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## RESEARCH

# Molecular Cloning and RARE Cleavage Mapping of Human 2p, 6q, 8q, 12q, and 18q Telomeres

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Large terminal fragments of human chromosomes 2p, 6q, 8q, 12q, and 18q were cloned using yeast artificial chromosomes (YACs). RecA-assisted restriction endonuclease (RARE) cleavage analysis of genomic DNA samples from 11 unrelated individuals using YAC-derived probes confirmed the telomeric localizations of the half-YACs studied. The cloned fragments provide telomeric closure of maps for the respective chromosome arms and will supply the reagents needed for analyzing and sequencing these distal subtelomeric regions.

Telomeres are extraordinarily dynamic chromosomal structures, both at their most distal molecular ends (Cooke and Smith 1986; Moyzis et al. 1988; deLange et al. 1990; Blackburn 1992; Counter et al. 1992) and in their adjacent subtelomeric sequences (Brown et al. 1990; Levis et al. 1993; Lundblad and Blackburn 1993; Blackburn 1994). In lower eukaryotes, alterations of terminal repeat tract length and composition through mutation of telomerase-associated activities result in cellular senescence-like phenotypes (Lundblad and Szostak 1989; Yu et al. 1990). The loss of yeast terminal repeats in a strain deficient in the normal yeast telomere maintenance machinery activates a survival pathway whereby subtelomeric repeat elements are amplified and redistributed among the chromosome ends, amplifying and exposing otherwise buried copies of the terminal repeat motif (Lundblad and Blackburn 1993). This yeast subtelomeric recombination pathway is apparently distinct from the postulated mechanism of normal telomere maintenance in the *Drosophila* genome, which involves transposition of subtelomeric DNA elements (Beissmann et al. 1992a,b; Levis et al. 1993).

There is an almost universal association of low-copy, subtelomere-specific repeat elements with molecular telomeres. Variable amounts of subtelomeric repeats are known to exist in some human subtelomere regions (Brown et al. 1990;

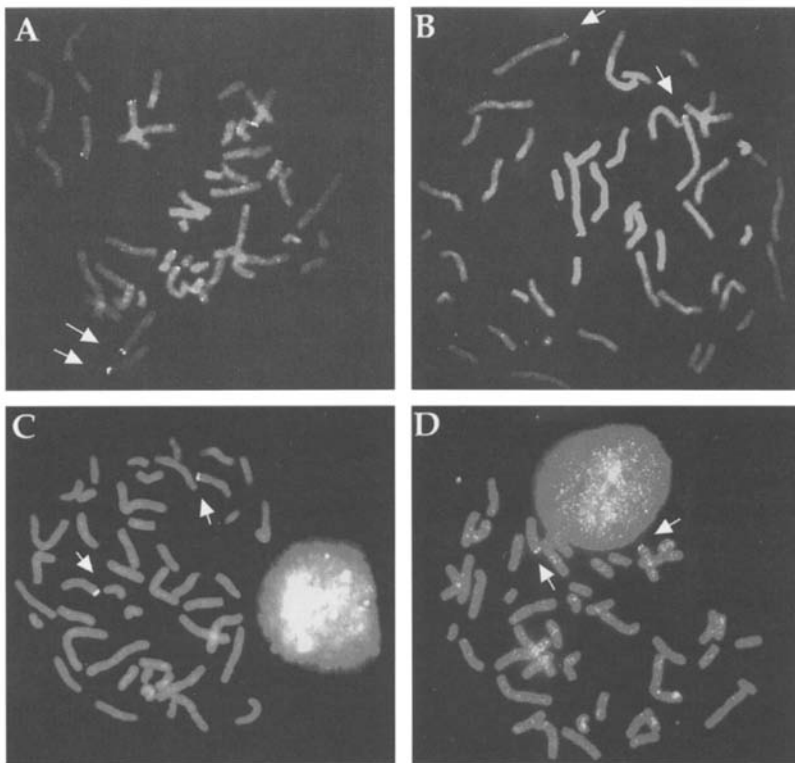
Wilkie et al. 1991; Ijdo et al. 1992), and may account, in part, for the remarkable chromosome length polymorphisms found at the human chromosome 16p telomere (Wilkie et al. 1991). Large chromosome length polymorphisms such as these would place subtelomeric genes at widely disparate allele-specific distances from the molecular telomere. Position effects of telomere proximity upon transcriptional activity have been demonstrated in yeast (Gottschling et al. 1990), and position effects have likewise been observed in *Drosophila* when euchromatic genes are juxtaposed to subtelomeric heterochromatin (Karpen and Spradling 1992). It is possible that the expression of genes located within human subtelomeric regions (Vyas et al. 1992; Saccone et al. 1993; Cook et al. 1994; Reston et al. 1995) may also be affected by telomere proximity and the variable presence of heterochromatin-like structures, although at present no direct experimental evidence exists to support this notion.

Terminal repeat length reduction and an increase in dicentric chromosome formation (Counter et al. 1992) have been correlated with cellular aging and immortalization in human cells. The fate of subtelomeric DNA during these processes has not been followed, primarily because of a lack of well-mapped subtelomeric probes. For example, it is not known whether recovery from human telomere reduction and loss during cellular immortalization mimics the yeast pathway characterized by subtelomeric DNA amplifications and rearrangements. Finely mapped, discrete subtelomeric probes are required for these sorts of studies.

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**Figure 1** FISH localization of half-YAC DNA. G-banded metaphases from leukocytes of a normal human male were photographed, then Alu-PCR products from the indicated clones were biotinylated and hybridized with these metaphases. Hybridization sites were detected using FITC-avidin, and compared with the original G-banded karyotypes. Chromosomes were counterstained with DAPI and propidium iodide. The major hybridization sites are indicated by arrows; only the FITC localizations on propidium iodide counterstained chromosomes are shown. For each probe,  $\geq 20$  metaphases from three separate experiments were examined; in every metaphase, the site with the strongest, paired signals corresponded to the indicated telomere. (A) Clone yRM2158 (6q). Secondary hybridization sites for yRM2158 included 8p23, 5q35, 11p15, 1p36, 1q42, 1q44, 16p13.3, 16q24, and Yq12. (B) Clone yRM2052 (2p); (C) clone yRM2196 (12q); (D) clone yRM2053a (8q).

Cloning, mapping, and sequencing of human telomeres and the telomere-adjacent DNA are crucial for the complete understanding of this peculiar region of the human genome. The molecular cloning of DNA fragments in yeast artificial chromosome (YAC) vectors modified to capture telomere-terminal genomic chromosome fragments (half-YACs) has provided one avenue toward the isolation of large segments of telomere-associated DNA (Riethman et al. 1989; Bates et al. 1990). Recently introduced site-specific genomic cleavage techniques are an extraordinarily versatile and effective means for the direct analysis of genomic DNA structure over size ranges up

to several megabases (Ferrin and Camerini-Otero 1991, 1994), and have provided an efficient means of mapping DNA from half-YACs (Macina et al. 1994; Negorev et al. 1994; Reston et al. 1995). In this study telomeric fragments of human chromosomes 2p, 6q, 8q, 12q, and 18q cloned using the half-YAC system were analyzed using RecA-assisted restriction endonuclease (RARE) cleavage mapping.

## RESULTS

### FISH Localization of YAC DNA

Individual clones from an expanded library of half-YACs (Riethman et al. 1989) were localized to normal human metaphase chromosomes using fluorescence in situ hybridization (FISH) of their Alu-PCR products (Negorev et al. 1994). Each clone is expected to contain a large segment of human subtelomeric DNA flanked by functional human telomere sequences (telomeric end) and vector sequences (centromeric end) (Riethman et al. 1989). The Alu-PCR products from four clones containing inserts roughly in the 200- to 300-kb size range gave strong FISH signals at specific human telomeres (Fig. 1). The 6q-ter signal was consistently the strongest for the yRM2158 probe ( $>20$  metaphases analyzed), although significant secondary hybridization signals were also observed consistently (see Fig. 1A). The Alu-PCR products from yRM2052 (Fig. 1B), yRM2196 (Fig. 1C), and yRM2053a (Fig. 1D) each yielded clear, discrete signals at the telomeres of 2p, 12q, and 8q, respectively. The FISH localization for the fifth half-YAC analyzed in this study (yRM2050a, 18q) has been described previously (Strathdee et al. 1994). A minimum of 20 metaphases from three separate experiments were examined for each probe. These experiments suggested likely genomic origins for the insert DNA from the five half-YACs, but the low resolution of metaphase FISH (1–3 Mb), the presence of subtelomeric repeats in many of the clones (Table 1), and arti-

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#### Half-YAC Probe Isolation and PCR Assay Development

The region of cloned DNA farthest from the molecular telomere is typically least likely to contain subtelomeric repeats and most likely to contain chromosome-specific sequences (Riethman et al. 1989; Macina et al. 1994; Negorev et al. 1994; Reston et al. 1995). Therefore, DNA from the centromeric end of each of the five half-YAC inserts was isolated and used to develop both a hybridization probe and a PCR assay (see Methods; summarized in Table 1). Sequences amplified by the respective PCR assays were localized to individual human chromosomes using a panel of rodent–human hybrid cell lines (Table 1); when combined with the cytogenetic localization data (Fig.

1; Table 1), this information provided strong evidence that the cloned fragments were not chimeric. We then sought molecular evidence that the cloned DNA was physically linked to genomic telomeres.

#### RARE Mapping of Genomic Telomeres

To test whether DNA from the centromeric regions of the respective YACs were closely linked with a genomic telomere, a series of RARE cleavage mapping experiments were conducted (Ferrin and Camerini-Otero 1991, 1994; Macina et al. 1994; Negorev et al. 1994). A single genomic cleavage event is predicted to result in a discrete fragment if the cleavage site is close to the genomic telomere. Therefore, we targeted the *Eco*RI cloning sites derived from the respective YACs for cleavage in genomic DNA and tested whether a liberated restriction fragment in the broad size range of the respective YACs could be detected using the vector-adjacent insert DNA probes.

Specific examples of RARE cleavage experiments for the different genomic telomeres are shown in Figure 2, and the accumulated results are summarized in Table 2. For each telomere, one RARE cleavage fragment in the expected size range of the YACs was detected when genomic DNA samples from each of 11 unrelated individuals were analyzed, providing direct evidence that the cloned fragments were in fact derived from telomere-terminal genomic DNA (Table 2).

The complete RARE cleavage experiment is shown for the 6q telomere (Fig. 2A). RARE cleavages of DNA from human peripheral blood cells

**Table 1. Cytogenetic and chromosomal localization of telomeric half-YACs**

Clone name	GDB designation	YAC size (kb)	Probes	Cytogenetic localization	Chromosomal localization <sup>a</sup>	Subtelomeric repeats content
yRM2158	D6S1062	280	2158V-I <sup>b</sup>	6q27 (+)	6	TH14, <sup>c</sup> HC1103, <sup>d</sup> HC1208 <sup>e</sup>
yRM2196	D12S399, D12Z5	190	2196V-I	12q24.3	12, 6	TH14, TelBam 3.4 <sup>f</sup>
yRM2050a	D18S553, D18Z4	290	2050V-I	18q23	18	
yRM2052	D252146	330	2052V-I	2p25	2	HC1403 <sup>e</sup>
yRM2053a	D8S595, D8Z6	170	2053V-I	8q24.3	8, 20	

<sup>a</sup>Somatic hybrid panel localization of vector-adjacent insert sequences.

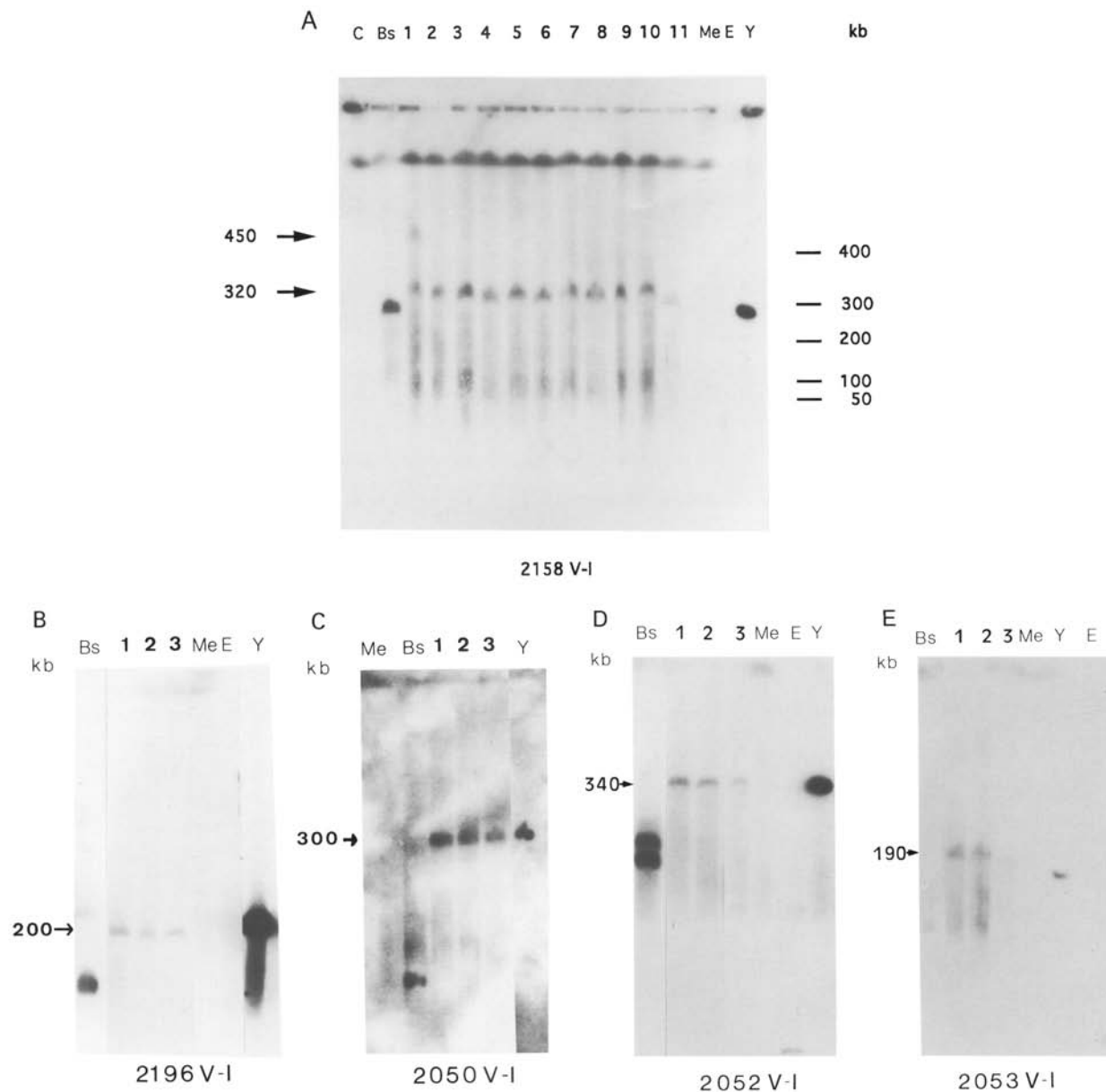
<sup>b</sup>The subterminal repeat HC1208 (Negorev et al. 1994) was also used as a probe.

<sup>c</sup>deLange et al. (1990).

<sup>d</sup>Martin-Gallardo et al. (1995).

<sup>e</sup>Negorev et al. (1994).

<sup>f</sup>Brown et al. (1990).



**Figure 2** RARE cleavage mapping of 6q, 12q, 18q, 2p, and 8q telomeres. (A) Analysis of the 6q telomere. (Lanes 1–11) Agarose plugs containing total human genomic DNA prepared using circulating leukocytes isolated from peripheral blood of 11 different people were treated with RecA protein and 400 ng of oligonucleotide for the vector-adjacent sequence of yRM2158. The RARE cleavage products were separated on a CHEF gel, and Southern blot analysis was carried out using the vector-adjacent insert probe 2158V-I. (Lane C) Control human DNA taken through all of the buffers and manipulations of the experiment but not exposed to any of the enzymes. (Lane Bs) Control human DNA digested with *Bss*HII; (lane Me) a methylase control (–*RecA*, –oligo, +methylase, +*Eco*RI digestion); (lane E) an *Eco*RI digestion control (–*RecA*, –oligo, –methylase, +*Eco*RI digestion). (Lane Y) Intact chromosomal DNA from yRM2158. (B–E) Human leukocyte DNA from three different individuals (lanes 1–3) was embedded in agarose and targeted for site-specific cleavage at the DNA sequences corresponding to the respective *Eco*RI cloning sites (using 400 ng of oligonucleotide per RARE cleavage reaction). The RARE cleavage products were analyzed by gel-transfer hybridization using vector-adjacent insert probes. Lanes Bs, Me, and E are as in A. Lane Y contains intact chromosomal DNA from the appropriate half-YAC clone. (B) (12q telomere): RARE experiment using the oligonucleotide for yRM2196, hybridized with the probe 2196V-I. (C) (18q telomere): RARE experiment using the oligonucleotide for yRM2050a, hybridized with the probe 2050V-I. (D) (2p telomere): RARE experiment using the oligonucleotide for yRM2052, hybridized with the probe 2052V-I. (E) (8q telomere): RARE experiment using the oligonucleotide for yRM2053a, hybridized with the probe 2053V-I.



**Table 2. RARE cleavage summary**

RARE oligo/ V-I probe (YAC size)	Sample	
	1	2-11
yRM2158 (280)	450, 320	320
yRM2196 (190)	200	200
yRM2050a (290) <sup>a</sup>	300	300
yRM2052 (330)	340	340
yRM2053a (175)	190	190
yRM 2000 (240) <sup>b</sup>	240	240
yRM 2123 (270) <sup>c</sup>	290	290

The sizes of the RARE fragments are shown in kb.

<sup>a</sup>From Strathdee et al. (1994).

<sup>b</sup>From Macina and Riethman (1994).

<sup>c</sup>From Negorev et al. (1994).

were carried out using a 60-mer oligonucleotide that spans the junction of vector and insert DNA at the *Eco*RI cloning site in yRM2158. The oligonucleotide contained 50 bases of sequence homologous to the insert adjacent to the cloning site and 10 bases corresponding to vector sequence which, in genomic DNA samples, should serve as a RecA-binding "tail" to assist in the protection of the *Eco*RI site from methylase activity. *Bss*HII-digested samples from a similar genomic DNA preparation (Fig. 2A, lane Bs) served as a positive control for probe hybridization. A 320-kb fragment liberated by RARE cleavage of total genomic human DNA targeted with the yRM2158 oligonucleotide hybridized to the 2158 V-I probe (Fig. 2A) in each of the 11 DNA samples (lanes 1-11). A second RARE cleavage fragment, 130 kb larger than the first, could be detected in DNA from individual 1 (Fig. 2A, lane 1). Both fragments also hybridized with the subterminal repeat HC1208 probe (not shown). The sizes of the principal RARE fragments approximated that of the intact YAC (Fig. 2A, lane Y), indicating that the targeted *Eco*RI cloning site was the expected distance from the 6q terminus on all but one of the 22 chromosomes analyzed. The slightly slower migration of genomic fragments relative to YACs of the same size is typical of a DNA concentration effect seen in pulsed-field gels and can be shown to be artifactual in many cases. For example, the sample in Figure 2A, lane 11, contains about one-half the concentration of DNA as the other samples, and the migration of the RARE cleavage fragment is nearly identical to that of the YAC (Fig. 2A, lane Y, for a discussion of this

artifact, see also mixing experiments of Riethman et al. 1993). We conclude from this experiment that yRM2158 contains a telomere-terminal fragment of one allele of 6q DNA. The simplest explanation for the 450-kb band in sample 1 is that an additional allele, 130 kb larger than the cloned one, exists in the population sampled.

The results of RARE cleavage experiments are also shown for 3 of the 11 DNA samples for the 12q telomere (yRM2196, Fig. 2B), the 18q telomere (yRM2050a, Fig. 2C), the 2p telomere (yRM2052, Fig. 2D), and the 8q telomere (yRM2053, Fig. 2E). The complete RARE cleavage analysis for the 11 DNA samples is summarized in Table 2, which also includes results from the 7q and 1q telomeres (analyzed previously using RARE cleavage analysis of a single genomic DNA sample; Macina and Riethman 1994; Negorev et al. 1994). No evidence for large-scale subtelomeric polymorphism was detected at the 1q, 2p, 7q, 8q, 12q, or 18q telomeres (Table 2).

## DISCUSSION

Analysis of the RARE mapping results for the telomeres studied are straightforward; in each case, both the hybridization probe and the sequences amplified by the PCR assay were single copy (see Methods; data not shown). Therefore, the RARE experiments described in this paper prove at the molecular level that these half-YACs are derived from the expected genomic telomere-terminal fragments and, thus, represent telomeric closure for overlapping clone maps of these chromosome arms.

Several possible explanations exist for the presence of the additional RARE fragment in the 6q-ter experiment. Partial digestion of a duplicated 6q-ter subtelomeric fragment containing the *Eco*RI site cannot be ruled out entirely but is unlikely given the stoichiometry of the two discrete bands in the sample and the absence of similar bands in all of the other samples. A simple mutation in the genomic *Eco*RI site of one allele of the sample has been ruled out by conventional Southern blot analysis; a single *Eco*RI fragment was hybridized with this probe (not shown). On the basis of the precedent of subtelomeric DNA polymorphisms detected at several other human telomeres (Wilkie et al. 1991; Ijdo et al. 1992; Martin-Gallardo et al. 1995; R.A. Macina, K. Morii, D.G. Negorev, and H.C. Riethman, unpubl.), the simplest explanation may be a chromosome length polymorphism on a relatively

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rare 6q-ter allele. However, additional experiments are required to prove this.

Integration of cloned DNA maps with uncloned genomic DNA structures over the size ranges described in this paper are essential for reliable physical maps. There is a fairly high fraction of artifactual clones in most YAC libraries, including this one (>50% of the clones are either chimeric or nontelomere derived; R.A. Macina, K. Morii, D.G. Negorev, and H.C. Riethman, unpubl.). Genomic DNA regions rich in low-copy repeats are especially problematic for conventional physical mapping strategies and are also often difficult to clone. RARE cleavage mapping can relate cloned DNA fragments efficiently to native genomic structures in this size range, a step that is essential in understanding the dynamics of large DNA segment variations in populations (Wilkie et al. 1991) and large-scale somatic rearrangements involving subtelomeric DNA (Cook et al. 1994).

Our results validate the half-YAC cloning system as an effective method for isolating large stretches of telomere-terminal DNA in an intact form and provide telomeric closure for sequence-tagged site (STS) content maps (Olson et al. 1989) of human chromosomes 2p, 6q, 8q, 12q, and 18q. Modification of yeast host genotypes to increase clone stability (Kouprina et al. 1994) and continuing refinement of RARE cleavage protocols (Gnirke et al. 1993; Ferrin et al. 1994; Macina et al. 1994; Negorev et al. 1994) might permit very efficient cloning strategies, both random and directed, for the molecular dissection of what are likely to be among the most rapidly evolving and variable regions of eukaryotic genomes (Martin-Gallardo et al. 1995).

## METHODS

### YAC Characterization

Methods for the molecular analysis of YAC clones and the cytogenetic localization of YAC DNA were as described previously (Negorev et al. 1994), except that photographic slides of FISH metaphases were scanned into a computer using a Sprint-scan (Polaroid), labeled using Adobe Photoshop software, and printed using a Color-Ease printer (Kodak). The Genome Data Base (GDB) designations for the YACs are given in Table 1. The host strain for yRM2196 is CGY2516 (Smith et al. 1990). The host strain for yRM2158, yRM2050a, yRM2052, and yRM2053a is AB1380 (Burke et al. 1987). yRM2050a, yRM2052, and yRM2053a were constructed using DNA from circulating leukocytes (Riethman et al. 1989), whereas yRM2158 and yRM2196 were prepared with DNA from CGM-1, a lymphoblastoid cell line derived from leukocytes of the same

individual who donated the DNA for the first group of clones.

### DNA Probes

Vectorette PCR methods (Riley et al. 1990) were used to recover 2053V-I (663 bp) from the vector-insert junction of yRM2053a. 2158V-I (1300 bp), 2196V-I (600 bp), 2050V-I (2200 bp), and 2052V-I (1400 bp) were isolated by plasmid-rescue methods from the vector-insert junctions of yRM2158, yRM2196, yRM2050a, and yRM2152, respectively. Each of the aforementioned hybridization probes were characterized using Southern blot analysis of human genomic DNA digested with either *EcoRI* or *BamHI* (5 µg of human DNA per lane). Hybridization of these blots with each probe yielded a single band, easily detectable after a 2- to 3-day autoradiographic exposure with a conventional enhancer screen.

### DNA Sequencing and PCR Assay Development

Double-stranded DNA templates for sequencing were obtained directly from the rescued plasmids or from vector-ette-rescued junction DNA subcloned into pBluescript IIS. Sequence analysis was performed using the GCG sequence analysis package (Devereux et al. 1984) version 7.0 and MacVector (IBI)<sup>4</sup>.

PCR conditions were as described previously (Negorev et al. 1994). The PCR primers for sV-I 2158 were 5'-TTTTCACCAACATTATCAAGG-3' and 5'-GCTGTGCAG-GTCCTTGTG-3'; the assay generated a 95-bp product (2.5 mM MgCl<sub>2</sub> at 65°C annealing temperature). The primers for sV-I 2196 were 5'-GATGAGGGAGTTGGGGG-3' and 5'-AAGCCATTTCCACTCCTCCT-3'; the assay generated a 116-bp product (1.5 mM MgCl<sub>2</sub> at 65°C annealing temperature). The primers for sV-I 2050 were 5'-GTGCCAC-GAGAACGTGAAC-3' and 5'-ATTCCATCACCTAAAA-CATGGC-3'; the assay generated a 151-bp product (1.5 mM MgCl<sub>2</sub> at 65°C annealing temperature). The primers for sV-I 2052 were 5'-GATCTCACTGCAATTTCTACA-3' and 5'-TCCATTTTCTCCAAGTTATCA-3'; the assay generated a 96-bp product (2.5 mM MgCl<sub>2</sub> at 60°C annealing temperature). The primers for sV-I 2053a were 5'-ATTCTCCTAT-GTTTCCTGGTGC-3' and 5'-GTTCCACCACITCCCACTCTTG-3'; the assay generated a 75-bp product (1.5 mM MgCl<sub>2</sub> at 65°C annealing temperature).

DNA samples comprising the National Institute of General Medical Sciences (NIGMS) Human/Rodent Somatic Hybrid Mapping Panel 2 were purchased from the Coriell Cell Repositories. Recent publications (Drwina et al. 1993; Dubois et al. 1993) describe the known inconsistencies present in this panel of mainly monochromosomal human-rodent hybrid cell lines.

### RARE Cleavage

RARE cleavage and detection of RARE cleavage fragments on blots of pulsed-field gels were as described previously (Ferrin and Camerini-Otero 1994; Macina et al. 1994; Negorev et al. 1994). Oligonucleotides with perfect homology to *EcoRI* half-sites are sufficient to direct RARE cleavages of DNA (Ferrin and Camerini-Otero 1991). The efficiency of these cleavages can be increased by including a short region of random sequence at the end of the oligonucleotide, adjacent to the perfect match at the *EcoRI* site



(Ferrin and Camerini-Otero 1991). We have shown that oligonucleotides containing 50 nucleotides of homology to genomic DNA adjacent to an *EcoRI* site and 10 bases of "random" sequence beyond the targeted *EcoRI* site can be used to produce very efficient single cleavages of mammalian complexity genomic DNA (Macina et al. 1994; Negorev et al. 1994). When significant purine versus pyrimidine DNA strand biases were present on the two potential candidate RARE oligonucleotides for a given *EcoRI* site, the purine-rich strand was selected to direct RARE cleavage. The oligonucleotides used for the RARE experiments were R-2158 (60-mer), 5'-CGTCTTCAAGAATTCTACACTGTAAATGTGCTGAGTGC GGCCGAATTCTACACTGTAAAT-3'; R-2196 (60-mer), 5'-TGGAGGTTAGGCAATGTGTCTAAGTAGTAACAGTGAGAGTTTTCAGAAATCTTGAAGACG-3'; R-2050a (60-mer), 5'-CGTCTTCAAGAATTCTAACAGAGGCAAACTACCACATAAAGCTCAGTGCCACGAGAACG-3'; R-2052 (60-mer), 5'-CGTCTTCAAGAATTCTTAACAAGATTGAATGTCTAAATTAATGAAAATAGTTATATCTCC-3'; and R-2053a (60-mer), 5'-TCCTTGCTGTATACATATGCAAGAGTGGGAAGTGGTGAACATAGGAATTCATGTTTTCGA-3'. The *EcoRI* sites are underlined. The 10 bases beyond the *EcoRI* sites correspond to the vector sequence in the YAC adjacent to the *EcoRI* cloning site, except for the yRM2053a RARE oligonucleotide, which spans an internal *EcoRI* site located 128 bp from the *EcoRI* cloning site.

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## NOTE

GenBank accession numbers for the sequenced fragments are U11835 and U11834 (2158V-I), U11838 (2196 V-I), U11828 (2050V-I), U32389 (2052V-I), and U11829 (2053V-I).

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## Molecular cloning and RARE cleavage mapping of human 2p, 6q, 8q, 12q, and 18q telomeres.

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