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Cloning human telomeric DNA fragments into *Saccharomyces cerevisiae* using a yeast-artificial-chromosome vector

(genome mapping/*in situ* hybridization/human repetitive DNA)

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ABSTRACT **Telomeric fragments of human DNA ranging in size from 50 to 250 kilobases were cloned into *Saccharomyces cerevisiae* using a yeast-artificial-chromosome (YAC) vector. Six human-telomeric YAC (HTY) strains were selected by virtue of the specific hybridization of their DNA with the human telomeric terminal-repeat sequence $(TTAGGG)_n$, and the telomeric localization of this sequence within each YAC was demonstrated by its sensitivity to nuclease BAL-31. *In situ* hybridization of DNA from three of these HTY strains with human metaphase chromosomes yielded discrete patterns of hybridization signals at the telomeres of a limited number of human chromosomes, different for each clone. DNA from selected cosmid subclones of one of the HTY strains was used to localize the origin of the cloned telomeric DNA by *in situ* hybridization to the tip of the long arm of chromosome 7.**

Specialized telomeric DNA sequences are required for the replication and stability of linear chromosomes (for review, see ref. 1). In many organisms, telomeric DNA is also associated with recombinational events that result in frequent rearrangements of telomere-proximal DNA (for examples, see refs. 2–4). Two classes of repeated DNA elements have been implicated in these functional attributes of telomeres. The first is a simple repeat sequence with a consensus motif containing a G-rich strand (1); this sequence motif has been found at the termini of linear chromosomes of representative plants, animals, protists, and fungi (1, 5, 6). Particularly striking is the recent demonstration that the terminal-repeat sequence in the human, $(TTAGGG)_n$, is identical to that in trypanosomes and similar to that in yeast, $(TG_{1-3})_n$, and *Tetrahymena*, $(TTGGGG)_n$ (6).

The terminal-repeat sequence is apparently maintained in *Tetrahymena* macronuclei and in *Oxytricha* by the template-independent addition of repeat nucleotides through the enzymatic activity of a ribonucleoprotein termed telomerase (7–9). The *Tetrahymena* telomerase can recognize oligonucleotides corresponding to the G-rich strand of DNA from terminal-repeat sequences of many organisms, suggesting similar mechanisms of telomere maintenance among these organisms and a strong evolutionary conservation of this process (7, 8). Recombination may also be involved in the maintenance of the terminal-repeat sequences in *Saccharomyces cerevisiae* (10).

The second class of repeated telomeric DNA element is localized just proximal to the terminal repeat and is species-specific. These subtelomeric repeats are not required for telomere replication and maintenance but are involved in the enhanced recombination often associated with telomeres. In *S. cerevisiae*, two subtelomeric repeat elements have been characterized, X and Y′. X is present at most of the telomeres in a single copy. Y′ is present on about half of the telomeres, is sometimes present in multiple copies on a single telomere, and is always distal to X (refs. 11 and 12; A. Link and M.V.O., unpublished). *S. cerevisiae* has a strain-specific distribution of these subtelomeric repeats among its 32 telomeres; they are probably responsible in part for the chromosome-length polymorphisms that have been noted among strains (ref. 13; A. Link and M.V.O., unpublished). Protists such as *Plasmodium* and *Trypanosoma* can apparently exploit the enhanced recombination of subtelomeric DNA to generate diversity in genes encoding antigenic determinants involved in host–parasite interactions (3, 14). Repeat elements that have been shown by cytogenetic methods to be specific for telomeric regions of chromosomes of several animals and plants have been cloned and characterized (15–17), but their proximity to the chromosomal termini has not been determined at the molecular level. Studies of the single example of a human telomeric region that has been mapped at the molecular level suggest a high level of DNA sequence variation and the involvement of subtelomeric repeats in the generation of this diversity (4, 18).

A universal deficiency in genetic maps of higher organisms is the absence of markers that identify the ends of chromosomes. Unique, physically defined genetic markers associated with individual telomeres would provide the boundaries for genetic maps. Cloned sequences specific for individual telomeres may also be in close proximity to some disease-associated genes that appear to map near the ends of particular human chromosomes (19). We describe here a cloning system that can be used to isolate large telomeric fragments of human chromosomes in yeast. The system is similar in principle to that described by Burke *et al.* (20) but has been modified to exploit the structural and functional similarity of the telomeric terminal-repeat sequences of human and yeast chromosomes.

MATERIALS AND METHODS

Human-Telomeric-Yeast-Artificial-Chromosome (HTY) Library Construction. High molecular weight human DNA was prepared from circulating leukocytes, digested partially with *Eco*RI, and size-fractionated on sucrose density gradients (21). The gradients, which were exponential (although not strictly isokinetic), were prepared using the gradient maker described by Noll (22); the starting sucrose concentration in the mixing chamber was 5% and the concentration in the high-sucrose reservoir was 25% [sucrose solutions contained 0.8 M NaCl, 0.01 M EDTA, and 0.02 M Tris·HCl (pH 8)].

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Abbreviations: YAC, yeast artificial chromosome; HTY, human-telomeric YAC; YTY, yeast-telomeric YAC.

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Gradient fractions containing *Eco*RI fragments of the desired size were pooled and then concentrated and dialyzed using a collodion-bag concentrator (Schleicher & Schuell UH 100/75). The vector segment containing the functional yeast-artificial-chromosome (YAC) elements was prepared by digesting 100 μg of pTYAC1 DNA with *Bam*HI and *Eco*RI and then purifying the 8-kilobase (kb) fragment using agarose gel electrophoresis. Approximately 75 μg of the purified fragment was treated with excess calf intestinal phosphatase (Boehringer Mannheim, molecular biology grade), extracted with phenol and chloroform, and then precipitated with ethanol. Ten micrograms of size-selected *Eco*RI partial-digestion fragments was added to 75 μg of phosphatase-treated vector in a 200-μl ligation reaction mixture containing 15 units of T4 ligase, 50 mM Tris·HCl, 10 mM $MgCl_2$, 50 μg of bovine serum albumin per ml, 20 mM dithiothreitol, and 1 mM ATP (pH 7.5). After 14 hr at 15°C, the ligation mixture was extracted gently with phenol and then with chloroform. Unligated vector was removed from high molecular weight material using sucrose density gradient centrifugation (21); gradient fractions containing the large DNA fragments were pooled and then concentrated and dialyzed as before. *S. cerevisiae* strain AB1380 was transformed as described (20, 23), using at least 0.1 μg of transforming DNA per 5×10^7 cells and omitting carrier DNA. Transformants were picked individually from the minus-uracil regeneration agar onto selective media.

Analysis of HTY Strains. Growth of yeast strains, preparation of chromosome-sized DNA in agarose, pulsed-field gel electrophoresis, and gel-transfer hybridization procedures have been described (20, 24, 25). Hybridization probes were prepared from total yeast DNA and total human DNA by using the random-hexamer method (26); hybridization was at 65°C in a solution containing 0.5 M NaCl, 0.1 M sodium phosphate (pH 7), 5 mM EDTA, 1% (wt/vol) sodium-*N*-lauroyl sarkosinate, and 0.1 mg of calf thymus DNA per ml. A hybridization probe specific for the human-telomere repeat, $(TTAGGG)_n$, was prepared by using the *Pst* I insert of pHuR93 (6) as template for the extension of the complementary synthetic 18-mer $(CCTAAC)_3$ in the absence of α-dGTP. *Sau*3AI-digested pTYAC1 (1.5 μg/ml) was included in prehybridization and hybridization solutions that employed this probe and calf thymus DNA was omitted. Hybridization was carried out at 68°C for 14–24 hr in the same buffer that was described above.

HTY Recloning in sCos-1. DNA from HTY colonies was partially digested with *Sau*3AI and ligated into the *Bam*HI site of cosmid sCos-1 (27). Following *in vitro* packaging and transformation of *Escherichia coli* strain HB101, human-recombinant cosmids were detected by colony hybridization to ^{32}P-labeled C_0t 50 DNA [where C_0t 50 indicates the initial concentration of DNA (moles of nucleotide per liter) × time (sec)] (28). The fraction of positive colonies detected (0.3–2%) was consistent with the ratio of human to yeast DNA present in HTY strains containing artificial chromosomes of various sizes. DNA from positive cosmid clones was digested with *Eco*RI, separated on 1% agarose gels, and blotted to nitrocellulose. Confirmation of human DNA sCos-1 recombinants was conducted by hybridization of these blots to ^{32}P-labeled human C_0t 50 DNA and oligomeric GT·AC repetitive DNA (29). Yeast DNA recombinants containing GT·AC repeats, representing 5–10% of the initially selected colonies, were eliminated by this double screening procedure.

***In Situ* Hybridization.** *In situ* hybridization to metaphase chromosomes from human lymphocytes was conducted as described (6, 30) with the following modifications. After the RNase treatment, slides were prewarmed to 40°C and then incubated with proteinase K (0.6 μg/ml) in a solution containing Tris·HCl (pH 7.5) and 2 mM $CaCl_2$ for 2–3 min. The slides were rinsed once in Tris buffer, rinsed once in phosphate-buffered saline with 50 mM $MgCl_2$, and then fixed in 4% paraformaldehyde in phosphate-buffered saline/$MgCl_2$ for 10 min at room temperature. After a quick rinse in phosphate-buffered saline, the slides were dehydrated and air-dried before denaturation. The hybridization solution contained 20 μg of biotinylated cosmid DNA per ml (from 40 to 150 kb of overlapping contiguous DNA subcloned from HTYs into sCos-1) in 50% formamide, 0.3 M NaCl/30 mM sodium citrate, and 10% dextran sulfate. Cross-hybridization to interspersed repetitive DNA was blocked by using 500–700 μg of C_0t 1 or C_0t 50 human DNA per ml (28).

RESULTS

The Telomeric YAC Cloning System. The vector pTYAC1 (Fig. 1) contains the yeast replication origin ARS1 (33), the yeast centromere sequence CEN4, which confers mitotic and meiotic stability on large linear plasmids (34, 35), and the *Tetrahymena* telomere-repeat sequence TrTEL, which heals into a functional telomere when present at the end of a linear DNA molecule that is introduced into yeast by transformation (31). In addition, pTYAC1 contains the selectable marker URA3 for positive selection of transformants in ura3 hosts as well as the amp and ori regions of pBR322 for growth and amplification of the plasmid in *E. coli*.

Digestion of pTYAC1 with *Eco*RI and *Bam*HI yields an 8-kb linear fragment containing all of the essential components of the vector. This fragment is treated with alkaline phosphatase and ligated to source-DNA fragments prepared by partial digestion with *Eco*RI followed by size-selection of fragments larger than ≈50 kb. When this ligation mixture is used to transform a population of yeast cells, the desired products are clones that contain artificial chromosomes with the structure shown in Fig. 1. In these chromosomes, one of the telomeres is formed by the healing of a TrTEL terminus into a yeast telomere, whereas the other is formed by the healing of a source-DNA telomere into a yeast telomere. Expected background events would include circularized products and linear chromosomes in which a nontelomeric source-DNA terminus had healed by recombination or telomerase action into a yeast telomere.

Pilot Experiments Cloning Natural Yeast Telomeres. The feasibility of this cloning system was first tested by using *S.*

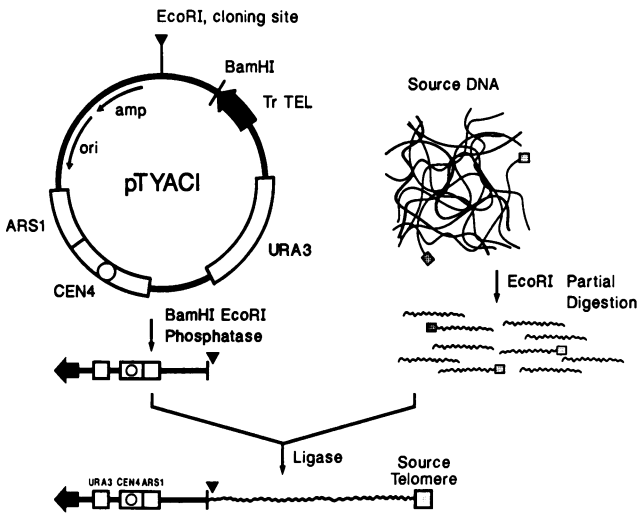

FIG. 1. Telomeric YAC cloning system. The plasmid pTYAC1 was constructed by the insertion of a 700-base-pair (bp) *Bam*HI/*Xho* I fragment of pYAC4 (20, 31) into a *Bam*HI/*Sal* I fragment produced by double-digestion of YCp50 (32). The rationale for the cloning procedure is described in the text.

cerevisiae source DNA. Partially digested high molecular weight DNA from *S. cerevisiae* contains a high proportion of telomere-terminal DNA fragments since the yeast genome contains approximately one telomere per 500 kb. Size-selected *Eco*RI fragments of *S. cerevisiae* DNA ranging from 80 to 250 kb were ligated to the vector as described, and the ligation mixture was used to transform the host strain AB1380. Eighty-four transformants were analyzed by separating their chromosomal-DNA molecules by pulsed-field gel electrophoresis and carrying out gel-transfer hybridization with probes to the pBR322 sequences in pTYAC1 and to the yeast subtelomeric repeats X and Y′. The latter probes are diagnostic of natural yeast telomeres since most or all such telomeres contain one or both of these repeats (12, 36, 37). Only 20 of the 84 transformants analyzed contained an artificial chromosome of the expected size, as visualized by ethidium bromide staining and hybridization with pBR322 sequences; in all 20 cases, this chromosome hybridized with either the X sequence, the Y′ sequence, or both. These transformants will be referred to as YTY (yeast-telomeric YAC) clones. Most of the other 64 transformants contained a pair of pBR322-hybridizing bands that migrated at the same position in each strain; these bands did not hybridize with X or Y′ sequences and appear to represent supercoiled and relaxed forms of vector molecules that had circularized, presumably *in vivo*. This background almost certainly arises from excess vector present in the ligation mixture (38). In subsequent experiments, these molecules were removed prior to transformation by fractionating the ligation mixture on a sucrose density gradient.

Isolation of Human Telomeric Candidate Clones. The cloning protocol optimized during the pilot experiments with yeast source DNA was used to prepare a HTY library. The first 48 transformants obtained were analyzed by gel-transfer hybridization following the separation of their chromosomes on pulsed-field gels; total human DNA, pBR322, X, and Y′ sequences were used as probes. All 48 transformants contained bands that hybridized to pBR322, most contained bands that hybridized with total human DNA, but none of the artificial chromosomes in the 48 transformants hybridized with the X or Y′ sequences (data not shown). The latter result indicates that the human ends of the transforming molecules have been stabilized by some mechanism other than illegitimate recombination with a natural yeast telomere. Overall, 24/48 transformants analyzed in this way contained a single artificial chromosome of the expected size that hybridized to human DNA and to pBR322. A preliminary screen of these 48 transformants with a probe for the human terminal-repeat sequence $(TTAGGG)_n$ indicated that only one clone, HTY146, showed strong, specific hybridization (data not shown). Because of this low frequency, an additional 352 transformants were screened with the $(TTAGGG)_n$-specific probe using a gel-transfer-hybridization protocol that involved pooling high molecular weight DNA from 10 independently grown strains on a single lane of a pulsed-field gel. Five additional HTY candidates were detected in this way.

Characterization of Human Telomeric Candidate Clones. Three of these HTY candidates were selected for more extensive analysis by using carefully controlled hybridization conditions. Cross-hybridization between human telomere sequences and the $(TTGGGG)_n$ sequences present in the TrTEL region of the vector has been reported (39). To circumvent this potential problem, excess fragmented pTYAC1 DNA was added to the prehybridization and hybridization solutions as an unlabeled competitor for $(TTGGGG)_n$ hybridization sites. Also, YACs that were known to contain TrTEL sequences but not human telomeres were included in all hybridizations as controls. The results of these experiments are shown in Fig. 2. Lanes 3, 4, and 5 contain HTY candidates that show strong and specific hybridization to the $(TTAGGG)_n$ probe (Fig. 2*C*).

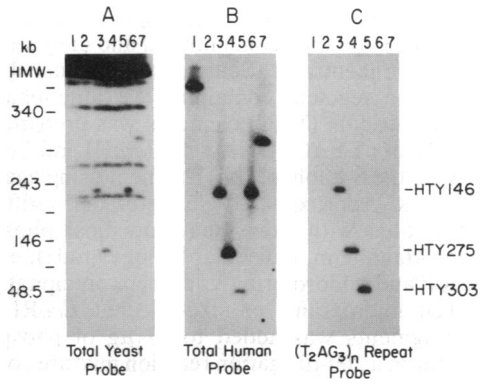

FIG. 2. Analysis of human telomeric YAC candidates by gel-transfer hybridization. The smaller chromosomal DNA molecules present in seven yeast strains containing artificial chromosomes were separated by pulsed-field gel electrophoresis and analyzed by gel-transfer hybridization using three different probes. Lanes 1 and 2, control YAC clones: a large human fragment cloned in pYAC4 (lanes 1) and a YTY strain containing a natural yeast telomere cloned in pTYAC1 (lanes 2). Lanes 3–7, HTY candidates, only three of which hybridize to the $(TTAGGG)_n$ probe: HTY146 (lanes 3), HTY275 (lanes 4), and HTY303 (lanes 5). HMW, high molecular weight.

The sensitivity of the $(TTAGGG)_n$-hybridizing sequences in these HTY candidates to the exonuclease BAL-31 was tested. This enzyme digests double-stranded DNA progressively inward from the ends of linear DNA molecules, including those terminated with the natural telomeres of a variety of organisms (5, 6, 18, 40). High molecular weight DNA isolated from each strain was digested with nuclease BAL-31 for various periods of time, the products of these digestions were cleaved by a restriction endonuclease, and the resultant fragments were analyzed by gel-transfer hybridization. Hybridization using the $(TTAGGG)_n$ probe yielded the pattern expected of a sequence at or near the end of a linear DNA molecule. The data are shown in Fig. 3 *A* and *C* for HTY146 and HTY255, respectively. From the mobility shift of the hybridizing band that accompanies the increasing BAL-31 digestion time, it appears that the entire $(TTAGGG)_n$-hybridizing tract in HTY146 is within 500 bp of one end of the artificial chromosome, whereas in HTY255 it is within 1000 bp of an end (the different rates of BAL-31 digestion in the two experiments are due to different incubation temperatures). The diffuse nature of the bands formed from small telomere-adjacent restriction fragments in these two strains is indicative of the heterogeneity found in telomere-terminal repeat tracts in yeast and other organisms (11).

Control experiments showed that nonspecific degradation of DNA by nuclease BAL-31 did not account for the disappearance of the $(TTAGGG)_n$-hybridizing bands. The filters used in Fig. 3 *A* and *C* were stripped of the $(TTAGGG)_n$ probe and rehybridized with a DNA fragment containing the yeast ARS1 and TRP1 sequences (Fig. 3 *B* and *D*). The endogenous TRP1 and ARS1 sequences are located far from the natural yeast telomeres on chromosome IV and are expected to be insensitive to nuclease BAL-31, whereas the ARS1 sequence in the vector is 5 kb from the TrTEL terminus. In Fig. 3*B*, where the BAL-31 products have been digested with *Eco*RI, the expected 1.45-kb fragment carrying both TRP1 and ARS1 (41) is evident in all lanes and is insensitive to BAL-31 digestion. The band migrating at 8 kb in Fig. 3*B* is the vector-derived telomeric fragment that includes the ARS1 sequence (*Eco*RI site to TrTEL, Fig. 1). The mobility shift of this band caused by BAL-31 treatment is too small to be detected under these electrophoresis conditions; the greater decrease of this fragment's hybridization signal, in compar-

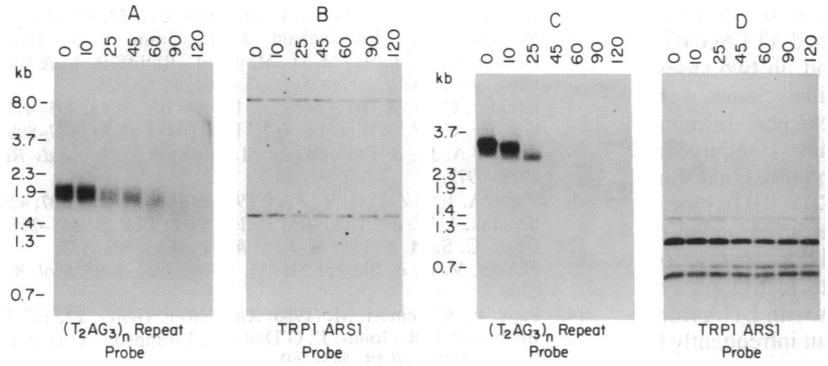

FIG. 3. Sensitivity of the $(TTAGGG)_n$-hybridizing sequences in HTY146 (*A* and *B*) and HTY255 (*C* and *D*) to digestion with the exonuclease BAL-31. High molecular weight genomic DNA isolated from HTY146 or HTY255 was treated with nuclease BAL-31 for the indicated time (in minutes; the incubation temperature was 22°C for HTY146 and 37°C for HTY255). The DNA was then cleaved with *Eco*RI (HTY146) or *Sau*3AI (HTY255) and analyzed by gel-transfer hybridization using the indicated probes.

ison with that from the 1.45-kb fragment, is presumably due to the 8-kb fragment's larger cross section for nonspecific endonucleolytic cleavage. The analogous control for HTY255 (Fig. 3*D*) gives a more complex banding pattern since the secondary digestion was carried out with *Sau*3AI, an enzyme that cleaves a number of times within the ARS1 and TRP1 sequences; however, the expected bands are seen (42) and none is sensitive to BAL-31. Thus, the BAL-31 sensitivity of the $(TTAGGG)_n$-hybridizing sequences in HTY146 and HTY255 demonstrates that these sequences are directly adjacent to one end of the artificial chromosome.

Confirmation of the Telomeric Origins of HTY146 by *in Situ* Hybridization to Human Metaphase Chromosomes. Direct confirmation that several of the HTY clones contain human DNA derived from the immediate vicinity of human telomeres was obtained by *in situ* hybridization. Total HTY DNA or subregions cloned into cosmid sCos-1 (27) were biotinylated and hybridized to human metaphase chromosomes (6, 30). Total DNA from HTY275 or cosmid subclones derived from HTY275 gave strong, discrete hybridization to the telomeric regions of multiple human chromosomes (Fig. 4*A*), which could not be competitively inhibited with excess repetitive DNA; sporadic signals from nontelomeric sites were not reproducible. Single cosmids derived from HTY275 exhibited similar hybridization patterns to that shown in Fig. 4*A*. DNA from pooled cosmids derived from either HTY146 or HTY243 gave a strong signal at the tip of a single chromosome (tentatively identified as chromosomes 7 and either 19 or 20, respectively) together with weak signals at the tips of multiple chromosomes. The identities of these weakly hybridizing chromosomes were not established by G-banding, but the sets of chromosomes recognized by HTY146, HTY243, and HTY275 were clearly distinct from one another, suggesting the presence of a complex arrangement of subtelomeric repeats within the human genome.

To determine the origin of the DNA present in HTY146, cosmid DNA from a limited internal subregion of this clone (representing ≈100 kb of the 240-kb human insert) was biotinylated and hybridized to metaphase chromosomes in the presence of excess C_0t 50 DNA. These internal single-copy sequences present in HTY146 were localized to the telomere of the long arm of chromosome 7 (Fig. 4*B*).

DISCUSSION

We have utilized the evolutionary conservation of telomeric terminal-repeat sequences to clone large, telomeric fragments of human chromosomes into *S. cerevisiae*. Szostak and Blackburn (11) used a similar strategy in their initial isolation of natural yeast telomeres on small linear plasmids containing a *Tetrahymena* telomere on one end. We selected for large fragments of human DNA bounded on one end by an *Eco*RI cloning site and on the other end by DNA sequences that could seed the formation of a stable yeast telomere; several of the

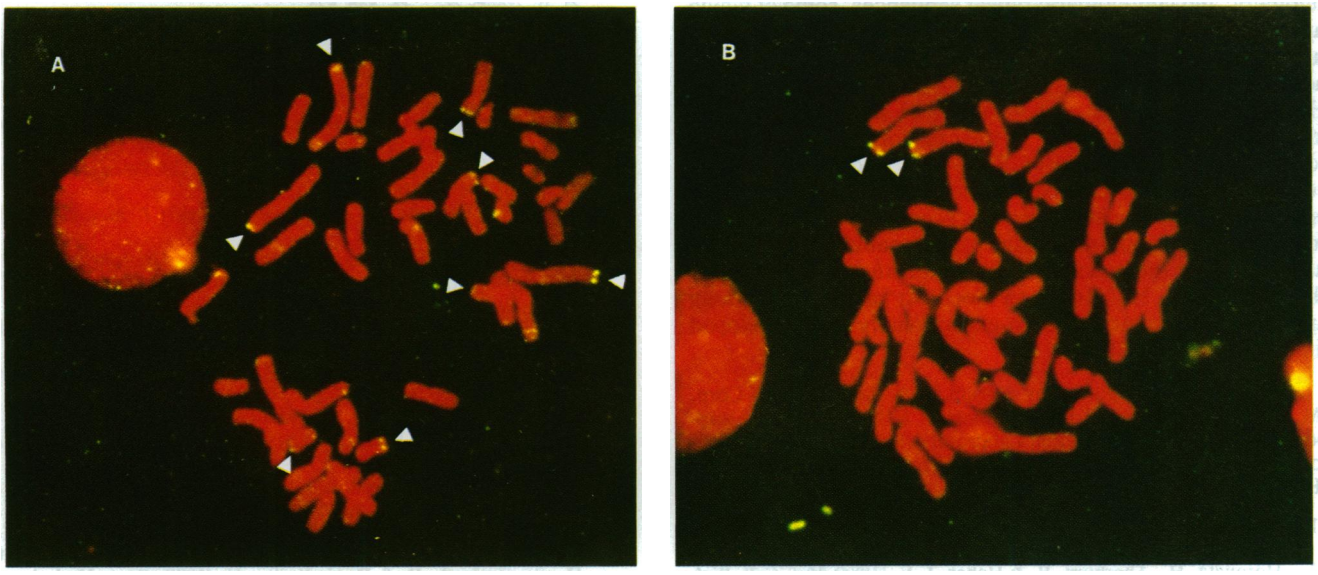

FIG. 4. *In situ* hybridization of sequences present in HTY275 and HTY146 to human metaphase chromosomes. (*A* and *B*) *In situ* hybridization of biotin-labeled cosmid clones derived from HTY275 (*A*) and HTY146 (*B*). Major sites of hybridization of HTY275 DNA to the telomeres of 1p, 2q, 7p, 8p, 16p, and 20p are indicated by white arrowheads in *A*; minor hybridization sites were sometimes evident at additional telomeres. Pooled cosmid clones representing ≈100 kb of internal DNA from HTY146 exhibited specific hybridization to 7qtel, indicated by white arrowheads in *B*; the chromosome identity was confirmed by banding analysis (30).

resultant clones had the properties expected of authentic telomere-terminal fragments. They contained $(TTAGGG)_n$ sequences at the end distal to the vector and, in two cases, single-copy sequences contained in individual clones were mapped by *in situ* hybridization to the extreme terminal regions of two different human chromosomes. Unequivocal proof that the cloned fragments represent sequences that start at an internal *Eco*RI site and end within the $(TTAGGG)_n$ repeat of a natural human telomere will require the development of single-copy probes from the HTY clones and the construction of detailed physical maps using appropriate restriction enzymes. Comparison of genomic source DNA with DNA from HTY clones using restriction enzymes that cut infrequently is difficult because most or all of these enzymes are sensitive to the methylation of their restriction sites. All of these sites are expected to be cleavable in yeast, which has no cytosine methylation, whereas only a subset of these sites is cleavable in human genomic DNA.

The most obvious alternative explanation for our results would be that a nontelomeric $(TTAGGG)_n$ repeat has healed into a yeast telomere after exonucleolytic degradation of a large, broken ligation product. From the results of BAL-31 sensitivity assays and *in situ* hybridization experiments, it has been demonstrated that there are no detectable nontelomeric tracts of $(TTAGGG)_n$ repeats in the human genome (6). In addition, our *in situ* hybridization data indicate that if such an event did occur, the $(TTAGGG)_n$ repeat sequence must have originated in the immediate vicinity of a natural human telomere.

One to two percent of the clones that we isolated hybridized with the human $(TTAGGG)_n$ repeat sequence, and each of the hybridizing clones contained a $(TTAGGG)_n$ tract several hundred base pairs in length. It is notable that the $(TG_{1-3})_n$ tracts normally present at the ends of yeast chromosomes are also a few hundred base pairs in length (43, 44). Consequently, if the system in yeast that monitors the lengths of these tracts does not distinguish between $(TG_{1-3})_n$ and $(TTAGGG)_n$, one would expect to obtain clones of the type that we recovered.

The protocol that we have described circumvents a number of problems inherent in efforts to characterize telomeric regions using conventional cloning vectors. The presence of telomeric and subtelomeric repeat sequences, some of which appear to be difficult to clone in *E. coli*, makes the ends of the human chromosomes unattractive regions in which to characterize large blocks of DNA by chromosome walking (4). However, single HTY clones ranging in size from 50 to 250 kb are likely—as appears to be the case for the 7q terminus—to bridge from the terminal $(TTAGGG)_n$ tract to single-copy sequences characteristic of a particular chromosome end. It is from these chromosome-specific sequences that restriction fragment length polymorphism probes could be sought for use in defining the boundaries of the human genetic map. For these purposes, HTY146 is an ideal test case since the long arm of chromosome 7 has an exceptionally well-developed linkage map (45, 46).

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