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Evolution

Conservation of the human telomere sequence $(TTAGGG)_n$ among vertebrates

(vertebrate evolution/synthetic oligodeoxynucleotides/*in situ* hybridization/repetitive DNA)

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ABSTRACT **To determine the evolutionary origin of the human telomere sequence $(TTAGGG)_n$, biotinylated oligodeoxynucleotides of this sequence were hybridized to metaphase spreads from 91 different species, including representative orders of bony fish, reptiles, amphibians, birds, and mammals. Under stringent hybridization conditions, fluorescent signals were detected at the telomeres of all chromosomes, in all 91 species. The conservation of the $(TTAGGG)_n$ sequence and its telomeric location, in species thought to share a common ancestor over 400 million years ago, strongly suggest that this sequence is the functional vertebrate telomere.**

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 (1). All known eukaryotic telomeres consist of simple repeated sequences of G- and C-rich complementary strands, with the general structure $(T \text{ or } A)_m(G)_n$ (1, 2). The G-rich DNA strand, oriented $5' \rightarrow 3'$ toward the chromosome end, is synthesized by an RNA-dependent "telomerase" activity in *Tetrahymena* (3–6) and *Oxytricha* (7). Frequent recombination occurs during telomere formation in yeast genomic and *Tetrahymena* mitochondrial DNA, predicted by models of recombination-mediated telomere replication (8, 9). Either telomerase or recombination models for telomere replication explain the stability of the basic repeating sequence, yet infrequent evolutionary change in the telomere sequence could occur with either replication method.

Recently, our laboratory has identified and cloned the human telomere sequence $(TTAGGG)_n$ (10). To define the evolutionary origin of this repeat without molecular cloning from numerous species, we determined *in situ* hybridization conditions under which absolute sequence identity must be present in the complementary chromosomal DNA for significant hybridization to occur. A survey of 91 representative vertebrate species is presented in this paper, using biotinylated $(GGGTTA)_7 \cdot (TAACCC)_7$ oligodeoxynucleotides as probes. In all species, hybridization to the telomeres of all chromosomes was observed, strongly suggesting that the sequence $(TTAGGG)_n$ is the functional vertebrate telomere.

MATERIALS AND METHODS

Oligodeoxynucleotide Synthesis and Thermal Denaturation Analysis. Oligodeoxynucleotides were synthesized on a Beckman system 1 DNA synthesizer; synthesis was followed by trityl-group removal and purification on a NENsorb prep cartridge by the procedure recommended by the supplier (NEN). Prior to lyophilization, ammonium hydroxide was added (final concentration, 0.5 M) to the purified oligonucleotides. This was required for the deprotonation of cytosine residues in the oligonucleotides, because protonation occurred during the trifluoroacetic acid detritylation step. Following heating to 95°C, oligomers were allowed to hybridize in 0.05 M NaCl; the concentration of each oligodeoxynucleotide was 0.5 A_{260} units/ml. Denaturation was monitored at A_{260} in a Beckman DU-8 spectrophotometer with a t_m (DNA duplex "melting" temperature) analysis system, at a heating rate of 1°C/min.

***In Situ* Hybridization.** A modification of the methods described previously (10, 11) was used for oligomer labeling and *in situ* hybridization. In brief, slides were pretreated with RNase (100 μg/ml) in 2× SSC (1× is 0.15 M NaCl/0.015 M sodium citrate, pH 7) for 1 hr at 37°C, rinsed four times in 2× SSC, and dehydrated through an ethanol series. The air-dried slides were denatured in 70% (vol/vol) formamide in 2× SSC for 2 min at 70°C and dehydrated. The biotinylated probe (0.4 μg of DNA per ml) was prepared in 2× SSC containing *E. coli* carrier DNA (500 μg/ml) and 30% formamide. After 16 hr of incubation at 37°C in a moist chamber, slides were washed first in 2× SSC plus 30% formamide, then in five changes of 2× SSC, all at 40°C.

Chromosomes from at least one species of each vertebrate order studied were hybridized using a "melt" wash protocol to prevent the $(TTAGGG)_n$ sequence from cross-hybridizing to any related telomere sequences. Hybridization was conducted in 0.4 M NaCl/30% formamide at 37°C and was followed by three washes in 0.33 M NaCl (2× SSC or SET; 1× SET is 150 mM NaCl/1 mM EDTA/20 mM Tris, pH 7.8) at 40°C. A final melt wash was conducted in 33 mM NaCl by raising the temperature from 37°C to 50°C at a rate of ≈1°C/min. The 50°C temperature was maintained for 5 min. This protocol has been successful for both Southern DNA blots and *in situ* hybridization. A minor modification of this protocol for Southern blots includes washing the filters in 33 mM NaCl rather than 0.33 M NaCl at 37°C (unpublished results). Fluoresceinated avidin and a single round of amplification with anti-avidin antibody were used to detect hybridization of the probe. Chromosomes were counterstained with propidium iodide (0.5 μg/ml) and 4′,6-diamidino-2-phenylindole (DAPI, 0.06 μg/ml) in antifade solution (11).

RESULTS AND DISCUSSION

In order to use *in situ* hybridization to define the evolutionary origin of the human telomere, synthetic oligodeoxynucleotides were used to determine the sequence dependence for cross-hybridization of the $(TTAGGG)_n$ repeat. We synthesized oligomers of the known telomeric repeats of human, *Tetrahymena*, *Paramecium*, *Oxytricha*, *Saccharomyces*, and

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Table 1. Synthetic repetitive oligodeoxynucleotides

Oligomers	Organism	t_m, °C	Ref(s).
$(GGGTTA)_7 \cdot (TAACCC)_7$	Human	70	10
$(GGGGTT)_7 \cdot (AACCCC)_7$	*Tetrahymena*	74	12, 13
$(GGGTTT)_7 \cdot (AAACCC)_7$	*Paramecium*	72	14
$(GGGGTTTT)_5 \cdot (AAAACCCC)_5$	*Oxytricha*	68	15
$(TGTGTGGG)_5 \cdot (CCCACACA)_5$	*Saccharomyces*	70	16
$(GGGTTTA)_6 \cdot (TAAACCC)_6$	*Arabidopsis*	68	17
$(GGTA)_{10} \cdot (TACC)_{10}$	*Pagurus*	68	18
$(GGGTA)_8 \cdot (TACCC)_8$	*Physarum*	70	19

Oligodeoxynucleotides 40–42 nucleotides long were synthesized, hybridized, and denatured in 50 mM NaCl. The thermal denaturation (duplex "melting") temperature (t_m) is taken at the last linear height increase in hyperchromicity (see Fig. 1).

Arabidopsis, as well as the closely related repetitive sequences $(GGTA)_n$ and $(GGGTA)_n$, reported from the hermit crab (*Pagurus*) and *Physarum* genomes, respectively (Table 1). These oligomers were used in various annealed combinations for thermal denaturation analysis.

As shown in Fig. 1*A*, the human telomere sequence cross-hybridizes with both the *Tetrahymena* and *Paramecium* sequences, even though there is a base mismatch every six nucleotides in these duplexes. In 50 mM NaCl, the human–*Tetrahymena* complex is more stable than the human–*Paramecium* complex, presumably because G–T base mismatches are more stable than T–T base mismatches (Fig. 1*A*; refs. 20 and 21). The *Tetrahymena* sequence has been reported (22) to cross-hybridize to human telomeres. The melting temperature of the human–*Tetrahymena* complex is only 5°C lower than that of the human $(TAACCC)_7 \cdot (GGGTTA)_7$ complex (Fig. 1*A*; Tables 1 and 2). Interestingly, the human telomere sequence $(TAACCC)_7$ also formed complexes with the plant $(GGGTTTA)_6$, yeast $(TGTGTGGG)_5$, and *Oxytricha* $(GGGGTTTT)_5$ telomere sequences, as well as the related repetitive sequences $(GGTA)_{10}$ and $(GGGTA)_8$, although the duplexes were 10–28°C less stable and exhibited lower hyperchromicities than the human–*Paramecium* telomere complexes (Table 2). The ability of these divergent telomere sequences to form stable hydrogen-bonded complexes under physiologically relevant conditions (Table 2) may explain their functional interchangeability as telomeres in yeast artificial chromosomes (23, 24), and as primers for telomerase activity (4).

To define conditions under which the human sequence would not cross-hybridize to other telomeric sequences, thermal denaturations in various concentrations of salt and formamide were conducted with the most stable human–*Tetrahymena* and human–*Paramecium* complexes. It is not expected that other sequences with single base changes from the human $(TTAGGG)_n$ sequence would form more stable mismatched duplexes than these sequences (Fig. 1*A*; refs. 20 and 21). Fig. 1*B* shows one of these conditions, the thermal denaturation of complexes in 0.4 M NaCl/30% formamide. Under these conditions, the cross-species complexes are less stable than in normal salt solutions. The human–*Tetrahymena* complex, in particular, exhibits a dramatic reduction in thermal stability and a decreased hyperchromicity (Fig. 1*B*).

Table 2. Melting temperatures of mismatched oligodeoxynucleotide complexes

Oligomers	Organisms	t_m, °C
$(GGGGTT)_7 \cdot (TAACCC)_7$	*Tetrahymena*–human	65
$(GGGTTT)_7 \cdot (TAACCC)_7$	*Paramecium*–human	60
$(GGGTTTA)_6 \cdot (TAACCC)_7$	*Arabidopsis*–human	50
$(GGGTA)_8 \cdot (TAACCC)_7$	*Physarum*–human	50
$(GGGGTTTT)_5 \cdot (TAACCC)_7$	*Oxytricha*–human	38
$(TGTGTGGG)_5 \cdot (TAACCC)_7$	*Saccharomyces*–human	34
$(GGTA)_{10} \cdot (TAACCC)_7$	*Pagurus*–human	32

When *in situ* hybridization was conducted in 0.4 M NaCl/30% formamide at 37°C, some cross-hybridization of the *Tetrahymena* $(GGGGTT)_7$ sequence was observed on human telomeres (22). The fluorescent signal intensity was, however, considerably weaker and less consistent than the signal obtained with the human sequence $(GGGTTA)_7$. The difference between these hybridization signals was quite apparent, and hence metaphase spreads from 91 different species (Table 3; Fig. 2), including representative orders of bony fish, reptiles, amphibians, birds, and mammals, were hybridized to biotinylated $(GGGTTA)_7 \cdot (TAACCC)_7$ according to this hybridization protocol. A final melt wash, described in *Materials and Methods*, was used for at least one species of each order, to confirm the identity of the $(GGGTTA)_7$ hybridization. None of the other synthetic telomere sequences cross-hybridize to vertebrate telomeres under the melt wash conditions. Representative photomicrographs of hybridization to bird, reptile, amphibian, and fish chromosomes are shown in Fig. 3. In all 91 species (Table 3), hybridization to the telomeres of all chromosomes was observed, regardless of chromosome number or size (Fig. 3).

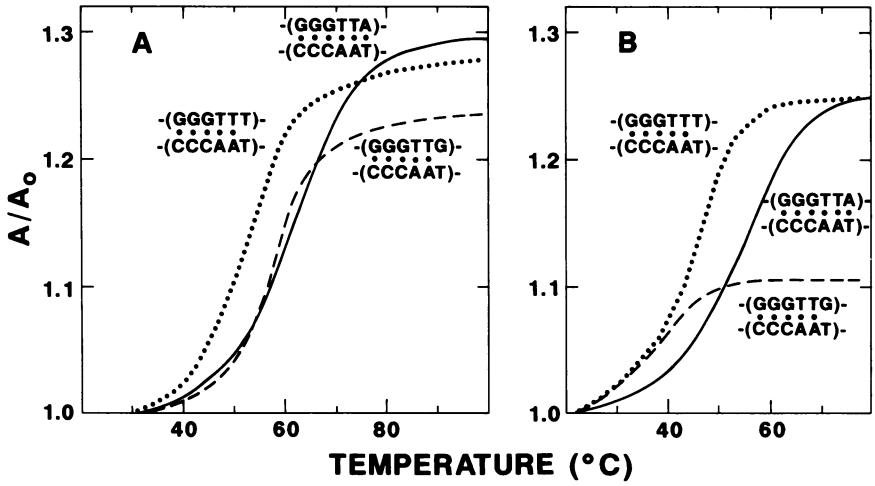

FIG. 1. Thermal hyperchromicity profiles of telomere oligodeoxynucleotide duplexes. Telomere oligodeoxynucleotides were synthesized, hybridized, and denatured as described in *Materials and Methods*. (*A*) Hyperchromicity profiles of $(GGGTTA)_7 \cdot (TAACCC)_7$ (solid line), $(GGGGTT)_7 \cdot (TAACCC)_7$ (dashed line), and $(GGGTTT)_7 \cdot (TAACCC)_7$ (dotted line) complexes in 50 mM NaCl. (*B*) As in *A*, except that thermal denaturation was performed in 0.4 M NaCl/30% formamide.

Table 3. Species used for *in situ* hybridization

Class: Mammalia	(Rodentia, continued)	(Mammalia, continued)
Order: Marsupialia	*Sigmodon fulviventer*	Order: Primates
Macropus rufogriseus	*Acomys spinosissimus*	*Homo sapiens*
Order: Insectivora	*Cricetulus griseus*	*Gorilla gorilla*
Condylura cristata	*Mesocricetus auratus*	*Pongo pygmaeus*
Scapanus latimanus	*Cavia porcellus*	*Pan troglodytes*
Scalopus aquaticus	*Spermophilus mexicanus*	*Pan paniscus*
Order: Edentata	Order: Lagomorpha	*Perodicticus potto*
Myrmecophaga tridactyla	*Sylvilagus aquaticus*	*Lemur macaco rufus*
Order: Chiroptera	Order: Cetacea	Class: Aves
Macrotus waterhousii	*Balaenoptera physalus*	Orders: Columbiformes, Galliformes,
Macrotus californicus	Order: Carnivora	Falconiformes, Passeriformes
Monophyllus redmani	*Ailuropoda melanoleuca*	*Gallus gallus domesticus*
Molossus molossus	*Mellivora capensis*	*Vireo bellii*
Pteronotus maceayii	*Fossa fossa*	*Passer domesticus*
Artibeus jamaicensis	*Prionodon linsang*	*Cyanocitta cristata*
Carollia perspicillata	*Panthera pardus*	*Buteo jamaicensis*
Eumops glaucinus	*Felis pardalis*	*Scarddafella inca*
Chiroderma sp.	*Melogale* sp.	Class: Reptilia
Order: Rodentia	*Mustela frenata*	Orders: Sauria and Serpentia
Mus musculus	*Vulpes vulpes*	*Cnemidophorus sexlineatus*
Mus poschiavinus	*Urocyon cinereoargenteus*	*Cnemidophorus gularis*
Mus dunni	*Nyctereutes procyonoides procyonoides*	*Sceloporus olivaceus*
Peromyscus boylei	*Nyctereutes procyonoides viverrinus*	*Cophosaurus texanus*
Peromyscus leucopus	Order: Pinipedia	*Phrynosoma cornutum*
Peromyscus eremicus	*Phoca vitulina*	*Crotalus* sp.
Reithrodontomys fulvescens	Order: Proboscidea	Class: Amphibia
Reithrodontomys montanus	*Loxodonta africana*	Order: Salientia
Reithrodontomys megalotis	*Elephas maximus*	*Bufo woodhousei fowleri*
Reithrodontomys sumichrasti	Order: Hyracoidea	*Bufo terrestris*
Aethomys chrysophilus	*Procavia capensis*	*Xenopus laevis*
Aethomys namaquensis	Order: Perissodactyla	*Hyla versicolor*
Uromys caudimaculatus	*Equus caballus*	*Hyla squirella*
Microtus agrestis	*Diceros bicornis*	*Hyla chrysoscelis*
Microtus montanus	Order: Artiodactyla	Class: Pisces
Rattus norvegicus	*Cervus timorensis*	Orders: Cypriniformes and Salmoniformes
Rattus rattus	*Gazella spekei*	*Gambusia affinis*
Sigmodon hispidus	*Muntiacus muntjak*	*Eigenmannia virescens*
Sigmodon mascotensis	*Cervus albirostris*	*Eigenmannia* sp.

While the number of species studied (Table 3) is a small percentage of the total number of extant vertebrate species, it represents a diverse range of this subphylum. It is likely, therefore, that if a variety of telomere sequences were utilized by vertebrates, evidence of this variation would have been detected in this study. The human sequence does not cross-hybridize to insect or plant telomeres under stringent hybridization conditions, however (data not shown), indicating that telomere sequence evolution has occurred during animal evolution. The average rates of DNA sequence evolution differ between taxonomic groups, varying between 0.25% and 1.25% per million years (26). The conservation of the $(TTAGGG)_n$ sequence and its telomeric location, in species thought to share a common ancestor over 400 million years ago (Fig. 2), strongly suggest that this molecular "fossil" (2) is the functional vertebrate telomere.

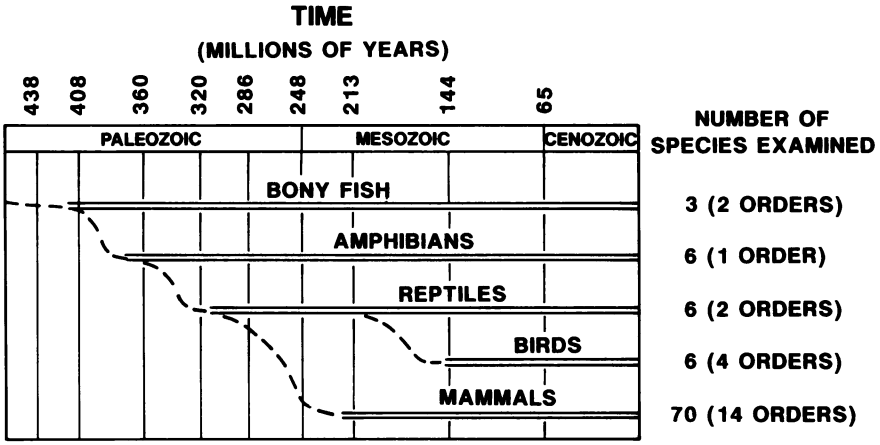

FIG. 2. Temporal distribution of vertebrate classes. The probable relationship among vertebrate classes is diagrammed, modified from ref. 25. The number of species of each major vertebrate group examined in this study is indicated at right. Species names are given in Table 3.

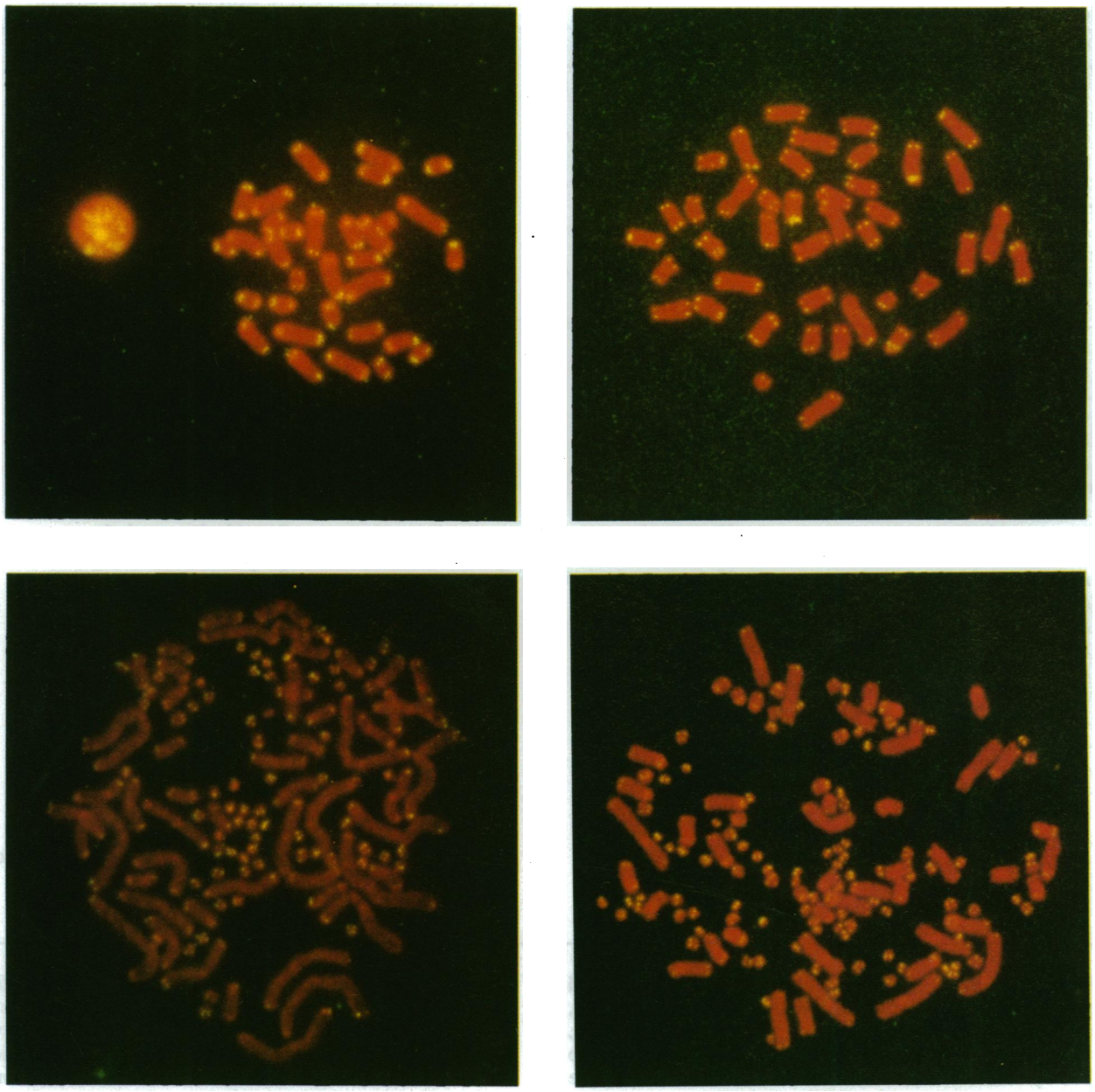

FIG. 3. *In situ* hybridization of biotin-labeled $(GGGTTA)_7$·$(TAACCC)_7$ oligomers to metaphase chromosomes. Shown are representative hybridization patterns observed on chromosomes of *Eigenmannia virescens* (glass knifefish; *Upper Left*), *Xenopus laevis* (African clawed frog; *Upper Right*); *Crotalus* species (rattlesnake; *Lower Left*), and *Vireo bellii* (Bell's vireo; *Lower Right*). Note that even the microchromosomes of reptiles and birds have a hybridization signal at each telomere. The intensity and size of the hybridization signal are not changed by washing with melt wash conditions described in *Materials and Methods*.

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