer's recommended buffer. After circularization the DNA was ethanol precipitated and electroporated into DH5α competent cells. Primers were designed from the *Eco*RI and *Nde*I ends of pBR322 sequence present in the rescued plasmid, in order to obtain sequence from both ends of the human insert. Sequencing was performed on double-stranded templates using Sequenase v2.0 (United States Biochemicals Inc.). The EMBL and GenBank databases were searched using the program BLAST-N.

Pulsed-field gel electrophoresis

Tissue culture cells were trypsinized, washed twice with phosphate buffered saline and resuspended at 2×10^{-7} cells/ml in SE (75 mM NaCl, 25 mM EDTA, pH 7.5). Agarose blocks were prepared and digested according to standard methods. Digested DNA was electrophoresed through 1% ultrapure agarose (Bio-Rad) gels in 0.5× TBE using a Bio-Rad Chef-DR II apparatus. Uncut DNA was separated using a Biometra Rotophor and 0.7% chromosomal grade agarose (Bio-Rad) in 0.25× TBE. PFGE run conditions were as indicated in the figure legends. Markers used were *S.pombe* chromosomes, *S.cerevisiae* chromosomes and a lambda ladder (Bio-Rad).

In situ hybridization

In situ hybridization to metaphase chromosomes and interphase nuclei from exponential cultures was as described (Farr *et al.*, 1991). Probes were labelled by standard nick translation with either biotin-labelled or digoxigenin-labelled dUTP. Hybridization of biotinylated probes was detected with FITC- or Cy5.29 (Biological Detection Systems Inc.)-conjugated avidin. Digoxigenin-labelled probes were immunolocalized by means of fluorescently labelled anti-digoxigenin antibodies. Slides were examined using either: (i) a Bio-Rad MRC 600 laser-scanning confocal microscope. Images were collected using a trichroic filter block (Bio-Rad), merged using Nexus software (Bio-Rad), then opened in Photoshop (Adobe); or (ii) a Zeiss Axioplan fluoresence microscope equipped with a 100 W mercury source and a Photometrics CCD camera. Slides were imaged using a Chroma P1 filter set with excitation filters mounted in a rotating wheel giving exact registration between images from different fluorochromes. Images were acquired and processed using software from Digital Scientific (Cambridge, UK).

Preparation of extended DNA fibres

Agarose-embedded DNA (PFGE blocks) was used as a target for high resolution FISH following the method of Heiskanen *et al.* (1994). A small piece of PFGE block was melted on a poly-L-lysine-coated microscope slide, the DNA extended with the aid of another slide and air-dried.

Immunofluorescent staining

Indirect immunofluorescent staining of cytospun cell preparations with CREST antisera was as described in Nicol and Jeppeson (1994). The slides were then un-crosslinked in 0.1 N NaOH for 30 s and neutralized in 0.1 M Tris–HCl, pH 7.5 for 30 s before fixation in 3:1 methanol: acetic acid for 10 min and air-dried, ready for *in situ* hybridization.

Accession numbers

The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession numbers X87325, X87326 and X87327.

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