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Isolation and characterization of a human telomere

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ABSTRACT

A method is described that allows cloning of human telomeres in *S. cerevisiae* by joining human telomeric restriction fragments to yeast artificial chromosome halves. The resulting chimeric yeast-human chromosomes propagate as true linear chromosomes, demonstrating that the human telomere structure is capable of functioning in yeast and suggesting that telomere functions are evolutionarily conserved between yeast and human. One cloned human telomere, yHT1, contains 4 kb of human genomic DNA sequence next to the tandemly repeating TTAGGG hexanucleotide. Genomic hybridizations using both cloned DNA and TTAGGG repeats have revealed a common structural organization of human telomeres. This 4 kb of genomic DNA sequence is present in most, but not all, human telomeres, suggesting that the region is not involved in crucial chromosome-specific functions. However, the extent of common features among the human telomeres and possible similarities in organization with yeast telomeres suggest that this region may play a role in general chromosome behavior such as telomere-telomere interactions. Unlike the simple telomeric TTAGGG repeats, our cloned human genomic DNA sequence does not cross-hybridize with rodent DNA. Thus, this clone allows the identifications of the terminal restriction fragments of specific human chromosomes in human-rodent hybrid cells.

INTRODUCTION

The term telomere has been used to specify the end structure of linear eucaryotic chromosomes for over half a century (1). This structure is different from any normal duplex DNA by its ability to protect DNA ends from degradation and aberrant end-to-end interactions which result in the loss or rearrangement of the genetic material (2, 3, 4). To maintain the true integrity of the chromosome, this structure must also ensure complete DNA replication at the ends of chromosomes (5, 6, 7; reviewed in 8).

Blackburn and Szostak (8) classified telomeric sequences into two types: telomere-associated sequences and telomeric DNA sequences, based on their structural complexities and very likely different functional roles. Telomere-associated sequences are DNA sequences located near the ends of the chromosomes, and are often composed of large, tandemly repeated DNA segments in complex forms (9, 10, 11, 12). Cytological studies suggest that some of these sequences are probably involved in both intrachromosomal and interchromosomal telomere-telomere interactions (12, 13).

Telomeric DNA sequences are simple, tandemly repeated DNA sequences located at the very ends of eucaryotic chromosomes (reviewed in 14). Detailed DNA analyses in lower eucaryotes revealed that the copy number of this type of telomeric sequences fluctuates markedly (15, 16, 17, 18). In some cases, the lengths of the simple repeats are regulated in response to cell growth conditions and cell developmental stages (19, 20). Little is known

Table 1. List of the human , hamster, and hybrid cell lines used for telomere studies.

Cell Line	Cell Type	Karyotype, Origin and Description	Reference
GM3468	Human fibroblast	46,XY, normal, foreskin	Jacobs and Demars, (1978)
GM6167	Human fibroblast	46,XY, normal, lung biopsy	NIGMS* Cell Repository, Camden, NJ
GM3657	Human lymphoblast	46,XY, normal,	NIGMS Cell Repository, Camden, NJ
GM3658	Human fibroblast	46,XY, normal, same person as GM3657	NIGMS Cell Repository, Camden, NJ
GM5920	Human lymphoblast	46,XX, normal,	NIGMS Cell Repository, Camden, NJ
GM5879A	Human fibroblast	46,XX, normal, same person as GM5920	NIGMS Cell Repository, Camden, NJ
Gus4066	Human lymphoblast	45,XX,-4,-21,+der(4), t(4;21) (4qter-4p16::21q21-21qter)	Carpenter et al., (1987)
Gus3365A	Human lymphoblast	46,XX,t(4;21) (p16;q21)	
CHP134	Human neuroblast	Normal	Balaban-Malenbaum and Gilbert, (1977)
LA-N-5	Human neuroblast	Normal	Kohl et al., (1983)
CHO-K1	Chinese hamster ovary	CHO auxotroph for purine synthesis	Puck and Kao, (1982)
UCW56	Chinese hamster ovary	CHO with triple mutations	Wasmuth and Chu, (1980)
EyeF3A6	CHO-K1/Human hybrid	Containing single human chromosome 22	Van Keuren et al., (1987)
29-1F-3a	Hamster/Human hybrid	Containing single human chromosome 21	Francke et al., (1985)
HHW416	UCW56/Human hybrid	Containing single human chromosome 4	MacDonald et al., (1987)
HHW599	UCW56/Human hybrid	Containing single human chromosome 5	MacDonald et al., (1987)
72532X-6	CHO-K1/Human hybrid	Containing single human chromosome 21	Patterson et al., (1981)
153E7bx	CHO-K1/Human hybrid	Containing partially deleted human chromosome 21	Moore et al., (1977)
R2-10	CHO-K1/Human hybrid	Containing partially deleted human chromosome 21	Patterson, (1987)
ACEM	CHO-K1/Human hybrid	Containing partially deleted human chromosome 21	Patterson, (1987)
21q+	CHO-K1/Human hybrid	Containing partially deleted human chromosome 21	Patterson, (1987)

* NIGMS stands for National Institute of General Medical Science

about the cellular signals that regulate the length variation of this telomeric sequence. DNA sequence comparisons and cross-hybridization experiments with telomeric DNAs show remarkable evolutionary con-servation among lower eucaryotes (14), monocot and dicot plants (21), human (22), and many other organisms (23). In yeast, *Tetrahymena* telomeric sequences allow a linear plasmid DNA to act as a linear self-replicating DNA molecule. This linear DNA was also used as a vector to clone yeast chromosomal telomeres (24). In a similar study, Pluta et al. (25) showed that the terminal restriction fragments from *Oxytricha* chromosomes, whose telomeres share no common structural feature with yeast except short repeating sequences, are still capable of converting a yeast plasmid to act as an extrachromosomal linear DNA molecule. These results demonstrate two important facts that encouraged the cloning of human telomeres described in this paper: First, these telomeric simple repeating sequences contain the structural information essential for the recognition of telomere functions. Second, these telomere functions are highly conserved even in nonhomologous systems.

Isolation of human telomeres and adjacent DNA sequences is of great interest for several reasons. First, it will accelerate the physical mapping of human chromosomes because these regions define the ends of the physical maps. Second, it provides critical probes to hunt for disease genes located near the telomeres, such as the Huntington's Disease gene on the short arm of chromosome 4 (26) and the polycystic kidney disease gene on the short arm of chromosome 16 (27). Third, since high frequency recombination occurs at the ends of chromosomes, it will be intriguing to learn what types of DNA structures or genes are located near the telomeres.

Clones containing telomeres are apparently not present in ordinary genomic DNA libraries because telomere end sequences are incompatible with conventional cloning. Most recent telomere studies have been limited to the lower eucaryotes or organisms with a high ratio of telomeres to total DNA content (21). We wished to set up a standard procedure for cloning telomeric restriction fragments from higher eucaryotes that contain low ratios of telomeres to DNA content. Chimeric yeast-human artificial chromosomes were generated by taking advantage of the yeast artificial chromosome (YAC) system developed by Burke et al. (28). These clones contain both the telomeric TTAGGG repeating sequences and DNA sequences adjacent to these repeats. Our studies on one cloned human telomere and its adjacent DNA sequence show that the human telomeric conserved sequences extend many kb beyond the simple repeats. One DNA segment within this cloned region appears to be human specific. This finding provides a useful probe for identifying the most distal restriction fragment of a specific human chromosome in human-rodent hybrid cells.

EXPERIMENTAL PROCEDURES

Cell Lines

All cell lines used in this paper are listed in Table 1. Cell lines GM3468, GM6167, GM3657, GM3658, GM5920, GM5879A, CHP134, LA-N-5, 29-1F-3a and CHO-K1 were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 mg/ml streptomycin (PS). EyeF3A6 cells were grown in the same RPMI medium using 10% dialyzed FBS. Cell lines Gus4066, Gus3365, UCW56, HHW416 and HHW599 were grown in Iscove's Modified Delbecco's Medium (IMDM) supplemented with 10% FBS and PS. Cell lines 72532X-6, 153E7bx, R2-10 and ACEM were grown in MEM Alpha and HAM F12 (1:1), supplemented with 10% dialyzed fetal calf serum (FCS) and PS. Cell line 21q+ was grown in MEM (without glycine) supplemented with

10% FCS, PS, 2 mM glutamine and 2 mM proline. All media and supplements were purchased from GIBCO and Cellgro. The incubation temperatures for HHW416 and HHW599 were 34°C and 39°C, respectively. All other cell lines were grown at 37°C.

Pulsed Field Gel Electrophoresis

Yeast transformants were grown overnight in a 5 ml synthetic medium lacking tryptophan at 30°C, and cell concentrations were determined using a hemocytometer. Cells were harvested at 3000 rpm for 10 min in a clinical centrifuge, and washed once with 3 ml of 50 mM EDTA (pH 7.5). Cells were pelleted, resuspended and incubated at 37°C in 1 ml of 1 M sorbitol, 0.1 M EDTA (pH 7.5), and 0.1 mg/ml zymolyase 60T (Seikagaku America, Inc.) for 1 hr. Spheroplasts were harvested at 1000 rpm for 5 min, and washed once with 3 ml 1 M sorbitol and 0.1 M EDTA (pH 7.5). Yeast DNAs were prepared in low gelling-temperature agarose at a concentration of 10^8 cells/ml (29). PFG electrophoresis of yeast DNAs was carried out using the Pharmacia-LKB Pulsaphor apparatus with 10-sec pulse times at an electric field strength of 10 V/cm for 30 hr.

Mammalian DNAs were prepared in low gelling-temperature agarose and digested with *Not* I restriction enzyme (29). DNA fragments were then separated by PFG using a 100-sec pulse time at 10 V/cm for 40 hr.

Genomic Hybridization

Southern blotting and hybridization of yeast and mammalian genomic DNAs employed nytran filters (Schleicher and Schuell) and a modified procedure of Church and Gilbert (30). The hybridization reactions were done at 65°C for 14−16 hr in 0.5 M $NaHPO_4$ (pH 7.2), 7% SDS, 1 mM EDTA, 100 mg/ml sheared salmon sperm DNA and approximate 5 ng/ml radiolabeled DNA probes. $(TTAGGG)_7$ was radiolabeled with [γ-^{32}P]ATP (3000 Ci/mM, Amersham) and T4 polynucleotide kinase (New England Biolabs, Inc.) to a specific activity of 1×10^7 cpm/mg. All other probes were labeled with [α-^{32}P]dCTP (3000 Ci/mM) in random priming reactions (31) to specific activities ranging from 10^8 to 10^9 cpm/μg. Filters were washed twice at 65°C in 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.5) and 1% SDS. Filters were reused after stripping off the probes by incubating them in H_2O for 30 min at 68°C and then in 50% deionized formamide for another 30 min at 68°C.

Molecular Cloning of Human Telomeres

High molecular weight genomic DNA was prepared from a human fibroblastoid cell line, GM3468 (32, 33), in 0.5% low gelling-temperature agarose (Seaplaque, FMC, Inc.) to minimize DNA breakage (29, 34). DNA prepared from 10^6 cells was completely digested with *Eco*R I in agarose. The agarose was melted at 65°C, and removed by phenol extractions and phenol/chloroform/isoamyl alcohol (25:24:1) extractions. The DNA fragments were precipitated with 2 M ammonium acetate and ethanol. Linearized *Eco*R I fragments were circularized at a concentration of 0.5 μg/ml with 5 units/ml T4 DNA ligase (New England Biolabs, Inc.) at 4°C for 12 hr, and precipitated with ethanol. The telomeric *EcoR* I fragments should retain their linear configurations.

pYAC4 is a bacterial plasmid containing all necessary DNA sequences for constructing yeast artificial chromosome (28). Plasmid pYAC4 was digested with *Eco*R I and *Bam*H I to generate a 6.0 kb *Eco*R I-*Bam*H I fragment. This fragment was isolated on a DEAE membrane (NA45, Schleicher and Schuell) following gel electrophoresis. The 5′ phosphates were removed by treatment with calf intestinal phosphatase (Boehringer Mannheim Biochemicals). Ligation of this 6.0 kb fragment with human *Eco*R I fragments was carried out at 10°C for 12 hr using 20 units/ml T4 DNA ligase at a human DNA concentration

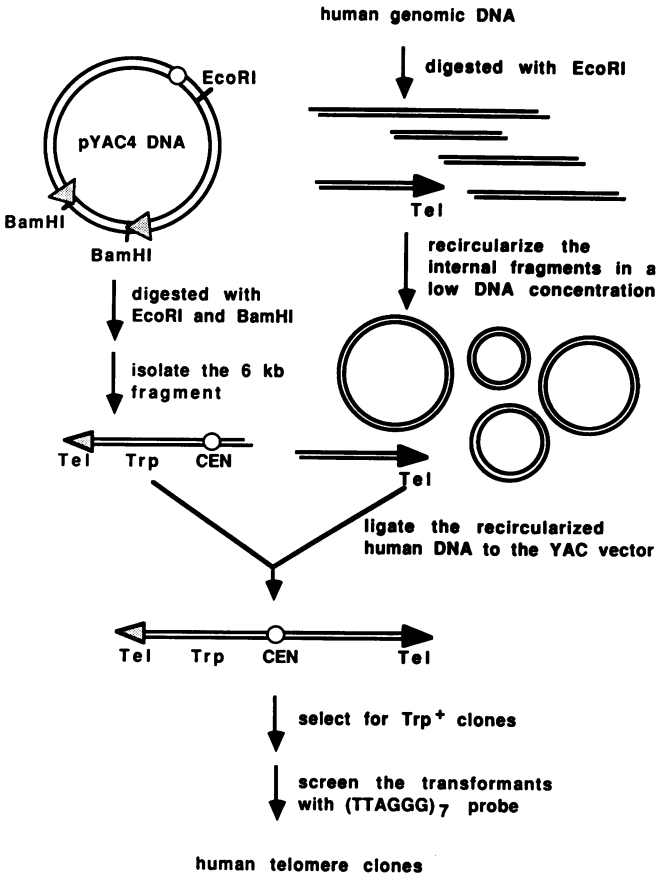

Figure 1. *Human Telomere Cloning Scheme*
A complete *Eco*R I digest of high molecular weight human genomic DNA was prepared in agarose (55). The resulting fragments were recovered, and treated with T4 DNA ligase at low DNA concentration. Fragments with two *Eco*R I ends are circularized by ligation while fragments with one *Eco*R I end and one telomere end (Tel) remain linear. A 6 kb *Eco*R I-*Bam*H I fragment containing a yeast centromere (CEN), a tryptophan-selectable marker (Trp), and one telomere end (Tel) was generated from plasmid pYAC4 (28) and ligated to the human DNA fragments to form yeast-human chimeric artificial chromosomes. These molecules were transformed into a Trp^- yeast strain AB1380 (28, 60) and clones were selected on a growth medium lacking tryptophan. The Trp^+ yeast cells were further screened by colony hybridization with $(TTAGGG)_7$ to detect human telomeric clones.

of 100 μg/ml. 0.5 μg of the ligated DNA was transformed into *S. cerevisiae* strain AB1380 (28) by the lyticase method of Burgers and Percival (35). All transformants were selected on plates containing synthetic medium lacking tryptophan (36). Tryptophan-positive yeasts were screened by colony hybridization using the protocol of Sherman et al. (36).

Subclones of the Human Telomere

Chromosomal DNAs of yeast cells containing cloned human telomeres were separated by PFG electrophoresis (37). The artificial chromosome, yHT1, was visualized by ethidium

bromide staining, and purified by electroelution (38). DNA fragments of gel-purified artificial chromosome were subcloned into a bacterial vector, Bluescript (Stratagene). Plasmid pPst2.7 contains a 2.7 kb *Pst* I fragment located next to the $(TTAGGG)_n$ simple repeating sequence at the end of the human telomere yHT1. Plasmid pEBg1.2 contains an 1.2 kb *Eco*R I-*Bgl* II fragment located distal to the human telomere end of yHT1.

Bal31 Reactions

High molecular weight genomic DNA from yeast and 29-1F-3a hybrid cells (39) was prepared as described, respectively, by Sherman et al. (36) and Maniatis et al. (38). One mg of yeast genomic DNA was digested with 30 units/ml of Bal31 nuclease (New England Biolabs, Inc.) at 30°C in 10 μl of 0.6 M NaCl, 12 mM $CaCl_2$, 12 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.0) and 1 mM EDTA. In case of 29-1F-3a DNA, 1 unit/ml of Bal31 nuclease and 10 μg/ml genomic DNA were incubated at 30°C. Reactions were stopped at the times indicated by addition of EGTA to a final concentration of 20 mM. DNA was then extracted with phenol and chloroform, and precipitated with ethanol.

RESULTS

Cloning Human Telomeres in Yeast

Our protocol for cloning human telomeres is based on the results of Szostak and Blackburn (24) and Pluta et al. (25) who showed that the telomeric sequences of *Tetrahymena* and *Oxytricha* macronuclear DNAs can support telomere formation in yeasts. The protocol was designed by assuming that human telomeres would serve the same functions in yeast as did the ciliated protozoan sequences.

The scheme for cloning telomeric DNA fragments from a human fibroblast cell line, GM3468 (32), is described in Figure 1. Human genomic DNA was prepared in low gelling-temperature agarose to minimize the mechanical breakage of large DNA molecules before enzymatic cleavage. This preparation step limits the number of DNA molecules carrying randomly broken ends which will interfere with the circularization step described below. The human DNA was digested in agarose with the restriction enzyme *EcoR* I, and the resulting fragments were isolated from the agarose plug as described in Experimental Procedures. Internal (non-telomeric) DNA molecules were circularized by ligation at a low DNA concentration so that self-ligation was more efficient than intermolecular ligation. This step removed most of the ligatable ends provided by the internal *Eco*R I fragments, and thus enriched the sample for ligatable telomeric *EcoR* I fragments. Plasmid, pYAC4, constructed by Burke et al. (28) for cloning large DNA fragments as yeast artificial chromosomes, contains a convenient 6 kb *Eco*R I-*Bam*H I fragment which includes one yeast centromere, an ARS sequence, a selectable Trp marker and one telomere end. Ligation of a human telomeric *Eco*R I fragment with this 6 kb *Eco*R I-*Bam*H I fragment will form a chimeric yeast-human artificial chromosome (Fig. 1). In this construct, the human telomeric fragment provides the other telomere end and protects this linear DNA molecule from integrating into yeast chromosomes. In principle, a yeast library containing different human telomeric fragments can be constructed using this protocol.

Moyzis et al. (22) hybridized human metaphase chromosomes with $(TTAGGG)_7$ and showed that all human chromosomes carry the simple repeating sequence $(TTAGGG)_n$ at their telomeres. We used this same oligonucleotide as a probe in colony hybridizations to screen for yeast transformants carrying human telomeres. One YAC clone, HY1, containing two 0.7 kb *Tetrahymena* telomeric TTGGGG repeats (28) was included as a control in the hybridizations to rule out possible cross-hybridizations between $(TTAGGG)_7$

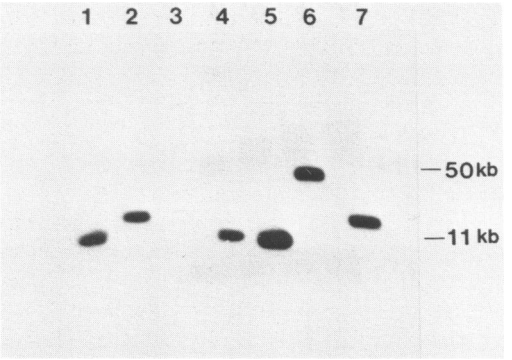

Figure 2. *Hybridization of $(TTAGGG)_7$ to Human Telomeric YAC Clones*
Intact yeast chromosomal DNAs from six Trp^+ clones (lanes 1, 2, and 4–7) were separated by PFG electrophoresis, transferred onto a nylon membrane, and hybridized with radiolabeled $(TTAGGG)_7$. The 50 kb size marker indicates the position of lambda monomer. Lane 3 contains HY1 DNA which includes a YAC with two *Tetrahymena* telomeres that contain TTGGGG repeats (28).

and the *Tetrahymena* TTGGGG repeats on the YAC vector DNA. Six out of one hundred Trp^+ yeast transformants carried DNA sequences that hybridized to the $(TTAGGG)_7$ probe. These clones contained yeast-human chimeric chromosomes that ranged in size from 11 to 50 kb as estimated by pulsed field gel (PFG) electrophoresis (Fig. 2). These small chromosomes seem to be stable when yeast cells are grown and maintained in selective medium (without tryptophan). However, when the clones are transferred into non-selective medium (YPD medium), the chimeric chromosomes are lost during subsequent cell divisions (data not shown). The instability of artificial chromosomes less than 150 kb in length is expected (40).

The other ninety-four Trp^+ transformants do not hybridize to $(TTAGGG)_7$ but do hybridize to pBR322 DNA, indicating they all contain parts of the original 6 kb *Eco*R I-*Bam*H I YAC fragment. Over 50% of these Trp^+ clones contain DNA sequences that hybridize to a human Alu probe. These clones have not been further characterized: they may have been generated by random integration of the 6 kb *Eco*R I-*Bam*H I YAC fragment and linear human DNA fragments into yeast chromosomes. Another potential way of generating Trp^+ clones is the joining of two 6 kb *Eco*R I-*Bam*H I YAC fragments by a linear human DNA molecule with two *Eco*R I ends. The resulting DNAs, carrying two yeast centromeres, will be subjected to a breakage-fusion-bridge cycle that will generate rearranged derivatives (1, 41). These derivatives may include centromere deletions and other DNA rearrangements (40), and may contribute to some of the background clones we observe. Given these possibilities for producing false Trp^+ clones, the recircularization of non-telomeric human fragments is probably a crucial step in our cloning procedure. Our protocol is likely to work in any organism with a low telomere to DNA ratio. The 6% frequency of human telomeric clones we observed is rather high considering that human cells possess only 46 telomeres in 3000 Mb. Thus, without purification or functional selection only about one clone in ten thousand should contain a telomere.

Structural Organization of One Cloned Human Telomere

The structure of one chimeric chromosome, designated yHT1, has been examined in detail. This chromosome contains a 5.5 kb human telomeric *Eco*R I fragment joined to a 6 kb

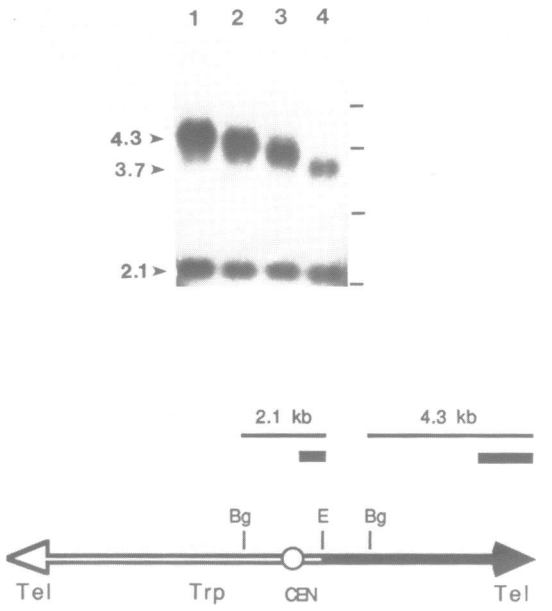

Figure 3. *Bal31 Analysis of a YAC Clone Containing a Human Telomere*
Genomic DNA from the human telomere-containing YAC clone, yHT1, was treated with Bal31 for 0 (lane 1), 5 (lane 2), 10 (lane 3), or 15 min (lane 4) and subsequently digested with *Eco*R I (E) and *Bgl* II (Bg). The resulting fragments were size fractionated by conventional gel electrophoresis and transferred to a nylon membrane. $(TTAGGG)_7$ and a 0.5 kb *Eco*R I-ClaI fragment isolated from pYAC4 were both radiolabeled and hybridized to the membrane. One kb size ladders ranging from 2 to 5 kb are marked as horizontal bars. A simple map of clone yHT1 is shown at the bottom; the filled arrow represents human DNA, and the open arrow represents yeast DNA. Other abbreviations are as in Figure 1. The positions of the probes and the fragments they detect are shown, respectively, by the thick and thin horizontal bars above the map.

*Eco*R I-*Bam*H I YAC fragment at the *Eco*R I site. Sensitivity of clone yHT1 to digestion by Bal31 exonuclease was used to show that this cloned DNA is a linear molecule. Total yeast genomic DNA was digested with Bal31 for various times, subsequently digested with *Eco*R I and *Bgl* II restriction endonucleases, size fractionated by agarose gel electrophoresis, and transferred to a nylon membrane. This membrane was then hybridized with two combined probes, telomere-specific $(TTAGGG)_7$ and an internal 0.5 kb *Eco*R I-ClaI fragment isolated from the 6 kb YAC fragment (Fig. 3). This 0.5 kb *Eco*R I-ClaI fragment hybridizes to a 2.1 kb internal fragment. In the absence of Bal31 treatment, the $(TTAGGG)_7$ probe hybridized to a diffuse 4.3 kb band (Fig. 3 lane 1). The size and intensity of this band decreased upon prolonged treatment with Bal31. After a 15 minute treatment, the 4.3 kb band became 0.6 kb shorter, and the hybridization was markedly less intense (Fig. 3 lane 4). In contrast, the size and intensity of a 2.1 kb internal fragment were unaffected by the same Bal31 treatment. The diffuse band seen with $(TTAGGG)_7$ is characteristic of a telomeric restriction fragment, which is heterogeneous in size during propa-gation in yeast (24, 42), and other eucaryotes (15, 16, 19, 20, 21). It is also likely that yeast DNA was added to the end of the human telomere just as has been observed at the ends of the *Tetrahymena* ribosomal DNA (43), but this has not yet been confirmed.

The 11.5 kb linear yHT1 molecule can be separated from other yeast chromosomes

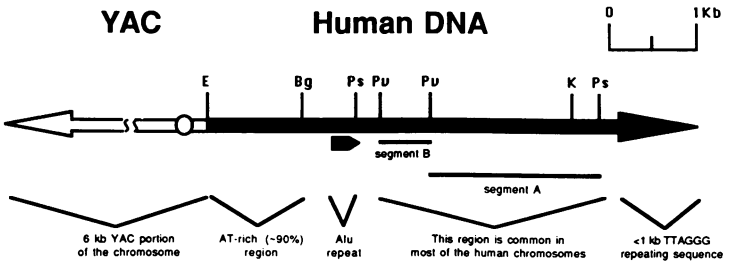

Figure 4. *Schematic Structure of the Chimeric Yeast-Human Clone yHT1*
Clone yHT1 is a chimeric minichromosome containing human DNA (filled arrow) and yeast DNA (open arrow). Arrowheads represent regions of telomeric simple repeats. The open circle represents a yeast centromere. Locations of *Eco*R I (E), *Bgl* II (Bg), *Pst* I (Ps), *Pvu* II (Pv), and *Kpn* I (K) restriction sites are shown above the map. The position of an Alu repetitive element is indicated by a solid arrowhead below the map, which points in the direction of the poly(dA) stretch. The positions of segments A and B used in subsequent experiments are indicated by the horizontal bars below the map.

by PFG and directly isolated from the gel. Several restriction enzymes have been used to map this cloned human telomere (Fig. 4). Both *Bgl* II and *Kpn* I have unique cleavage sites in the human 5.5 kb *Eco*R I fragment, located 4.3 kb and 1.4 kb from the tip of the chromosome, respectively. Two DNA segments of yHT1 were subcloned for detailed structural analysis. They are a 2.7 kb *Pst* I fragment located less than 400 bp from the terminal TTAGGG repeats, and a 1.2 kb *Eco*R I-*Bgl* II fragment located distal to the end

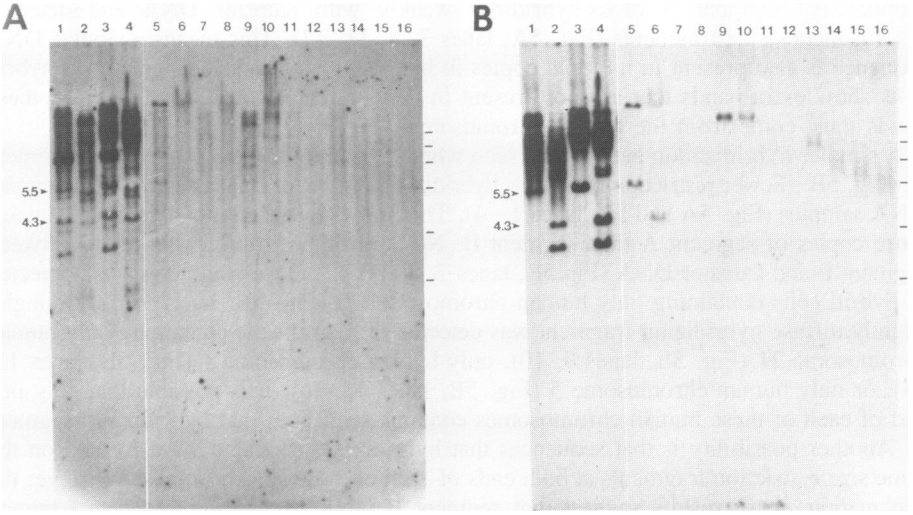

Figure 5. *Hybridizations of Human, Hamster, and Hybrid Cell DNAs with Human Telomeric Probes*
(A). Genomic DNAs isolated from cell lines GM3468 (lane 1 and 2), Gus4066 (lane 3 and 4), EyeF3A6 (lane 5 and 6), CHO-K1 (lane 7 and 8), 29-1F-3a (lane 9 and 10), UCW56 (lane 11 and 12), HHW416 (lane 13 and 14), and HHW599 (lane 15 and 16) were digested with *Eco*R I (odd numbered lanes) or *Bgl* II (even numbered lanes). The resulting fragments were size fractionated by conventional gel electrophoresis and transferred to a nylon membrane. The membrane was then hybridized with radiolabeled segment A (Fig. 4). Sizes markers are shown by horizontal bars, positioned every 2 kb. (B). The same membrane shown in (A) was rehybridized with radiolabeled segment B.

(Fig. 4). Both of these sequences were subcloned into a bacterial vector, and designated as pPst2.7 and pEBg1.2, respectively. Preliminary DNA sequencing data show that a long, tandemly repeated, AT-rich sequence is located in the middle of the 1.2 kb *Eco*R I-*Bgl* II fragment. In fact, the whole 1.2 kb *Eco*R I-*Bgl* II DNA segment contains AT-rich sequences. We estimate that the DNA in this region is over 80% A+T. Clone yHT1 contains one Alu repeat located 3.8 kb from the chromosome end. This proves, unequivocally, the human origin of the cloned sequence. DNA sequence analysis revealed that the poly(dA) stretch of this Alu repeat is oriented proximal to the telomere (unpublished results).

Clone yHT1 Contains DNA Sequences that Selectively Recognize Human Telomeres in Hamster-Human Hybrid Cells

Two DNA segments isolated from plasmid pPst2.7 were used as probes in hybridization experiment to study the genomic organization of yHT1. These segments are a 1.8 kb *Pvu* II-*Pst* I and a 0.6 kb *Pvu* II fragment, designated as segment A and segment B, respectively (Fig. 4). Segment A was used as a hybridization probe to a genomic blot containing DNAs from two human cultured cell lines, two hamster cell lines and a collection of hybrid cells containing single human chromosomes (see Table 1 for a description of cell lines). Many *Eco*R I and *Bgl* II fragments were detected in the human cell lines, indicating that multiple copies of segment A are present in the human genome. The sets of *Eco*R I and *Bgl* II restriction fragments that hybridized to segment A are quite similar in the two human cell lines, GM3468 and Gus4066 (Fig. 5A, lanes 1 and 3; lanes 2 and 4). This suggests that copies of segment A are located in regions of chromosomes highly conserved between individuals. Segment A cross-hybridized weakly with hamster DNA and detected heterogeneous size fragments (Fig. 5A, lanes 7, 8, 11, 12). This means a similar DNA sequence is also present in multiple copies in hamster genomes. Hamster-human hybrid cells show extra bands that are not present in their parental hamster cells. Thus, these bands must come from the human chromosomes.

A simpler hybridization pattern was seen when the same blot was probed with segment B. (Fig. 5B). Fewer restriction fragments hybridized to segment B than segment A in human DNA samples (Fig. 5A and 5B, lanes 1−4). This shows that the human genome contains more copies of segment A than segment B. No cross-hybridization was found between segment B and hamster DNA (Fig.5B, lanes 7, 8, 11, 12). Two fragments were detected in hybrid cells containing only human chromosome 22 (Fig. 5B, lanes 5, 6). A single, slightly diffuse hybridizing fragment was detected in hybrid cells containing only human chromosome 21 (Fig. 5B, lanes 9, 10), only human chromosome 4 (Fig. 5B, lanes 13, 14), or only human chromosome 5 (Fig. 5B, lanes 15, 16). It is possible that only one end of each of these human chromosomes contains sequences that hybridize to segment B. Another possibility is that sequences that hybridize to segment B are located on the same size restriction fragments at both ends of each of these chromosomes. Whatever the explanation, these results suggest that segment B DNA can be used to detect human restriction fragments in hybrid cells. Other results will show that these restriction fragments are telomeric (see below). Thus, segment B is particularly useful in mapping human chromosomes in hybrid cells because it can detect the end fragments of specific human chromosomes.

The telomeric location of segment B DNA was demonstrated by its sensitivity to Bal31 exonuclease treatment, a characteristic of telomeric sequences (17, 18, 21, 44, 45, 46). Bal31 sensitivity was first measured on a hybrid cell instead of a human cell because of

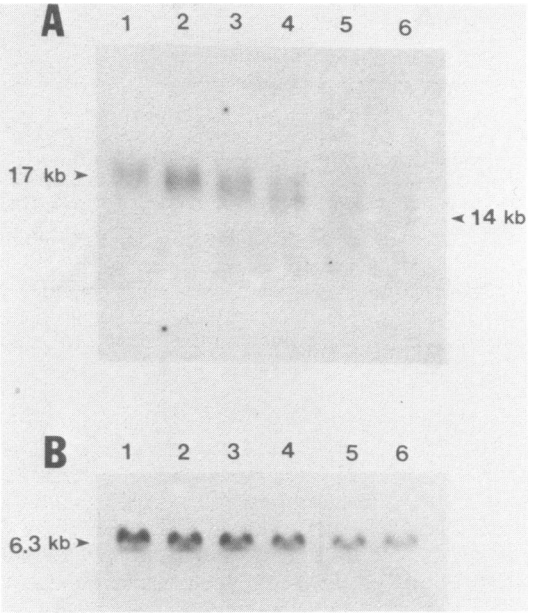

Figure 6. *Bal31 Analysis of EcoR I Fragment Containing Human Chromosome 21 Telomeres*
Genomic DNA isolated from the hybrid cell line, 29-1F-3a, was treated with Bal31 for 0, 20, 40, 60, 120, and 140 min (lanes 1 to 6, respectively) and subsequently digested with *Eco*R I. The resulting fragments were size fractionated by gel electrophoresis and transferred to a nylon membrane. The membrane was then probed with both radiolabeled segment B and pGSB3 a single copy probe assigned to human chromosome 21 (47). DNA sizes are shown in kb.

its simpler and predictable hybridization pattern to segment B. Genomic DNA prepared from cell line 29-1F-3a (Table 1), which contains a single human chromosome 21, was first digested with Bal31 for various lengths of time and then digested with the restriction enzyme *Eco*R I. The fragments were fractionated by conventional gel electrophoresis, transferred to a nylon membrane and hybridized with segment B DNA (Fig. 6). In the absence of Bal31 treatment, segment B hybridized to a slightly diffuse fragment about 17 kb in length. The size of this *Eco*R I fragment decreased upon prolonged treatment with Bal31. The rate of exonuclease digestion was roughly linear during the first 60 minutes of digestion (Fig. 6 lane 1−4). Approximately 3 kb of the sequence was removed after a 2 hour Bal31 treatment. The same membrane was then hybridized with pGSB DNA. This cloned interstitial single-copy chromosome 21 sequence has been mapped to 21q22.3 which is the distal portion of the human chromosome 21 q arm (47, 48, 49). The probe hybridized to a 6.4 kb *Eco*R I fragment which was unaffected by Bal31 digestion (Fig. 6). This result demonstrates that under the conditions used, the Bal31 digestion was telomere specific.

Structural Organization of Other Human Telomeres

Additional evidence that yHT1 is a telomeric clone came from comparative genomic hybridizations with $(TTAGGG)_7$ and yHT1 subclones. *Not* I restriction fragments of various human cultured cells were fractionated by PFG and blotted onto a nytran membrane. These human samples includes two neuroblastoma cell lines (CHP134 and LA-N-5), four

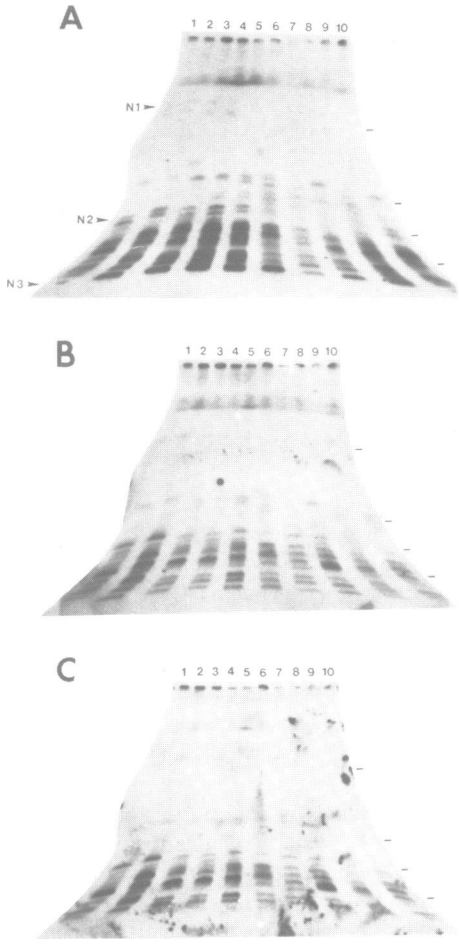

Figure 7. *Not I Telomeric Fragments of Various Human Cell Lines*
Genomic DNAs isolated from human cells CHP134 (lane 1), LA-N-5 (lane 2), Gus3365A (lane 3), Gus 4066 (lane 4), GM3468 (lane 5), GM3657 (lane 6), GM3658 (lane 7), GM5920 (lane 8), GM5879A (lane 9), and GM6167 (lane 10) were digested with *Not* I, size fractionated by PFG eletrophoresis, and blotted onto a nylon membrane. The membrane was (A) probed with radiolabeled $(TTAGGG)_7$; (B), reprobed with radiolabeled segment A; (C), reprobed with radiolabeled segment B. Sizes markers are shown by horizontal bars positioned every 250 kb.

lymphoblastoid cell lines (Gus3365A, Gus4066, GM5920 and GM3657), and four fibroblast cell lines (GM3468, GM3658, GM5879A and GM6167; see Table 1 for details). The pairs of lines, GM3657 plus GM3658, and GM5920 plus GM5879, are samples prepared from different tissues of the same individuals. Radiolabeled $(TTAGGG)_7$ was used to detect all the human telomeric *Not* I fragments in this genomic blot (Fig. 7A). At least 15 distinct telomeric *Not* I fragments (not including overlapping bands) were detected in each cell line ranging in size from less than 50 to 850 kb. Each cell line displayed a unique pattern

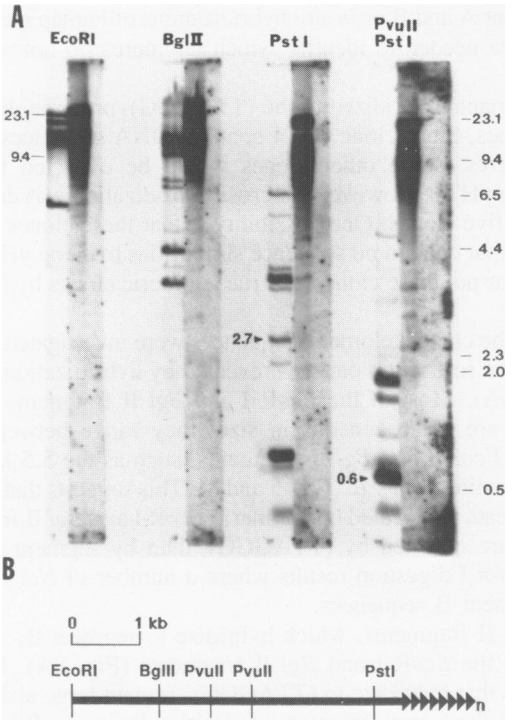

Figure 8. *Restriction Fragments Identified by Telomeric Probes*
(A). Genomic DNA isolated from a human cell line, GM3468, was digested with various restriction enzymes, size fractionated by conventional gel electrophoresis, and blotted onto a nylon membrane. Each enzyme-digested lane was sliced into two strips. The strips shown on the left were hybridized to radiolabeled segment B while the strips shown on the right were hybridized to radiolabeled $(TTAGGG)_7$. Arrowheads indicate the corresponding fragments in yHT1. Sizes are given in kb. (B). A proposed general structure distal to the human telomeric simple $(TTAGGG)_n$ repeats (consecutive arrowheads); five restriction sites indicated appear fairly well conserved in human chromosomes.

of *Not* I fragments detected by $(TTAGGG)_7$. Cell lines derived from different tissues of the same person also had unique hybridization patterns (lane 6 vs lane 7, lane 8 vs lane 9).

When the same blot was probed with segment A, fewer fragments were detected than with $(TTAGGG)_7$ (Fig. 7B). For example, the bands designated as N1 and N2 in Fig. 7A did not hybridize to segment A in Fig. 7B. Fig. 7C shows the same blot hybridized with segment B. Even fewer *Not* I fragments were detected. For example, the smallest *Not* I fragment, designated as N3 in Fig. 7A, was missing in Fig. 7C. However, all the fragments that hybridized to segment B also contain sequences that hybridized to segment A, and all the fragments that hybridized to segment A also contain sequences that hybridized to $(TTAGGG)_7$. These results suggest that all copies of segments A and B in the human genome are located on telomeric *Not* I fragments. They also indicates that DNA sequences proximal to the telomeric ends are somewhat more conserved in human chromosomes than distal DNA sequences. However, the number of telomeres that lack segment A and segment B sequences is not yet known. Hybridizations of genomic DNA from a panel of hybrid

cell lines with segment A and B or *in situ* hybridizations of human metaphase chromosomes with these probes are needed to identify which telomeres do not contain this conserved sequence.

Six yeast transformants hybridized to the $(TTAGGG)_7$ probe in the initial screening for human telomere clones. Since clone yHT1 contains DNA sequences also present in many other human telomeres, some other clones would be expected to share homologous sequences with clone yHT1. However, no cross hybridization was detected between clone yHT1 and the other five clones. One possibility is that these clones represent a subgroup of human telomeres that contain no sequence similarities to clone yHT1. Future work will distinguish whether or not these clones are true telomeric clones by measuring their Bal31 sensitivities.

The properties of the cloned telomeric sequences were investigated further by comparing four different genomic restriction patterns revealed by hybridizations with segment B and $(TTAGGG)_7$ (Fig. 8A). Most of the *Eco*R I and *Bgl* II fragments detected by segment B and $(TTAGGG)_7$ are heterogeneous in size; they range between 9.4 and 23.1 kb. Nevertheless, a few *Eco*R I and *Bgl* II fragments, such as the 5.5 kb *Eco*R I and 4.3 kb *Bgl* II fragments, are uniform in size (Figs 5 and 8). This suggests that not all of the segment B and TTAGGG repeats are located on telomeric *Eco*R I and *Bgl* II fragments. More *Eco*R I and *Bgl* II bands are detected by $(TTAGGG)_7$ than by segment B (Fig. 8A). This is consistent with the *Not* I digestion results where a number of *Not* I telomeric fragments did not contain segment B sequences.

The *Pst* I and *Pvu* II fragments, which hybridize to segment B, are smaller and more uniform in size than the *Eco*R I and *Bgl* II fragments (Fig. 8A). In contrast, the *Pst* I and *Pvu* II fragments that hybridize to $(TTAGGG)_7$ remain large and heterogeneous. This suggests that most of the human telomeres contain at least one *Pst* I and one *Pvu* II site located between the segment B sequence and the TTAGGG repeat. A 0.6 kb *Pvu* II fragment is the predominant genomic band detected by segment B. This suggests that the two *Pvu* II sites in yHT1 are also conserved in other telomeres. The internal 2.7 kb *Pst* I and 0.6 kb *Pvu* II fragments, present in yHT1, are seen in genomic hybridizations with segment B probe (arrowheads in Fig. 8) but not with $(TTAGGG)_7$ probe. These results suggest that no DNA rearrangement has occurred within this 2.7 kb *Pst* I fragment during the cloning of yHT1. However, the corresponding *Eco*R I and *Bgl* II fragments are not found in this genomic hybridization because most of the length of the human TTAGGG repeats were trimmed by yeast during DNA replication.

Most *Bgl* II fragments containing the TTAGGG repeats are approximately 1 kb shorter than most *Eco*R I fragments. The 1 kb size difference is seen even more distinctly with fragments that hybridize to segment B. These differences match with the distance seen between the *Eco*R I and *Bgl* II restriction sites in the yHT1 clone. This suggests that most human telomeres contain these conserved *Eco*R I and *Bgl* II sites. Similarly, most *Pst* I fragments detected by $(TTAGGG)_7$ were approximately 3 kb shorter than most *Bgl* II fragments. This difference in size matches the distance seen between the *Bgl* II site and the distal *Pst* I site in the yHT1 clone. This observation suggests that the distal *Pst* I site is also conserved in most telomeres.

A model for the organization of human telomeric sequences which is consistent with all of these observations is shown in Fig. 8B. The model applies to most, but not all of the human telomeres. In this model, conserved human telomeric sequences extend at least 4.4 kb beyond the TTAGGG repeats. The distance between the conserved *Pst* I site and

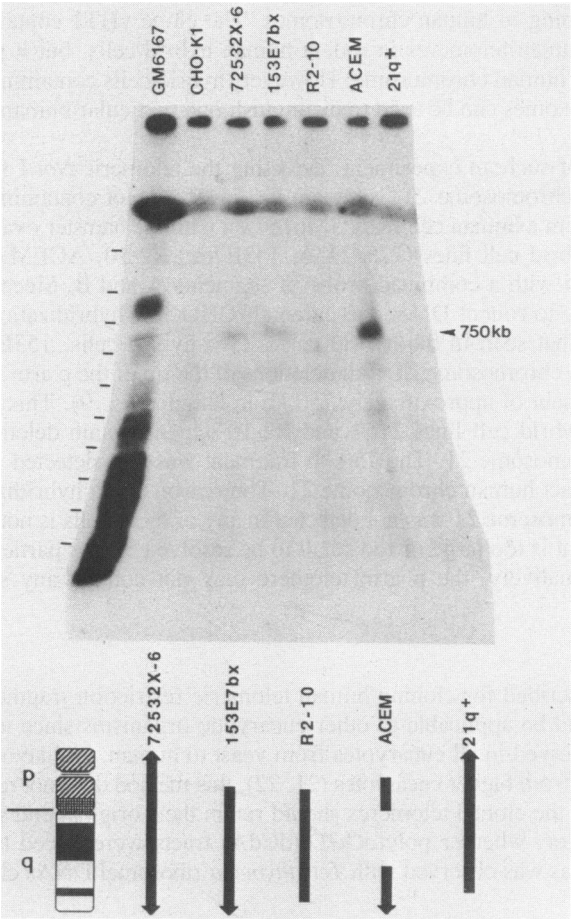

Figure 9. *Detection of the Telomeric Not I Fragment of the q Arm of Chromosome 21*
Genomic DNA isolated from human cells (GM6167), Chinese hamster ovary cells (CHO-K1), hybrid cells containing single intact human chromosome 21 (72532X-6), and hybrid cells containing various deletions of human chromosome 21 (153E7bx, R2-10, ACEM and 21q$^+$) were digested with *Not* I, size fractionated by PFG eletrophoresis, and blotted onto a nylon membrane. The membrane was then hybridized with combined segment A and B DNA. The portions of human chromosome 21 present in these hybrid cells are shown schematically by the vertical bars below the membrane, and the arrowheads indicate the intact telomeres. The size of the hybridizing fragment is 750 kb. The strong hybridization signal in the ACEM lane is due to an overloaded DNA sample. Size markers are shown by horizontal bars positioned every 100 kb.

the TTAGGG repeats is less than 400 bp. Thus, the sizes of regions containing TTAGGG repeats range from 6.5 kb to 20 kb based on the sizes of the heterogeneous *Pst* I fragments that hybridize to $(TTAGGG)_7$ (Fig. 8A). This length is twice that estimated by Moyzis et al. (22). The *Eco*R I, *Bgl* II, *Pst* I, and two *Pvu* II restriction sites next to the telomeric repeats are all conserved.

Application of the Human Specific Telomeric Probe to Chromosome Mapping

A probe that recognizes a specific telomeric restriction fragment could be extremely useful

for physical mapping of human chromosomes. The clone yHT1 contains sequences that recognize only human telomeres in rodent-human hybrid cells, but it potentially detects both ends of each human chromosome. However, hybrid cells containing partial deletions of human chromosomes can be used to distinguish one particular human chromosome end from the other.

One example of such an experiment, detecting the telomeric *Not* I fragment from the q arm of human chromosome 21, is shown in Fig. 9. A blot containing *Not* I digests of genomic DNA from a human cell line (GM6167), a Chinese hamster ovary cell line (CHO-K1), and five hybrid cell lines (72532X-6, 153E7bx, R2-10, ACEM, $21q^+$; see Table 1) was hybridized with a combined probe of segments A and B. Since this probe cross-hybridizes weakly to rodent DNA, the parental (CHO-K1) hybridization signals must be subtracted from that seen in the hybrid cells. Two hybrid cells, 153E7bx and ACEM, containing human chromosome 21 with deletions of the tip of the p arm showed a strongly hybridizing fragment of approximately 750 kb in length (Fig. 9). This fragment was not detected in the hybrid cell lines $21q^+$ and R2-10 which contain deletions of the end of the q arm of chromosome 21. The 750 kb fragment was also detected in 72532X-6 cells which contain intact human chromosome 21. The reason that a hybridization signal from the p arm of chromosome 21 was not detected in any of these cells is not known. Perhaps, this *Not* I fragment is too large or too small to be resolved by our particular PFG running conditions. Alternatively, the p arm telomere may not contain any segment A and B sequences.

DISCUSSION

A method is described for cloning human telomeric restriction fragments. In principle, this method should be applicable to other eucaryotic organisms since telomere functions appear to be conserved in all eucaryotes from yeast to human. Unlike other strategies for cloning telomeres from higher eucaryotes (21, 22), this method does not require exonuclease treatment, so that the cloned telomeres should retain their original end sequences. We do not know, however, whether poly(dGdT)·(dCdA) tracts were added to the ends of the human telomeres as was observed with *Tetrahymena* ribosomal DNAs cloned in yeast (43, 50).

A 5.5 kb human *Eco*R I fragment containing human telomeric simple repeats can form a 11.5 kb chimeric minichromosome with half of a YAC vector DNA. Apparently, cells must be kept under selective pressure in order to maintain an artificial chromosome of this size. Short yeast artificial chromosomes (< 42 kb) are known to be unstable because they segregate randomly at mitosis rather than disjoining from each other like natural chromosomes. The stability of these linear chromosomes increases with length (51). Thus, the telomere cloning scheme can presumably be improved by ligating larger restriction fragments, such as those generated by *Not* I or *Mlu* I, to the YAC DNA. Cloning of larger fragments should also allow isolation of single copy DNAs adjacent to the conserved telomere regions.

Isolation of single copy DNAs that recognize specific telomeres is important for several reasons. First, they can be used directly to identify the ends of chromosome restriction maps. Second, they can be used to detect chromosomal translocations between small DNA segments at chromosome ends, which can not be seen by low resolution chromosome banding (48). Third, they can be used to find restriction fragment length polymorphisms that can serve as genetic markers to determine linkage relationships of some inherited

disorders located at the tips of chromosomes. For example, special efforts have been made to isolate DNA probes from the short arm of chromosome 4, and to create a linkage map to position the gene responsible for Huntington's Disease near the telomere (26, 52, 53). Yet no distal, flanking marker for this disease has been identified. Isolation of clone yHT1 has allowed us to identify sequences next to the telomere of 4p (unpublished results). This narrows down the possible location of the Huntington's Disease gene considerably.

In clone yHT1, a 4.4 kb conserved DNA sequence is located next to the telomeric TTAGGG repeats. Part of this 4.4 kb DNA sequence (segment A) hybridizes weakly to rodent DNAs (Fig. 5A). These weak and heterodisperse cross-hybridizations could reflect short sequences that are evolutionally conserved because they serve similar functions. Cross-hybridizations may be due to a conserved family of autonomous replicating sequences (ARS). In yeast, a family of highly conserved ARS are located near the telomeres as part of the subtelomeric conserved sequences (54). These particular telomeric ARS elements replicate at the end of S phase (55). Similarly, telomeres in higher eucaryotes replicate late in S phase (56); presumably they are also initiated by a family of late-replicating ARS elements located near the telomeres. Identification of the DNA sequences responsible for the cross-hybridization and testing the ability of the sequences to self-replicate will be necessary to prove that a human ARS is part of this conserved telomeric region.

Another intriguing structural feature of the 4.4 kb telomeric conserved sequence is the greater than 1 kb segment of AT-rich DNA sequences. Such sequences are usually found in the non-transcribed spacer regions of 5S rDNA (57), transfer RNA genes (58), ribosomal RNA genes (59), and histone genes (60). Other data suggest that AT-rich sequences are chromosomal structures preferentially attached to the nuclear scaffold (61) or the nuclear matrix (62). It is not clear whether these subtelomeric AT-rich sequences serve any such functions in cells. However, the finding of these sequences close to telomeres is consistent with the observations that both AT-rich heterochromatin and telomeric DNA replicate late in S phase (63).

The yHT1 clone contains a human telomeric *Eco*R I fragment. The chromosomal origin of this telomere has not yet been determined because the DNA sequences in yHT1 are also present in other human telomeres. Genomic hybridizations and preliminary DNA sequence data of the region adjacent to TTAGGG repeats in yHT1 suggest a model for the DNA structures near the tips of human chromosomes. This model resembles the organization of yeast telomeres proposed by Chan and Tye (54) in several aspects. First, the DNA sequences next to the telomeric simple repeats are conserved in most, but not all, of the chromosomes in yeast (64) and in human (this work). Thus, these conserved regions do not appear to play any important roles in individual chromo-some-specific functions (64). Second, DNA sequences located further away from the telomeres are less conserved. For example, the telomere distal X elements in yeast are less conserved than more proximal Y elements (54). Although not enough is known yet to group the human telomeric sequences in a similar way, segment B which is less distal than segment A detects fewer distinct human sequences. These similar DNA organizations of telomeres in yeast and human may be general telomere characteristics of all eucaryotes.

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