De novo formation of several features of a centromere following introduction of a Y alphoid YAC into mammalian cells

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The DNA sequence requirements for mammalian centromere function have been investigated by re-introducing human YAC clones containing either centromeric or non-centromeric sequences into hamster and human cells. All YACs integrated into the host chromosomes. In most cell lines produced by spheroplast fusion into hamster cells, intact copies of the YAC and a large amount of yeast DNA were found. Cell lines produced by lipofection into human cells usually contained simple structures without yeast DNA. YACs containing Y alphoid DNA re-formed several of the properties of a centromere, including a cytogenetically visible constriction, CREST antiserum binding and disruption of anaphase chromosome movement. In contrast, YACs containing non-centromeric sequences produced none of these results. This work suggests that a few hundred kb of alphoid DNA is sufficient to reconstitute several important features of a centromere.

INTRODUCTION

The mammalian centromere is a specialised region of the chromosome which can be defined in several ways. Cytogenetically, it forms the primary constriction in condensed metaphase chromosomes and is the site of sister chromatid attachment. Biochemically, it contains specific chromatin proteins such as CENP-A (1) and CENP-B (2) which are identified by antisera such as those in CREST patients (3), and it binds to the kinetochore, a protein complex that contains additional proteins such as CENP-C (4) and CENP-E (5). Genetically, the centromere ensures stable inheritance of the chromosome during mitosis and meiosis, and controls its copy number, usually at one or two per cell.

Structural studies of mammalian centromeres have shown that they contain long stretches of tandemly repeated DNA sequences: initially, in situ hybridisation was used to locate satellite DNAs close to centromeres (6,7); subsequently, PFGE has been used to analyse the structures of satellite arrays (8,9). For a recent review, see ref 10. The human Y chromosome centromere has been analysed in detail (11,12). It consists of a large block of alphoid satellite DNA (240 kb–1600 kb in different individuals)

![Figure 1](http://hmg.oxfordjournals.org/Downloadedfrom/Bodleian%20Library%20on%20June%2015,2015)

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Figure 2. Introduction of YACs into hamster cells by spheroplast fusion. (a) DNA analysis by PFGE. DNA samples were digested and hybridised as indicated at the top of each track. Gel conditions were 1.5% agarose, 150 volts and a 5 sec pulse for 39 hr. (b) FISH analysis using Yac1 (I–IV) and YAC AMY8 (V) probes. The upper portion shows the merged hybridisation signal (yellow) indicated by an arrow and DNA stain (red). The lower portion shows the DNA stain in black and white with the position of the integrated site indicated by an arrow. I—α2F, II—αPic, III—α3L, IV—α4, V—A1P1.
flanked by at least seven other satellite arrays and several families of localised repetitive sequences (Figure 1). The analysis of rearranged Y chromosomes has allowed a mitotic centromeric interval to be defined: it consists of the alphoid satellite DNA and about 300 kb of adjacent short arm sequences (13).

Functional studies have been carried out by introducing candidate centromeric sequences into mammalian cells in order to analyse their biological properties. In early experiments Heartlein et al. (14) observed that a 0.34 kb fragment of human alphoid DNA introduced into hamster cells followed by amplification of the ingoing DNA resulted in an increase in the number of dicentric and ring chromosomes per cell. In more recent work Haaf et al. (15) introduced ~16 kb of human alphoid DNA into African Green Monkey cells and analysed in detail two lines where extensive amplification had taken place. CREST antiserum binding was seen at the site of integration in the marker chromosomes. Chromosomes containing both an endogenous monkey centromere and the integrated DNA showed abnormalities of segregation such as anaphase bridges, while marker chromosomes containing human alphoid DNA but lacking a detectable monkey centromere segregated normally. One interpretation of these experiments is that the alphoid DNA provides some or all of the information required in cis for the formation of a centromere. However, the observation of centromere formation following the introduction of XCM8 (16), a single copy non-centromeric human sequence from 9pter (17,18), into mouse cells suggests that the interpretation of experiments of this kind may not be simple. In particular, host DNA may be incorporated along with the ingoing sequences and it is therefore important to understand the entire structure of the integrated DNA.

The availability of longer stretches of centromeric DNA cloned as yeast artificial chromosomes (YACs) (19) and the development of techniques for re-introducing intact YACs into mammalian cells (20) provide an alternative experimental system for investigating centromere function. We have therefore introduced YACs containing human Y chromosome alphoid DNA or control non-centric sequences into hamster and human cells. In hamster cells the YACs integrated into the host chromosomes with a large amount of yeast DNA. In human cells no yeast DNA was detected and the Y alphoid YACs, but not the control YACs, re-formed several of the properties of a centromere at the site of integration and disrupted movement of the marker chromosome during mitosis.

RESULTS

Introduction of YACs into hamster cells

YAC α5 containing ~120 kb of Y alphoid DNA and control YAC AMY8 containing ~420 kb of non-centromeric Yp sequences were 'retrofitted' with a neomycin resistance gene to produce α5R, α5L and AMY8L (see Materials and Methods). Spheroplasts of yeast cells carrying these YACs were fused with human male HT1080 cells and hamster CHO-K1 cells. No neomycin resistant colonies were seen when human cells were used, but transformants were readily obtained using hamster cells. Eleven colonies containing Y alphoid DNA and three containing control Yp DNA were examined by filter hybridisation and fluorescence in situ hybridisation (FISH) to metaphase chromosomes. The structure of the alphoid DNA was analysed using the enzymes Accl and BstEII followed by pulsed field gel electrophoresis (PFGE). Both enzymes cut frequently in mammalian and yeast DNA but do not cut within Y alphoid DNA. BstEII has no site anywhere within α5R but does cleave within the LYS2 gene in α5L, while Accl has sites in both vector arms in both YACs. Figure 2a shows an analysis of four representative cell lines. In each sample a Y alphoid probe (Ya1) identified one or a small number of Accl and BstEII fragments similar in size to the parental YAC fragments, and present in one or a few copies as judged by signal intensity. The right vector arm probe hybridised to the same large Accl and BstEII fragments. The left arm probe detected a large BstEII fragment in each cell line but did not usually detect any Accl fragments because the fragments generated were too small to be resolved by PFGE. A probe containing a yeast interspersed repetitive Ty element hybridised to a large and variable number of small fragments in each track (data not shown). The structure of the control Yp YAC DNA was analysed by digestion with EcoRI and conventional electrophoresis (data not shown). Both right and left arm vector sequences were present in all three cell lines. The DNA analysis therefore indicates that at least one copy of the entire YAC has been incorporated into most cell lines. In some cell lines (e.g. α2F and αPIC) there has been no detectable rearrangement of the alphoid DNA, while in others (e.g. α3L and α4) there has been limited rearrangement.

FISH analysis of representative cell lines is shown in Figure 2b. Lines containing a Y alphoid YAC show one (III and IV) or two (I and II) sites of hybridisation embedded in a chromosomal region with an unusual narrow appearance. The control Yp YAC also shows a single site of hybridisation in a narrow region of the chromosome (Figure 2b, V). Similar looking regions have been seen previously (21,22) and are thought to be due to the large amount of yeast DNA integrated at the same site. The unusual region is easily distinguished from a centromeric constriction, but the narrowness makes it difficult to determine whether the alphoid DNA is further constricted. These experiments thus show that it is possible to introduce intact alphoid YACs into mammalian cells, but the co-introduction of yeast sequences at the same site as the alphoid DNA made interpretation of centromeric activity difficult. We have therefore carried out further experiments on transformants generated from purified YAC DNA and lacking yeast sequences.

Introduction of YACs into human cells

α5R and a different control non-centromeric Yp YAC ('retrofitted' with a neomycin resistance gene, CC1R ~165 kb), were purified by PFGE and introduced into human HT1080 cells by lipofection. Twenty colonies were produced from α5R and...
eight from CC1R. The resulting cell lines were analysed in the same way as the hamster transformants. Figure 3a shows a PFGE analysis of four cell lines containing α5R. The parental HT1080 cell line is male and contains large endogenous Y alphoid fragments (~1000 kb for AccI and ~1200 kb for BstEII). The transfectants contain additional alphoid fragments derived from α5R. AccI digests show one or more extra bands between ~140 kb and ~200 kb, similar in size to the YAC, and some small fragments. In contrast, BstEII digests show a single extra band which ranges in size from ~200 kb to ~1100 kb in different cell lines. Both vector arm probes hybridise to the BstEII fragment. The right arm probe also hybridises to the alphoid AccI fragments, while the left arm probe hybridises to smaller AccI fragments. No hybridisation was seen to the Ty element probe.

Figure 5. Anaphase analysis of Ap21 and Ap11. (a) Early anaphase of Ap21, confocal image. The Yα1 hybridisation is shown in yellow and the DNA is shown in red (scale bar = 2 μm). (b) Late anaphase of Ap11, combined image from three standard microscope photographs. The predominantly blue areas are DNA, the green shows the enhanced signal from the Yα1 hybridisation to the lagging chromosome, and the purple region shows the microtubules.
(not shown). These results show that the ingoing alphoid YAC DNA is assembled into a structure of ~200 kb to ~1100 kb in size consisting of YAC multimers with no detectable yeast DNA and, since no BstEII sites have been introduced, little or no additional host DNA. The 8 CC1R colonies hybridised to the R arm, but only 3 hybridised to the L arm (data not shown).

FISH analysis of metaphase spreads from the parental HT1080 cells using a Y alphoid DNA probe showed one or two copies of a Y chromosome with a normal appearance (results not shown and Figure 4). Exogenous alphoid DNA has integrated at a single site in a different chromosome in each transfected cell line (Figure 3b). The morphology of the chromosome is seen most clearly in the propidium iodide stained images, reproduced in black and white. A constriction is formed at the site of integration, even when as little as ~200 kb of alphoid DNA is present (Figure 3b, II). The control Yp YAC CC1R also showed integration at a single site but did not form a constriction (Figure 3b, V). It thus appears that information provided specifically by the alphoid DNA is sufficient to form a new constriction.

**Binding of CREST antiserum at the site of alphoid DNA integration**

To investigate whether the new constriction was associated with the formation of a new site for centromere-associated protein binding, metaphase spreads were stained with a CREST antiserum. Each normal chromosome shows a single site of CREST antiserum binding, metaphase spreads were stained with a CREST antiserum. The chromosmes containing the YAC alphoid DNA in cell line Ap21 can be identified by FISH with a Y alphoid DNA probe (Figure 4d) and shows a second site of CREST antiserum staining at the integration site, indicating that one or more centromere-associated proteins have bound to the alphoid DNA. Similar results were obtained with cell line Ap11. In contrast, integration of the YAC containing control Yp sequences (Figure 4b) did not form a new CREST staining site (Figure 4a).

**Mitotic segregation analysis**

We next investigated whether the new constriction and centromere protein binding site were biologically active by determining whether their presence affected the segregation of the chromosome. Untreated exponentially growing cells were fixed and stained to visualise the host chromosomes, the integrated YAC (if present) and the microtubules. Anaphase cells were located and the frequencies of bridges between the two sets of daughter chromosomes early in anaphase and lagging chromosomes late in anaphase were determined. In the parental HT1080 cell line less than 3% of anaphase cells showed abnormal movement, and similar results were obtained with a cell line containing the control Yp YAC CC1R (Table 1). However, as in the experiments of Haaf et al. (15), a high proportion (11–55%) of anaphase cells from three cell lines containing integrated alphoid YACs showed abnormal movement. For example, a bridge which stains red for total DNA and yellow for alphoid sequences is seen stretched between the two sets of chromosomes during early anaphase (Figure 5a), and a chromosome was observed lagging between chromosome sets which had already segregated to each daughter cell in late anaphase (Figure 5b).

**DISCUSSION**

The availability of a map of the Y chromosome centromere and YAC clones spanning much of the region allows a systematic assessment of the function of DNA segments from this region. Initial experiments used YAC α5 which carries only alphoid DNA. The methods used for introducing YACs into cells, especially lipofection, produce simple integrated structures in contrast to the calcium phosphate precipitation method used in earlier studies. The organisation of the alphoid DNA can be understood from analysis of Actel and BstEII digests. The Actel digests show that the YAC is usually present in an intact form, including vector sequences, while the BstEII digests show that limited local multimerisation has taken place during lipofection to produce single structures 200 kb to 1100 kb in size, about the size of natural Y alphoid arrays (23). BstEII is expected to cleave on average every 5 kb in human DNA but did not cleave within any of these integrated alphoid YAC structures, suggesting that there is unlikely to be additional host DNA in the integrated structures.

A constriction was seen at the site of integration in all cell lines examined, although this was difficult to distinguish in the hamster cell lines due to the large amounts of yeast DNA present. However, alphoid DNA did not cause gross rearrangements such as the formation of ring chromosomes, which in earlier experiments may have been due to the selection for amplification of adjacent dihydrofolate reductase sequences (14). In human cell lines as little as 200 kb of Y alphoid DNA in the absence of yeast DNA is capable of forming a visible constriction. The integrated alphoid DNA thus has one of the main cytogenetic properties of a centromere.

CREST antiserum binding was seen at both constrictions of the resulting dicentric chromosomes in human cells. The antiserum used recognises CENP-A, CENP-B and CENP-C, indicating that one or more of these epitopes is present. As the Y chromosome centromere is reported not to bind CENP-B (24), the only specific alphoid binding protein known (25), possible explanations for the immunofluorescence are: 1. Y alphoid DNA could bind small amounts of CENP-B, perhaps detected here because of the high concentration of antiserum used. 2. It could bind directly to another known protein such as CENP-A or CENP-C recognised by CREST antisera. 3. It could bind to an unknown protein and form a centromere/kinetochore complex which includes known proteins like CENP-A or CENP-C. Further experiments will be required to evaluate these and other possibilities.

The marker chromosomes containing Y alphoid DNA showed abnormal behavior during anaphase. Bridges were visible during early anaphase and lagging chromosomes were observed during late anaphase in about half of the cell in cell line Ap21. Lower levels of abnormal movement were seen in Ap2 and Ap11. The lowest level, in Ap11, may be related to the small amount of alphoid DNA in this cell line or its closeness to a human.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Early Anaphase (%)</th>
<th>Late Anaphase (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT1080</td>
<td>3/140 (2.1%)</td>
<td>4/161 (2.5%)</td>
<td>7/301 (2.3%)</td>
</tr>
<tr>
<td>CC1</td>
<td>3/98 (3.1%)</td>
<td>2/86 (2.3%)</td>
<td>5/184 (2.7%)</td>
</tr>
<tr>
<td>Ap2</td>
<td>4/43 (9.3%)</td>
<td>3/88 (3.6%)</td>
<td>7/131 (29.0%)</td>
</tr>
<tr>
<td>Ap11</td>
<td>5/94 (11.7%)</td>
<td>24/224 (8.3%)</td>
<td>29/218 (13.5%)</td>
</tr>
<tr>
<td>Ap21</td>
<td>5/29 (17.2%)</td>
<td>62/292 (21.4%)</td>
<td>67/321 (25.4%)</td>
</tr>
</tbody>
</table>

*Figures are expressed as number of bridges or lagging chromosomes/number of anaphase cells
centromere. The anaphase observations can be explained in two ways. The integrated alphoid DNA could lead to the formation of a new site of sister chromatid attachment so that the chromatids are attached at two positions at the beginning of anaphase. Disjunction might then be delayed, resulting in the bridging phenotype. Alternatively, or in addition, a new kinetochore might form at the site of the integrated alphoid DNA. A chromatid could then attach to microtubules from both sides of the spindle and form a bridge. The presence or absence of a new kinetochore could be investigated by looking for proteins such as CENP-C (4) or by electron microscopy.

Abnormal anaphase movement does not appear to lead to a high frequency of chromosome rearrangement. Examination of metaphase spreads showed predominantly (although not entirely) a single type of marker chromosome in each cell line, and chromosomes examined 30 generations apart in one subline of Ap21 retained the marker chromosome shown in Figure 3b III. It therefore seems that the abnormal anaphase structures are often resolved without chromosome breakage. However, another subline of Ap21 did contain a different marker chromosome (Figure 4c, d).

In conclusion, our results show that a few hundred kb of Y alphoid DNA is sufficient to direct the formation of at least three properties of a human centromere: a cytogenetic constriction, CREST antiserum binding and disruption of anaphase movement in a dicentric chromosome. The absence of frequent chromosome breakage suggests that the new centromere may have been partially suppressed or may not have formed full activity, perhaps because other sequences are also required. Nevertheless, this work shows that alphoid DNA is an important functional part of the human centromere.

MATERIALS AND METHODS

Plasmids, YACs, probes and cell lines

Retrofitting vectors pRAN4 (26) and pLNAl (27) were used to introduce new markers into YACs by replacement or insertion into the right or left arm, respectively. An alphoid YAC a5 (130 kb), a subclone of aB3 (28), was retrofitted with plpA4 (YAC αSR) or with pLNAl (YAC αSL). Control YACs contained typical euchromatic sequences from intervals 6 and 7 of the Y chromosome short arm (13) and lacked the known satellite sequences. YAC CC1 (C22 C10 (29), 165 kb) was identified using the probe GMG XXY and YAC AMY8 (420 kb) (30) was identified by using the probes S2d and 927R. The two YACs overlapped by about 120 kb. Control YACs were retrofitted with plpA4 (YAC CC1R) and plpA1 (AMY8L) respectively. Alphoid YACs αSR and αSL containing the neo gene were transferred to a recombination deficient strain L1V2 (31) by yeast spheroplast transformation in order to increase their stability. 4.5 x 10^7 yeast cells/ml were spheroplasted with lytase as described (33), and resuspended in STC (1M sorbitol, 10 mM Tris-HCl pH 7.4, 10 mM CaCl2) at 1 x 10^6 cells/ml. Yeast cells were fused with 3 x 10^6 CHO-K1 cells (washed 3 x in serum free medium) in 50% FEG 1500 (Boehringer Mannheim) containing 50 μM B-mercaptoethanol and 5 mM CaCl2, and plated at a density of 5 x 10^5 cells/10 cm dish. After 48 hr cells were selected in G418 (400 μg/ml).

Concentrated YAC DNA for lipofection was prepared by the following procedures. Yeast cells (approximately 4 x 10^9/ml) were embedded in low melting point (LMP) agarose (at a final concentration of 0.75%) in SCE (1M sorbitol, 0.1M sodium citrate pH 5.8, 10 mM EDTA) and 8 mg/ml Novozyme (Novabiochem) and cells were spheroplasted in SCE and 10 mM DTT at 37°C. Cells were lysed in 10 mM Tris, 1% lithium dodecyl sulphate, 100 mM EDTA. YAC DNA was gel purified by PFGE (Walther) on a 1.5% LMP agarose gel run at 8000 rpm for 44 hrs. The gel slice containing YAC DNA was excised (34) and equilibrated in 10 mM Tris-HCl pH 7.4, 1 M EDTA (1 x TE), and 100 mM NaCl, melted at 68°C in 240 μl aliquots, cooled to 37°C and 40 units of agarose (Sigma, 10 units/μl) was added for at least 1 hr. NaCl was then added to 300 mM final concentration prior to transfection. Tissue culture cells at 30–60% confluence in 6 cm dishes were washed in 1 x PBS, and 3 x in serum free medium immediately prior to transfection. Lipopolyamine (Transfectam, Northumbria Biochemicals) in dH2O was mixed with YAC DNA in agarose in a 5:1 to 10:1 (μl/μg) ratio, added to cells and incubated at 37°C for 6 hrs. Cells were washed 3 x in PBS and incubated in medium with serum at 37°C. After 48 hr cells were selected in G418 (200–400 μg/ml) for 10–12 days. Colonies were expanded for preparation of metaphase spreads and high molecular weight DNA in agarose blocks. Transfecting 1 μg of pRAN4 into human HT1080 cells by lipofection produced 4 fold more G418 resistant colonies than 1 μg of pLNAl.

DNA analysis

Genomic DNA was prepared at 1 x 10^6 cells/ml by standard methods (34) and in 10 mM Tris-HCl pH 7.4, 50 mM EDTA at 4°C. DNA in agarose was equilibrated in 1 x TE prior to restriction enzyme digestion. Restriction digests were analysed either by conventional electrophoresis or PFGE (Walther). DNA was transferred to Hybond N+ membranes, processed and hybridised to probes in formamide buffer (35).

FISH analysis

Metaphase spreads were prepared from yeast cells at 50–60% confluence. CHO-K1 cells were either co-cultured treated (Gibco, 0.3 μg/ml) for 1–1.5 hr or pretreated with thymidine (Sigma, 300 μg/ml) for 16 hrs prior to colcemid treatment for 30 min. HT1080 cells were pretreated with bromodeoxyuridine (1 mg/ml, Sigma) for 16 hr, washed twice in PBS and then treated with thymidine for a further 8 hr prior to colcemid treatment for 20 min. Biotinylated (BRL, bionick kit) DNA probes pYAC4, pRAN4, pLNAl, YAC DNA, or DJ23 (Y alphoid, Oncor) were resuspended in appropriate hybridisation buffer (Oncor, containing either 50% or 65% formamide depending on the stringency, 2x SSC, 0.1% dextran sulphate). Chromosomes were stained with propidium iodide (1 μg/ml) and analysed by standard fluorescence microscopy (Olympus BX-2) or confocal microscopy. Single optical sections or 3-D images were collected on a BioRad MRC 600 confocal scanning laser microscope with excitation at 488 nm and simultaneous collection of emission signals at 540 nm +/- 30 nm (FITC) and >600 nm (PI) using a Nikon 60 x 1.4 N.A. oil immersion lens with a pixel spacing between 0.07-0.23 μm and a z-increment of 0.3 μm. Images were averaged over eight frames during collection and further median filtered using a 3 x 3 or 5 x 5 kernel. Average projections of 5–9 optical sections through the metaphase spread were used to visualise constrictions on the PI stained chromosomes. Sites of in situ hybridisation were visualised from a maximum projection of the corresponding images from the FITC channel. 3-D images at anaphase were collected as a stack of 40 dual channel optical sections at 0.3 μm increments and visualised as a maximum projection of the 6–12 sections containing the lagging chromosome. Conventional microscopic images for reproduction were digitised. Images were normalised using the COMOS™ v6.05 and SOM v4.8 MPL™ software (Biorad microsciences Ltd., UK), 24-bit montages were created using Adobe Photoshop™ v2.5 (Adobe Systems Inc., CA) and output to a dye sublimation printer.

Indirect immunofluorescence with CREST autoantibodies

Cells at 60–70% confluence were colcemid treated as described above except that cells were swollen in hypotonic for 10 min at room temperature. Cells were resuspended at 1 x 10^6 cells/ml and 0.5 μl aliquots were spun at 1k for 5 min and made into chamber slides (Nunc). Cells were imaged immediately (Nikon). Cells were treated with 0.1% detergent (saponin, in cold methanol for 30 min, rinsed briefly in cold acetone, and then washed 3 x in PBS prior to detection. Slides were incubated with human autoimmune CREST antisera (detecting CENP-A, CENP-B and CENP-C as determined by Antibodies Inc., CA) diluted 1:2 in PBS at 37°C and then incubated with FITC conjugated anti-human immunoglobulin G (Amersham Inc., diluted 1:2 in PBS) as described (15). Slides were stained with propidium iodide (1 μg/ml) prior to examination by fluorescence microscopy as described above. After collecting the
immunocytochemical data, slides were processed for FISH as described above. If CREST staining was still apparent the FISH procedure was repeated.

Anaphase analysis
(Modified from an unpublished protocol of Karen Brown, Oxford) Cells at 80–90% confluency (seeded on coverslips) were permeabilised and fixed in PBS containing 10 mM EGTA, 2 mM MgCl2, 0.1% Triton X-100, 3.7% paraformaldehyde for 20–25 min at room temperature. Cells were washed 3 x in PBS and then incubated 2 x 10 min in 0.3M glycine. Cells were washed 3 x in PBS and then incubated in 10% goat serum in PBS for 30 min at 37°C. For detection of microtubules, cells were incubated with rat monoclonal antibodies (Jackson ImmunoResearch Labs, diluted 1:100 in 10% goat serum in PBS) for 1 hr at room temperature, washed 5 x 2 min in PBS, and then reacted with anti-rat IgG antibodies (Jackson, diluted 1:100 in 10% goat serum in PBS) conjugated to Texas Red for 1 hr at room temperature. Cells were again washed 5 x 2 min in PBS and then incubated with the crosslinking reagent ethylene glycol bis(succinimidylsuccinate) (Pierce, 5 mM) for 30 min at 37°C to preserve the microtubule morphology. Cells were washed 5 x 2 min in PBS and treated with 100 µg/ml RNase in 2X SSC for 30 min at 37°C. Cells were then washed 3 x in PBS and denatured in 0.2M NaOH for 4 min. Cells were neutralised 3 x in PBS and then hybridised to biotinylated DNA probes (DY22, YAC DNA) previously denatured at 90°C in 12 µl of hybridisation buffer containing 65% formamide. Cells were hybridised for 16 hr prior to FITC-avidin detection as described in FISH analysis. Chromosomes were stained with DAPI or propidium iodide at 1 µg/ml.

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ABBREVIATIONS
CREST, calcinosis, Raynaud’s phenomenon, esophageal hypomotility, sclerodactyly, telangiectasia; FISH, fluorescence in situ hybridisation; kb, kilobase pairs; PFGE, Pulsed field gel electrophoresis; YAC, Yeast artificial chromosome.

REFERENCES