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A highly conserved repetitive DNA sequence, $(TTAGGG)_n$, present at the telomeres of human chromosomes

(human repetitive DNA/*in situ* hybridization/trypanosome telomeres/BAL-31 nuclease/flow cytometry)

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ABSTRACT **A highly conserved repetitive DNA sequence, $(TTAGGG)_n$, has been isolated from a human recombinant repetitive DNA library. Quantitative hybridization to chromosomes sorted by flow cytometry indicates that comparable amounts of this sequence are present on each human chromosome. Both fluorescent *in situ* hybridization and BAL-31 nuclease digestion experiments reveal major clusters of this sequence at the telomeres of all human chromosomes. The evolutionary conservation of this DNA sequence, its terminal chromosomal location in a variety of higher eukaryotes (regardless of chromosome number or chromosome length), and its similarity to functional telomeres isolated from lower eukaryotes suggest that this sequence is a functional human telomere.**

The human genome contains a variety of DNA sequences present in multiple copies (1). These repetitive DNA sequences are thought to arise by many mechanisms, from direct sequence amplification by the unequal recombination of homologous DNA regions to the reverse flow of genetic information (2). While it is likely that some of these repetitive DNA sequences influence the structure and function of the human genome, little experimental evidence supports this idea at present.

We reasoned, however, that evolutionary conservation of a particular repetitive DNA sequence family might imply that the sequence is essential to cellular function. To isolate highly conserved repetitive DNA sequences, we constructed a recombinant human repetitive DNA library (pHuR library, for plasmid human repeat) and isolated clones that shared a high degree of sequence identity with rodent repetitive DNA. Four of the six most conserved cloned sequences isolated in this manner consisted of tandem arrays of the alternating (dG-dT)·(dA-dC) sequence, known to be ubiquitously interspersed in eukaryotic genomes and capable of forming the alternative Z-DNA conformation (3).

The remaining two highly conserved cloned DNA sequences consisted of tandem arrays of the hexanucleotide sequence $(TTAGGG)_n$,¶ identical to the hexanucleotide sequence known to be at the telomeres of trypanosome chromosomes (4, 5). A telomere is functionally defined as a region of DNA at the molecular end of a linear chromosome that is required for replication and stability of the chromosome (6). Replicating a linear DNA molecule presents unique challenges, since all known DNA polymerases require a polynucleotide primer bearing a 3′-hydroxyl group. A variety of mechanisms are used to circumvent this replication problem, from the production of concatemeric genomes (7) to the evolution of specific telomere terminal transferase enzymes (8). In addition to their role in chromosome replication, functional telomeric DNA sequences are believed to confer stability to chromosomes, preventing the end-to-end fusions and DNA degradation normally observed after breakage of chromosomes by x-irradiation or physical rupture (6).

In this paper, we present the results of fluorescent *in situ* hybridization (9) and BAL-31 nuclease digestion experiments (4, 5), indicating that the major clusters of the $(TTAGGG)_n$ sequence occur at the extreme termini of all human chromosomes. The evolutionary conservation of this DNA sequence, its terminal chromosomal location in a variety of eukaryotes (regardless of chromosome number or chromosome length), and its similarity to functional telomeres isolated from lower eukaryotes (6, 8) suggest that the sequence $(TTAGGG)_n$ is a functional human telomere.

MATERIALS AND METHODS

Construction of a Human Repetitive DNA Library. Human placental DNA (Calbiochem) was sheared to a single-strand length of 3.6 kb (10, 11), denatured in 0.1× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), and reassociated in 0.3 M Na^+ (Cl^- anion in all cases) at 63°C to an equivalent C_0t value of 50 (mol of nucleotide·liter^{-1}·sec) (10). Unreassociated DNA was digested with S1 nuclease (Boehringer Mannheim) to a digestion estimate value (DIG) of 0.9 (11), and the S1 nuclease-resistant DNA was isolated by Sephadex G-100 and hydroxylapatite column chromatography (10, 11).

The reassociated and S1 nuclease-treated human repetitive DNA was tailed with oligo(dC) and ligated to oligo(dG)-tailed pBR322 DNA by standard procedures (12). Transformation, using *Escherichia coli* strain HB101, yielded 11,000 recombinant clones. Statistically, this library has a 0.99 probability of containing sequences from the abundant class of human repetitive DNA sequences but only a 0.83 probability of containing sequences from the less abundant repeat class (13, 14). All work was carried out under the prevailing National Institutes of Health guidelines for recombinant DNA research.

Library Screening. Colonies from the human repetitive DNA library were grown on nitrocellulose filters and screened by standard procedures with ^{32}P-labeled human or hamster C_0t 50 repetitive DNA in 0.95 M Na^+ at 68°C (9, 10, 15). Under these conditions, hybridization should occur between DNA sequences sharing at least 70–75% identity (16). For higher stringency, prehybridization and hybridization were performed in either (*i*) 0.95 M Na^+ at 80°C (≈85% identity) or (*ii*) 0.17 M Na^+ at 80°C (≈95% identity). These

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¶The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J04078).

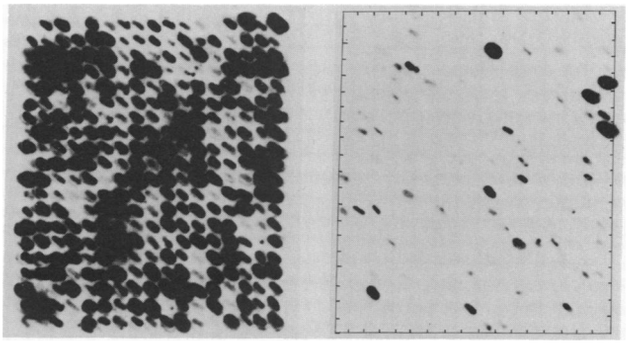

FIG. 1. Human repetitive DNA library screening. (*Left*) Colonies containing human repetitive DNA were grown on a nitrocellulose filter and hybridized to ^{32}P-labeled human C_0t 50 repetitive DNA. (*Right*) Replicate filter of the same colonies present in *Left* hybridized to ^{32}P-labeled hamster C_0t 50 repetitive DNA. Both hybridizations were carried out at 68°C in 0.95 M Na^+ (9, 10, 15). Autoradiographic exposures were 14 hr at −70°C with an intensifying screen.

calculated hybridization identities were derived from solution hybridization experiments and are not corrected for the well-known length effects on thermal stability (16). For short DNA duplex regions (i.e., under 50 nucleotides), the "85% identity" condition is at the melting temperature, t_m, for perfectly matched DNA duplexes, and the "95% identity" condition is ≈10°C above the t_m (17).

Oligomer Synthesis and Labeling. The deoxynucleotide polymers $(GGGTTA)_7$ and $(TAACCC)_7$ were synthesized on a Beckman System 1 DNA synthesizer. Oligomers were labeled in separate 100-μl reaction mixtures containing 600 ng of oligomer DNA, 200 mM potassium cacodylate at pH 7.2, 1 mM $MnCl_2$, 2 mM 2-mercaptoethanol, 100 units of terminal deoxynucleotidyltransferase (a gift from Ratliff Biochemicals, Los Alamos, NM), and a 200-fold excess of biotin-11-dCTP (biotin coupled to C5 of cytosine with an 11-carbon linker arm) over the number of 3′ ends (9).

Clone Isolation, Sequencing, Restriction Enzyme Digestion, Southern Blotting, Chromosome Blotting, and *in Situ* Hybridization. All methods have been described previously (9).

RESULTS

Human Repetitive DNA Library Screening. A search for highly conserved repetitive DNA sequences was initiated, utilizing the pHuR human recombinant repetitive DNA library. This library was constructed from randomly sheared and reassociated DNA, a method that minimizes the potential loss of sequences, such as centromeric repetitive DNA arrays, that are devoid of a given restriction enzyme site (9,

Table 1. Human repetitive DNA library screening with ^{32}P-labeled hamster C_0t 50 repetitive DNA

Hybridization signal	% positive clones	
	0.95 M Na^+, 68°C	0.95 M Na^+, 80°C
Strong	1.9	0.2
Medium	2.1	0.3
Weak	15.8	1.2

The DNA pHuR library was screened with ^{32}P-labeled hamster C_0t 50 repetitive DNA at two different hybridization conditions. The 1400 clones screened represent 12% of the pHuR DNA library. For short DNA duplexes (i.e., under 50 nucleotides), the 0.95 M Na^+ 68°C conditions are approximately 10°C below the t_m for perfectly matched DNA duplexes, and the 0.95 M Na^+ 80°C conditions are approximately at the t_m (16, 17). The percent of colonies giving strong, medium, and weak hybridization signals under these two hybridization conditions is indicated.

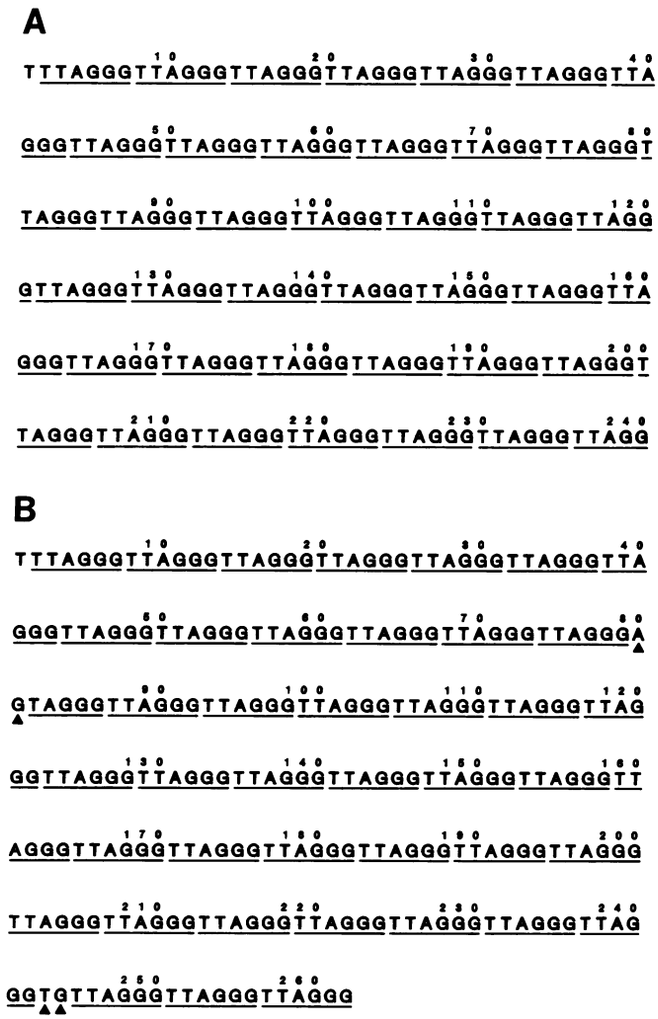

FIG. 2. Nucleotide sequences of clones pHuR 93 and pHuR 143. The nucleotide sequences of clones pHuR 93 (*A*) and pHuR 143 (*B*) are shown, minus the oligo(G·C) tails used for cloning. The conserved TTAGGG hexanucleotide sequence is underlined. Rare insertions or substitutions are indicated by ▲.

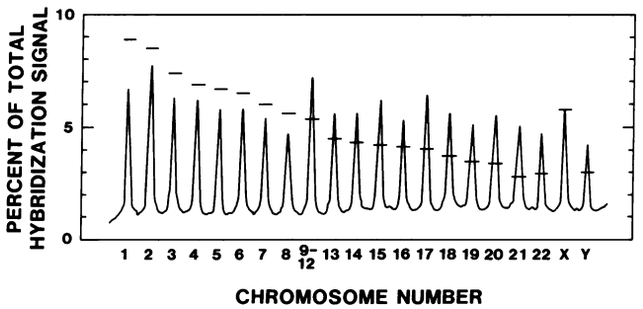

FIG. 3. Hybridization of ^{32}P-labeled pHuR 93 DNA to flow-sorted human chromosomes. Human chromosomes were flow sorted, bound to nitrocellulose filters, and hybridized to ^{32}P-labeled clone pHuR 93 DNA [which contains 40 copies of the TTAGGG repeat (Fig. 2)], as described previously (9). The DNA from 15,000 chromosomes was denatured and bound per slot. Densitometry of the autoradiographic signal was obtained on a Beckman DU-8 spectrophotometer. The signals from four separate hybridization experiments were normalized (each chromosome signal expressed as percent of total signal) and summed to produce the final densitometry plot shown. The expected normalized signal heights for a repetitive sequence distributed randomly throughout the genome (and hence proportional to chromosome length) is indicated by horizontal lines.

18). Likewise, the ends of linear DNA molecules will, by definition, be unclonable after restriction enzyme digestion, but they should be represented in this library if they consist of repetitive DNA arrays. Hybridization with a ^{32}P-labeled human C_0t 50 repetitive DNA probe indicated that at least 95% of the plasmid clones in this library contain human repetitive DNA inserts (Fig. 1 *Left*). A portion of this library was screened with ^{32}P-labeled hamster C_0t 50 repetitive DNA as a probe. Under standard hybridization conditions (0.95 M Na^+, 68°C), 20% of the bacterial colonies gave positive signals (Fig. 1 *Right*; Table 1). Many of these clones contained *Alu* repetitive sequence elements, known to be found in high abundance in mammalian genomes (ref. 2; data not shown). When the stringency of the hybridization conditions was increased to 0.95 M Na^+ at 80°C, 0.5% of the colonies still gave a strong or moderate hybridization signal (Table 1). No detectable hybridization signal to any bacterial colony was observed when hybridization was conducted in 0.17 M Na^+ at 80°C.

Isolation and Sequence Analysis of Highly Conserved Repetitive DNA Sequences. Six plasmid clones that produced strong high-stringency hybridization signals with hamster repetitive DNA were isolated for further analysis. Four of these recombinants contained small DNA inserts (39–48 nucleotides), each containing a variation of the same alternating (dG-dT)·(dA-dC) sequence (data not shown). This sequence, with the capacity to form the alternative Z-DNA configuration, is known to be ubiquitously interspersed in eukaryotic genomes and to be highly conserved (3). The other two clones, designated pHuR 93 and pHuR 143, consisted of 40 and 43 copies, respectively, of highly conserved tandem arrays of the hexadeoxynucleotide sequence TTAGGG (Fig. 2). This hexanucleotide sequence is identical to the hexanucleotide sequence known to be at the telomeres of trypanosome chromosomes (4, 5).

Chromosomal Localization of the Conserved $(TTAGGG)_n$ Sequence. Clone pHuR 93 DNA was used to determine the chromosomal distribution of the $(TTAGGG)_n$ sequence in the

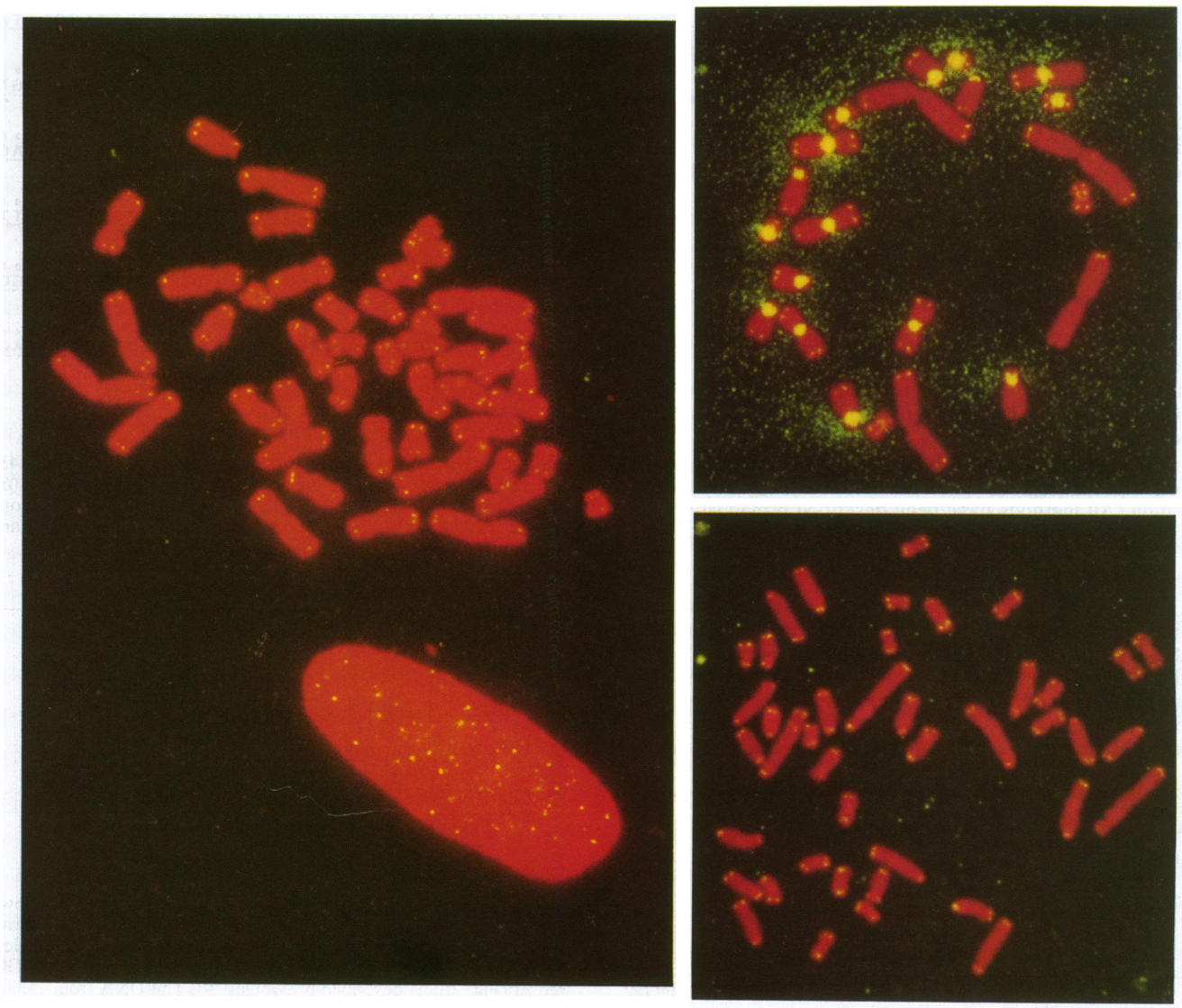

FIG. 4. *In situ* hybridization of biotin-labeled $(GGGTTA)_7$·$(TAACCC)_7$ oligomers to metaphase chromosomes. *In situ* hybridization was conducted in 2× SSC/30% (vol/vol) formamide at 37°C as described previously (9). Chromosomes were counterstained with propidium iodide after reaction with fluorescein-labeled avidin, and one amplification with avidin antibody, to detect the biotinylated probe DNA. The $(GGGTTA)_7$·$(TAACCC)_7$ oligomer duplex has an anomalously high t_m (66°C in 50 mM Na^+), and these hybridization conditions are ≈20°C below the t_m (17). Comparable hybridization results were obtained at hybridization condtions ≈7°C below the t_m, sufficient to prevent hybridization of this sequence to other known telomeric repeats (data not shown; refs. 6 and 8). Hybridization to human (*Left*), Chinese hamster (*Upper Right*), and rat (*Lower Right*) chromosomes is shown.

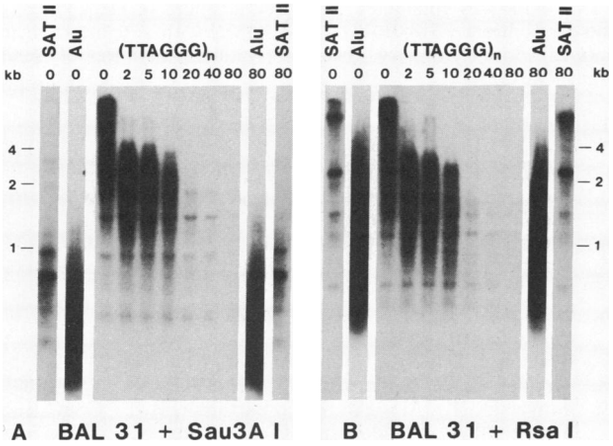

FIG. 5. The TTAGGG repeat is located at the terminus of human telomeres. High molecular weight [>400-kilobase-pair (kb)] human DNA was digested for the times shown in minutes above each lane with BAL-31 nuclease (4, 5). Average shortening by ≈0.4 kb (2 min), 1 kb (5 min), 2 kb (10 min), 4 kb (20 min), 8 kb (40 min), and 16 kb (80 min) occurred under these digestion conditions, as determined by control experiments with linear pHuR 93 plasmid DNA and confirmed by postdigestion analysis of genomic DNA sequences complementary to clone pHuR 93. Aliquots of the DNA from each time were then digested to completion with either *Sau*3AI (*A*) or *Rsa* I (*B*) restriction nuclease and electrophoresed on duplicate 1.0% agarose gels. After transfer to nitrocellulose filters, the size-fractionated genomic DNA was hybridized to either ^{32}P-labeled pHuR 93 DNA, indicated by $(TTAGGG)_n$, or ^{32}P-labeled pHuR 195 DNA (indicated as SAT II), a chromosome-16-specific centromeric satellite II DNA sequence (9). The filters hybridized to pHuR 93 DNA were rehybridized, after removal of the first probe, to ^{32}P-labeled pHuR 3 DNA (9), a human *Alu* repetitive sequence (indicated by *Alu*). Only the 0- and 80-min BAL-31 nuclease digestion aliquots hybridized to SAT II and *Alu* DNA are shown, since no detectable change in the hybridization pattern occurred during the course of BAL-31 digestion. The migration positions of 1-, 2-, and 4-kb DNA fragments, determined from *Hin*dIII-digested phage λ and *Hae* III-digested phage φX174 DNA markers are indicated.

human genome. Quantitative slot blot analysis, using flow-sorted human chromosomes (9), indicates that similar amounts of this repetitive DNA sequence are present on each human chromosome, regardless of the absolute chromosome length (Fig. 3). This pattern of hybridization contrasts with that observed for other families of human tandem repetitive DNA sequences, which are localized to distinct chromosomes (9) or interspersed repeat families, such as *Alu* sequences (2), that give signals proportional to chromosome length (data not shown). Estimates of the amount of $(TTAGGG)_n$ sequences present in the human genome, determined from both quantitative hybridization analysis (Fig. 3 and unpublished experiments) and the frequency of this sequence in the pHuR library indicate that 3000–12,000 base pairs (bp) (500–2000 hexamers) are present on each human chromosome.

The genomic location of the $(TTAGGG)_n$ sequences was further characterized by fluorescent *in situ* hybridization (Fig. 4). To better control the *in situ* hybridization conditions, heptamers of the hexamers (GGGTTA) and (TAACCC) were synthesized and end labeled with biotin-11-dCTP. A mixture of these two probes was hybridized to denatured human metaphase chromosomes *in situ*, and fluorescein-labeled avidin was used to detect the biotinylated DNA (9). While either strand alone gives observable hybridization signals, the fluorescent intensity increases when the two are mixed, presumably due to out-of-register concatenation. Fluorescence signals were observed at the telomeres of all human chromosomes. As shown in Fig. 4, about 80–90% of the telomeres are clearly labeled in most metaphases. Some telomeres have very faint hybridization, but the intensity of label appears to be a random variation. The fluorescent label is at the very end of prometaphase chromosomes, but, as the chromosomes condense, counterstained chromosomal DNA can be seen beyond the labeled site (Fig. 4). Whether this is the result of technical manipulation of the chromosomes or a function of chromosomal condensation remains to be determined.

The $(TTAGGG)_n$ sequence was originally isolated because of its ability to hybridize to rodent repetitive DNA at high-stringency conditions (Fig. 1 and Table 1). *In situ* hybridization to Chinese hamster and rat metaphase chromosomes indicated that sequences complementary to the synthetic $(GGGTTA)_7 \cdot (TAACCC)_7$ biotinylated probe are present at all telomeres in these two rodent species (Fig. 4 *Right*). In addition, small interstitial (Fig. 4 *Lower Right*) and large centromeric (Fig. 4 *Upper Right*) blocks of sequences complementary to the $(GGGTTA)_7 \cdot (TAACCC)_7$ probe are present in the rat and Chinese hamster genomes, respectively.

BAL-31 Nuclease Sensitivity of the $(TTAGGG)_n$ Telomeric Repeats. To determine if the $(TTAGGG)_n$ tandem repeats are directly at the ends of human chromosomes, high molecular weight DNA was digested with BAL-31 nuclease for increasing amounts of time (4, 5). This enzyme progressively shortens DNA molecules from their ends, and hence sequences that are at the original chromosome termini will be progressively shortened, while internal DNA sequences will be unaffected by moderate digestion. Genomic DNA sequences complementary to clone pHuR 93 are devoid of most restriction enzyme recognition sites (Fig. 2; data not shown) and remain as high molecular weight fragments after *Sau*3AI or *Rsa* I digestion and gel electrophoresis (Fig. 5). Digestion with BAL-31 nuclease prior to restriction enzyme digestion, however, shows a progressive shortening and eventual loss of over 99% of the genomic DNA sequences complementary to clone pHuR 93 (Fig. 5). In contrast, genomic DNA sequences complementary to either an *Alu* repetitive sequence probe (Fig. 5) or a chromosome-16-specific centromeric repeat sequence (9) are unaffected by BAL-31 digestion (Fig. 5). The observed kinetics of BAL-31 digestion (200 bp/min) is consistent with our estimate that 250 to 1000 hexamers are present at each human telomere (Fig. 3). The telomeric $(TTAGGG)_n$ sequences completely disappear after the removal of approximately 4000 bp (Fig. 5).

DISCUSSION

A human repetitive DNA library was constructed from randomly sheared, reassociated, and oligo(G·C)-tailed DNA, a method that minimizes the potential loss of sequences devoid of a given restriction enzyme site (9, 18). Sequences too large to clone efficiently in cosmid or λ vectors, such as centromeric repeats (9), or telomeric sequences with an end incompatible for cloning (6) should be present in this library. Screening this library at high hybridization stringency with ^{32}P-labeled hamster C_0t 50 repetitive DNA resulted in the isolation of four clones containing the sequence (dG-dT)·(dA-dC) (3) and two clones, designated pHuR 93 and pHuR 143, containing tandem repeats of the sequence $(TTAGGG)_n$ (Fig. 2). The identity of this sequence to that reported previously for the DNA present at trypanosome (4, 5) and acellular slime mold (19) telomeres suggested that the $(TTAGGG)_n$ sequence may be present at human telomeres as well.

In situ hybridization to human metaphase chromosomes localized the major clusters of this sequence at the telomeres of all chromosomes (Fig. 4). Quantitative hybridizations to flow-sorted human chromosomes indicated that similar amounts of this sequence are present on each chromosome,

regardless of chromosome length (Fig. 3). BAL-31 nuclease digestion experiments indicate that the major clusters of this sequence are near or directly at the chromosome termini (Fig. 5). Sequences complementary to a synthetic $(TTAGGG)_7$·$(TAACCC)_7$ probe are also present at the telomeres of Chinese hamster and rat chromosomes (Fig. 4), as well as at the telomeres of other primates, mammals, birds, and reptiles (J.M. and R.K.M., unpublished results). The evolutionary conservation of the $(TTAGGG)_n$ sequence (Fig. 2), its terminal chromosomal location in a variety of higher organisms (regardless of chromosome number or chromosome length) (Fig. 4), and its similarity to functional telomeres isolated from lower eukaryotes (4–6), suggest that this sequence is a functional human telomere.

Interestingly, in some organisms, such as Chinese hamster (Fig. 4 *Upper Right*) and Syrian hamster (data not shown), large blocks of this sequence are found at nontelomeric locations on some chromosomes, as well as at the telomeres. The $(TTAGGG)_n$ repeat has been reported to be the major component of guinea pig centromeric α-satellite DNA (20) and kangaroo rat centromeric HS-α-satellite DNA (21). Whether these nontelomeric blocks result from the historical fusion of two chromosome telomeres (22), the integration and amplification of autonomous telomere sequences, or other mechanisms remains to be determined. The maintenance of a "library" of potentially amplifiable repetitive DNA sequences has been proposed to explain the reoccurrence of centromeric $(TTAGGG)_n$ repeats in divergent rodent species (21). If this sequence is a functional eukaryotic telomere, then occasional genomic amplifications and rearrangements of the $(TTAGGG)_n$ repeat might be expected to accompany the chromosomal changes associated with speciation.

The recent finding that telomeric DNA oligonucleotides form intramolecular structures containing guanine·guanine base pairs (23) has suggested that telomere function may involve novel DNA–DNA or DNA–protein conformations and interactions (6, 8, 23, 24). The $(TTAGGG)_n$ repeat, present at the telomeres of trypanosome (4, 5), acellular slime mold (19), and human (Figs. 4 and 5) chromosomes, can prime, *in vitro*, the addition of *Tetrahymena* specific $(TTGGGG)_n$ repeats (25, 26) when the telomere terminal transferase of *Tetrahymena* is used (8). This priming ability suggests that the construction of mammalian cell artificial chromosome vectors, in a manner analogous to yeast artificial chromosome vectors (27), can now be attempted. Indeed, the production of stable acentric chromosome fragments in cancer cells may involve the natural yet inappropriate addition of telomeres to "tag" a fragmented region of DNA. Further, the identification of these $(TTAGGG)_n$ sequences at the extreme termini of human chromosomes will allow accelerated physical mapping of the human genome (28) by using pulsed-field gel electrophoresis (29, 30) and partial digestion strategies (31). Strategies to isolate sequences directly adjacent to all human telomeres can now be pursued (4, 5), which should facilitate the completion of a genetic linkage map of *Homo sapiens* (32, 33).

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