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In vitro and *in vivo* reconstitution and stability of vertebrate chromosome ends

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ABSTRACT

Telomeres are essential repetitive sequences at the ends of chromosomes that prevent chromosome fusion and degradation. We have successfully recapitulated these two protective functions *in vivo* and *in vitro* by incubating blunt-end DNA constructs having vertebrate telomeric ends in *Xenopus* eggs and egg extracts. Constructs with telomeric ends are stable as linear molecules; constructs with non-telomeric ends undergo intramolecular fusion. In extracts, 99.8% of the telomeric constructs from 78 to 700 bp in length are assembled into 'model telomeres' in <5 min and have an extrapolated half-life of >3.5 years. Non-telomeric constructs circularize with first order kinetics and a half-life of 4 h. In living eggs the telomeric constructs are protected from fusion and degradation. The stability of the telomeric constructs is not due to covalent processing. Extract can protect ~100 pM telomeric ends (equivalent to 1.7×10^7 ends/egg) even in the presence of a 20-fold excess of double-stranded telomeric DNA, suggesting that protection requires end-specific factors. Constructs with $(TTGGGG)_n$ repeats are unstable, suggesting that short tracts of this and other telomere-like sequences found within human telomeres could lead to genome instability if exposed by partial telomere erosion during aging.

INTRODUCTION

Telomeres are structurally and functionally specialized sequences at the ends of eukaryotic chromosomes, usually consisting of a simple tandem repeat (reviewed in 1,2), $(TTAGGG)_n$ in vertebrates. The G-rich strands are invariably oriented 5′→3′ toward the terminus. The terminal structure of telomere DNA is now known for a number of eukaryotes. Short 3′ single-strand extensions have been found in macronuclei of ciliates (3,4) and transiently at the end of S phase in yeast (5). Recently it has been found that human telomeres have 100–200 nt 3′ overhangs throughout most of the mitotic cycle (6–8). Little is known about the nucleoprotein structure of telomeres. *Oxytricha* has tightly bound end-specific proteins, which probably function to protect the ends (9). Yeast and *Tetrahymena* have less stable large end-complexes (10,11). In human, two double-strand-specific telomere binding factors, TRF1 and TRF2, have been isolated and cloned (12–16). The factor XTEF binds *in vitro* to the junction of double- and single-stranded telomere DNA and is a candidate telomere-protective factor in *Xenopus* and humans (17). Unusually compact and regular nucleosome arrays are also present on animal telomeres (18–21).

The significance of telomeres was first discovered by McClintock (22) and Muller (23). In yeast, chromosomes with broken ends are unstable, fusing with sister chromatids to form dicentric chromosomes that undergo a breakage–fusion–bridge cycle during cell division, leading to exonucleolytic degradation, arrest of the cell cycle and chromosome instability (24).Telomeres of human lymphocytes shorten by 20–90 bp/year (25,26) and telomeres of cultured mortal human cells shorten an average of 40–200 bp/cell doubling (27). The fact that primary and immortal human cells have 5′-terminal gaps 100–200 nt long suggests that an endogenous 5′→3′ exonuclease activity might be responsible for the rapid shortening (6). Most human cancer cells and immortal cell lines maintain telomere length, usually coincident with telomerase activity (28,29). Erosion of telomeres during aging of humans and human cell cultures is correlated with increases in the frequency of dicentric chromosomes and genome instability, which could contribute to cancer (30). The telomere hypothesis for cell senescence and transformation states that shortening of telomeres eventually leads to arrest of the cell cycle or chromosome fusion, causing abnormal control of cell growth or chromosome instability (30). Because human cells senesce or die as the average length of the telomeres approaches 1–4 kb (28), the validity of the telomere hypothesis depends on some chromosome ends being significantly shorter than others or on a mechanism whereby chromosome integrity is dependent upon long telomeric tracts. In principle, long telomeres could be necessary for assembly of a stable nucleoprotein 'cap', for correct regulation of genes present in the subtelomeric region or correct chromosome localization in the cell (30).

To understand the molecular mechanisms of telomere stabilization it is important to understand the behavior of telomeric and non-telomeric DNA ends in cells. Manipulation of telomere sequences with mutant telomerase RNA leads to cellular senescence in *Tetrahymena* (31). Transfection of human cells using linear constructs with $(TTAGGG)_n$ ends can seed the formation of new human telomeres, whereas $(TTGGGG)_n$ and other heterologous ends failed (32). Non-telomeric linear DNA

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transfected into mammalian cells integrates at random locations in the genome or undergoes intramolecular and intermolecular end-joining (33). End-joining in cells and extracts is very permissive, capable of joining blunt or even non-compatible overhanging ends. This process is likely to be responsible for the repair of double-strand breaks and for fusion of broken chromosomes. In the only controlled use of end-joining as a functional test of chromosome protection from fusion, linear constructs containing a *Paramecium* telomere tract at one end were injected into *Paramecium* macronuclei (34). Only the telomeric ends were protected from fusion and degradation.

Xenopus eggs and oocytes should be useful to investigate vertebrate telomere structure and function. They and their extracts are capable of gene expression, DNA replication, DNA repair, chromatin assembly, nuclear assembly and cell cycle regulation and should be good sources for activities for telomere protection, because they have 10^4- to 10^6-fold excesses of many nuclear factors (35). Most importantly, egg cytoplasm can efficiently recombine non-homologous DNA ends (36,37). However, earlier attempts to recapitulate telomere function in *Xenopus* have given ambiguous results on the degradation and fusion of telomeric and non-telomeric ends. Artificial constructs with *Tetrahymena* telomeric ends were not protected from degradation in *Xenopus* oocytes or eggs (38–40). In contrast, linear DNA consisting of intact isolated *Tetrahymena* rDNA or constructs passaged through yeast to acquire native yeast telomeres were not degraded in oocytes unless first treated with heat and S1 or *Bal*31, suggesting that unusual DNA structure is necessary for protection (38,39). In the only test of vertebrate telomere ends in *Xenopus*, eggs replicated a small fraction of degraded linear molecules with telomeric tracts of unspecified length having non-telomeric termini, although end-joining was not observed even with non-telomeric constructs (40).

We have studied the assembly of model telomeres in *Xenopus* eggs and extracts using very low concentrations of blunt-end constructs short enough to be studied on sequencing gels yet nearly as short as the shortest viable telomeres in human cell lines (≤500 bp) (41). The behaviors of the constructs mimicked those expected of telomeres. The assembly and stability of the model telomeres were quantified by kinetic analysis. Several hypotheses about the role of covalent processing of the DNA in telomere protection were tested by characterizing the structure of the DNA before and after incubation in the egg extract.

MATERIALS AND METHODS

Construct preparation

Linear DNA constructs are shown in Figure 1. The vertebrate telomeric construct vT_{700}–vT_{700}, with 700 bp telomeric tract at both ends, was constructed from plasmid Sty11, derived from pSp73 (a gift from Dr T.de Lange, with a 800 bp telomeric tract). Sty11 was cut with *Cla*I, dephosphorylated with alkaline phosphatase, cut with *Bgl*II, dimerized with *Escherichia coli* ligase, purified by agarose gel electrophoresis, precipitated and resuspended, digested with *Bal*31 (50 s at 37°C using 3 U enzyme with 3 µg DNA in 100 µl 600 mM NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 20 mM Tris–HCl, pH 8.0, 2 mM EDTA), precipitated and resuspended and digested with mung bean nuclease (20 min at 37°C using 60 U enzyme in 100 µl 50 mM NaCl, 30 mM sodium acetate, pH 4.5, 1 mM $ZnCl_2$, 10% glycerol) to expose blunt

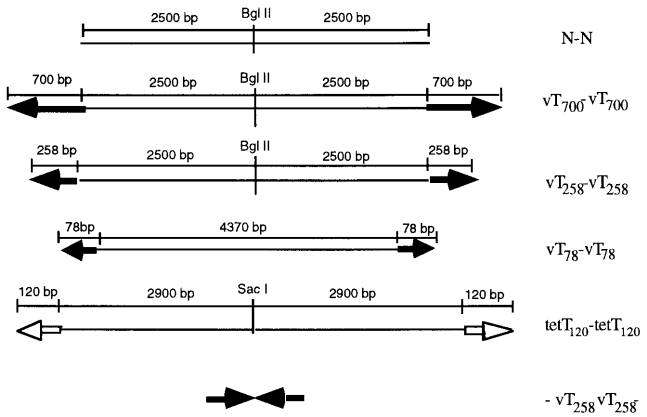

Figure 1. Linear blunt-end DNA constructs. N–N, with non-telomere ends; $tetT_{120}$–$tetT_{120}$, with 120 bp *Tetrahymena* telomere $(TTGGGG)_n$ ends; vT_{700}–vT_{700}, with 700 bp telomere tracts with $(TTAGGG)_n$ ends; vT_{258}–vT_{258}, with 258 bp vertebrate telomere ends; vT_{78}–vT_{78}, with 78 bp vertebrate telomere ends. All were oriented with G-rich 3′ termini. -$vT_{258}vT_{258}$-, fusion construct with internal telomere–telomere junction. Thin line, vector sequence; thick line and arrow, vertebrate telomere sequence; hollow line and arrow, *Tetrahymena* telomere sequence.

telomeric ends. The length of the telomeric tract was estimated by *Eco*RI restriction and electrophoretic analysis. Non-telomere construct N–N was obtained by *Sma*I removal of the telomere tracts from vT_{700}–vT_{700}. The vertebrate telomere construct vT_{258}–vT_{258}, with 258 bp telomere DNA at both ends, was constructed from pHuR93 (purchased from American Type Culture Collection, Rockville, MD). *Nla*IV and *Pst*I restriction released a fragment with a blunt telomeric end (-TTAGGGT), which was purified and ligated to both ends of a non-telomeric Sty11 *Bgl*II–*Pst*I fragment dimerized at the *Bgl*II site. The vertebrate telomere construct vT_{78}–vT_{78}, with 78 bp telomere DNA at both ends, was constructed from pYMtel1 (derived from pGEM by J.Bedoyan and Y.Ming, with a 78 bp telomere tract). pYMtel1 was cut with *Csp*45I, dephosphorylated, cut with *Sac*I, dimerized with *E.coli* ligase and digested with mung bean nuclease. The *Tetrahymena* telomere construct, tetT–tetT, was constructed from pJP 11, a gift from Dr E.Blackburn. A *Sma*I–*Pst*I fragment was dimerized at the *Pst*I site, yielding the construct with a 120 bp TTGGGG repeat at both ends. The fused vertebrate telomere construct, -$vT_{258}vT_{258}$-, was made by dephosphorylating the *Pst*I telomere-containing fragment of pHuR93, restricting with *Nla*IV to expose a blunt telomere end and fusing the blunt ends with T4 ligase producing the junction -TTAGGTACCTAA-. All constructs were gel purified one to two times and quantified by ethidium bromide staining. DNA sequencing showed that vT_{78}–vT_{78} contained a single variant repeat, TTTAGGG, 36 bp from the distal end, and vT_{700}–vT_{700} consisted of perfect TTAGGG repeats over the distal 200 bp but contained a large number of islands of TTGGGG repeats near the proximal end. The vT_{258}–vT_{258} construct consisted of perfect vertebrate repeats.

Xenopus extract preparation

Xenopus laevis was obtained from *Xenopus* I (Ann Arbor, MI). Egg extract was prepared according to Schaal *et al*. (42), with modifications to minimize extract dilution and proteolysis.

Mature females were boosted with 50 U human chorionic gonadotropin 5 days before egg collection and primed with 1000 U 12 h before egg collection. Eggs were collected in Ca^{2+}-free saline, dejellied in 2% cysteine–HCl (pH adjusted to 7.9 with NaOH). Good quality eggs were sorted and treated with 0.5 µg/ml calcium ionophore A-23817 (Sigma) in 100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM HEPES, pH 7.8, 0.1 mM EDTA with 5% Ficoll. Eggs were then washed four times in the same buffer and four times with 90 mM KCl, 30 mM HEPES–KOH, pH 7.8, 10 mM Na-β-glycerophosphate, pH 7.0, 2 mM EGTA, 1 mM DTT. Eggs were centrifuged in a SW-41 rotor at 27 000 r.p.m. for 30 min at 4°C. The supernatant was recentrifuged for 30 min. The second supernatant was collected and made 10 µg/ml in pepstatin A, chymostatin and aprotinin. Final protein concentration was 30–40 mg/ml. Extract was divided into 100 µl aliquots, frozen in liquid nitrogen and stored at –80°C. The extract was evaluated for protein concentration, exonuclease activity and end-joining of non-telomeric DNA ends. Only the batches of extracts with non-detectable exonuclease activity and high ligation efficiency were used. Extracts with the highest ligation activity were isolated in December and used for all experiments described in this paper. Extracts prepared in May had ~3 times lower ligation activity and high exonuclease activity. Several batches of extract were not used because they had little or no ligation activity unless diluted 5- to 10-fold. The telomeric constructs were protected from ligation in all extracts, regardless of the level of exonuclease or ligation activities.

End-joining reactions

Extract aliquots were thawed in hand for 1 min and ATP and Mg^{2+} were added to final concentrations of 1 and 5 mM respectively. End-joining reactions were started by adding constructs at low (50 ng/ml) or high (500 ng/ml) concentration. After mixing for 10–20 s, the zero time point aliquots were withdrawn and quenched by adding 10 vol 20 mM Tris–HCl, pH 7.6, 0.3 M NaCl, 10 mM EDTA, 1% SDS, 1 mg/ml proteinase K and digesting at 37°C for 3 h. The remainder of samples were incubated at 16°C and quenched at different time points. The DNA was extracted twice with phenol/chloroform and ethanol precipitated. Isolated DNA was electrophoresed on 0.8% agarose with 0.5 µg/ml ethidium bromide for 10 h at 5 V/cm. The gel was vacuum blotted and hybridized with radioactively labeled plasmid probe.

Egg injection

Eggs were stabilized in Ca^{2+}-free buffer according to Wangh (43). Stabilized eggs were washed three times in 120 mM NaCl, 7.5 mM KCl, 22.5 mM HEPES, pH 7.4 with NaOH, and then incubated in the same buffer enriched with 400 µM EDTA, 485 µM Mg^{2+}, 114 µM Ca^{2+}. Injection needles were prepared from siliconized glass tubing (Drummond) with an inner diameter of 0.2 mm drawn to a tip with an outer diameter of ~15 µm. An aliquot of 15 nl solution containing 15 pg DNA was injected into the animal pole using a Drummond microinjector. Injected eggs were separately incubated in enriched buffer at 16°C for 10 h. At time points following injection, groups of 10 eggs were rinsed in 150 mM NaCl, 30 mM EDTA, 50 mM Tris–HCl, pH 8.0, homogenized by rapid pipetting and suspended in 800 µl 4.5 M guanidine–HCl, 0.1 M EDTA, 0.15 M NaCl, 0.05% sarkosyl, pH 8.0. Samples were then heated to 65°C for 10 min, vortexed briefly, cooled on ice and precipitated by addition of an equal volume of ethanol. The pellets were then digested in 300 µl 150 mM NaCl, 30 mM EDTA, 50 mM Tris–HCl, pH 8.0, 1% SDS, 250 µg/ml proteinase K at 37°C for 4 h. The DNA was extracted, precipitated, resuspended and electrophoresed as described above.

Ligation assays

To test the ability of once-protected telomeric ends to be religated in extract, vT_{700}–vT_{700} construct was incubated in the extract at 50 ng/ml for 4 h to fully protect the ends, digested with proteinase K/SDS, phenol extracted and ethanol precipitated. The purified DNA was reincubated in fresh extract for different times at 500 ng/ml with a parallel control with fresh construct. To test the ability of the once-protected DNA telomeric ends to be ligated by T4 ligase, 5 ng once-protected and 5 ng fresh vT_{258}–vT_{258} constructs were each ligated with 1 U T4 ligase in 100 µl T4 ligase buffer at 16°C overnight. The products were analyzed by electrophoresis in 0.8% agarose.

Analysis of the lengths of the 3′- and 5′-ends of vT_{78}–vT_{78} after extract incubation

The vT_{78}–vT_{78} construct was incubated in the extract at 16°C for 10 h, purified by proteinase K/SDS digestion, phenol/chloroform extracted and ethanol precipitated. The termini were released with *Eco*RI, electrophoresed in 10% denaturing polyacrylamide, electrotransferred onto Zeta Probe GT membrane in TBE and probed separately for the G-rich strand and C-rich strand using kinase-labeled $(CCCTAA)_4$ and $(TTAGGG)_4$.

RESULTS

Vertebrate telomere ends are specifically protected from fusion *in vitro*

We tested the abilities of *Xenopus* egg extracts to protect telomere, telomere-like and non-telomere DNA ends from fusion using conditions similar to those shown to result in end-to-end fusion of double-stranded DNA in *Xenopus* eggs and extracts (see for example 37,44) Figure 1 shows the four types of symmetrical blunt-ended DNA constructs used in this study, made by restriction of plasmids or T4 ligase treatment of plasmid restriction fragments: (i) N–N, with non-telomeric ends; (ii) vT–vT, with vertebrate telomeric ends, $(TTAGGG)_n$; (iii) $tetT_{120}$–$tetT_{120}$, with *Tetrahymena* telomeric ends, $(TTGGGG)_n$; (iv) -$vT_{258}vT_{258}$-, with an internal telomere–telomere junction. The terminal vertebrate telomeric constructs were made with telomeric tract lengths of 78, 258 and ~700 bp, denoted vT_{78}–vT_{78}, vT_{258}–vT_{258} and vT_{700}–vT_{700}. Each linear construct was incubated at 16°C in the high speed extract prepared from activated *Xenopus* eggs and the construct stability determined by electrophoretic analysis. Low concentrations of construct were used to prevent exceeding the capacities of the extract to join non-telomeric double-stranded ends by non-homologous recombination and to protect telomeric ends from such joining. The low concentrations also prevented concatenation of the DNA so that the solution species were limited primarily to monomeric linear reactants and circular products.

Figure 2 shows the results of the extract incubation experiments. Low concentrations of constructs with non-telomeric ends underwent circularization, first to relaxed and then supercoiled monomeric circles (Fig. 2A). Supercoiling indicates covalent

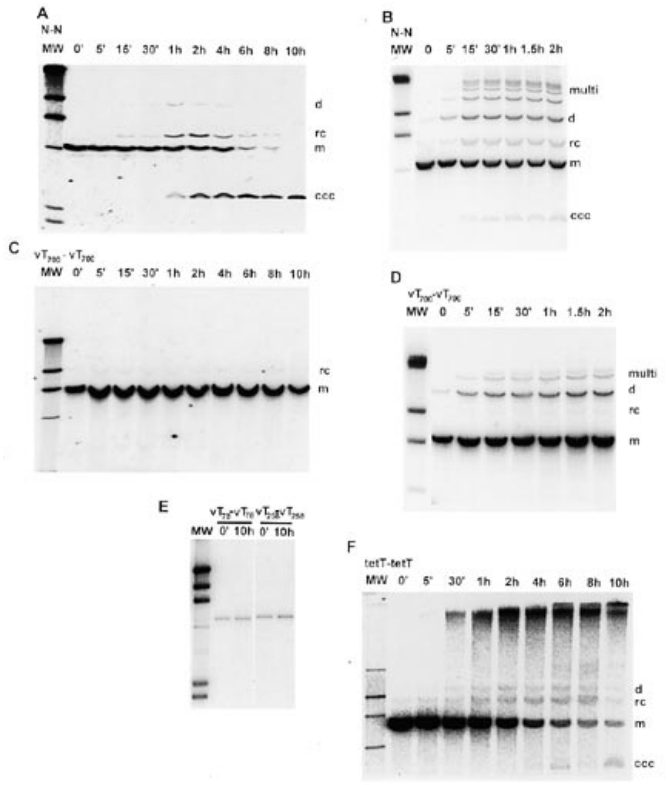

Figure 2. Vertebrate telomere ends specifically resist end-joining *in vitro* at low concentration. Constructs incubated in *Xenopus* extract at low or high concentration for various times and analyzed by electrophoresis. (**A**) N–N construct incubated at 14 pM. (**B**) N–N construct incubated at 140 pM. (**C**) vT_{700}–vT_{700} construct incubated at 12 pM. (**D**) vT_{700}–vT_{700} construct incubated at 120 pM. (**E**) vT_{258}–vT_{258} and vT_{78}–vT_{78} constructs incubated at 12 pM. (**F**) $tetT_{120}$–$tetT_{120}$ construct incubated at 12 pM. MW, λ/*Hin*dIII marker; remaining lanes labeled with time of incubation in extract. multi, multimers; d, dimer; rc, relaxed circle; m, linear monomer; ccc, covalently closed circle.

closure as well as assembly of nucleosomes, which occurs over ~4 h (see for example 45). Concatenation of a small percentage of the constructs occurred at a much slower rate. Higher concentrations of N–N constructs were extensively concatenated in a rapid reaction that slowed considerably after 15 min (Fig. 2B). Low concentrations of vT_{700}–vT_{700} constructs were stable as linear monomers throughout the 10 h assays, except for minor amounts of relaxed circular products produced within the initial 5 min (Fig. 2C). At high concentration, the vT_{700}–vT_{700} constructs reacted to a greater extent for a longer time (Fig. 2D). Paradoxically, the small fraction of vertebrate telomeric constructs that circularized did not supercoil during the 10 h incubations, perhaps because both strands were not ligated. To test whether shorter telomeric tracts could be protected constructs with telomeric tract lengths of 258 and 78 bp were also tested in the extract. Figure 2E shows that the vT_{258}–vT_{258} and vT_{78}–vT_{78} constructs were stringently protected.

To test whether the protection from fusion was highly sequence specific, we also tested the *Tetrahymena* telomeric sequence $(TTGGGGG)_n$, which is frequently found within human chromosomes. Figure 2F shows that the constructs with *Tetrahymena* telomeric ends were unstable, forming circular molecules as well as slowly migrating species of unknown structure, similar to those described before in *Xenopus* eggs, oocytes and extracts, previously attributed to homologous and non-homologous recombination (46–48). The rapid loss of linear, monomeric *Tetrahymena* constructs was reproducible, however, the ratio of slowly migrating to covalently circularized products varied considerably from reaction to reaction. Denaturing electrophoresis showed that most of the DNA in the slowly migrating band had not undergone ligation, suggesting that a stable intermediate or alternative product had formed (data not shown). On the other hand, constructs with one *Tetrahymena* telomeric end and one non-telomeric end underwent covalent circularization but did not form the slowly migrating species (not shown). Thus $(TTGGGG)_n$ terminal repeats were not protected from fusion in the extract.

Several potential artifacts in protection of the telomeric ends were ruled out. The protection of telomeres from both intramolecular and intermolecular ligation (Fig 2C) shows that the protection is not caused by factors that merely increase the persistence length of the DNA to prevent circularization. Intermolecular products are not detectable even after 10 h reactions of the vT_{700}–vT_{700} constructs. To rule out the possibility that vertebrate telomere ends were protected because they 'poisoned' or depleted critical factors for ligation, N–N constructs were added 3 h after incubation was begun with vT_{700}–vT_{700} constructs. The vT_{700}–vT_{700} constructs were protected throughout the reaction, but the N–N constructs were ligated at the same rate and to the same extent as if they had been added to fresh extract, showing that the ligation machinery had remained fully active (not shown). To control for the unlikely possibility that the vT_{700}–vT_{700} constructs were inherently unable to be ligated (e.g. due to damage during preparation), we used T4 ligase to ligate untreated telomeric and non-telomeric DNA in T4 ligase buffer. We found the same rates of reaction of the telomeric and non-telomeric ends (not shown), proving that the telomeric constructs were ligation competent. To exclude possible artifacts due to precipitation, we centrifuged extract reaction mixtures at 12 000 *g* for 20 min, after allowing the end-joining reactions to proceed for 2 h. Neither vT_{700}–vT_{700} nor the N–N constructs nor their ligation products were pelleted (not shown), indicating that the protection of telomere ends had not been caused by inhomogeneities in the reaction mixtures (e.g. removal of vT_{700}–vT_{700} constructs from contact with extract to decrease their reaction or aggregation of the N–N constructs to accelerate their reaction).

We conclude that the egg extracts have activities that mimic fusion of the ends of broken chromosomes and sequence-specific stabilization of the ends with intact vertebrate telomere repeats.

Vertebrate telomeric ends are specifically protected from degradation and fusion *in vivo*

To test the physiological significance of the extract results, 15 pg N–N and vT_{700}–vT_{700} constructs in 15 nl were injected into the animal poles of unactivated living *Xenopus* eggs, incubated for 10 h at 16°C and analyzed by electrophoresis. Figure 3 shows the results. The vertebrate telomeric constructs were reproducibly protected from end-joining and degradation, always remaining linear monomers. N–N constructs were consistently unstable, forming circular monomers, concatenates and degraded species, as observed by others (38–40,49). Different batches of eggs gave different amounts and conformations of the N–N products, however, the reason for the irreproducibility in behavior was not

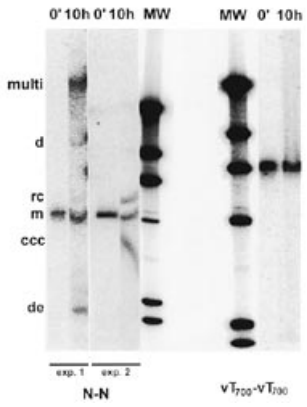

Figure 3. Vertebrate telomere ends resist end joining and degradation *in vivo*. Aliquots of 15 ng N–N or vT_{700}–vT_{700} constructs were injected into *Xenopus* eggs. In some cases, N–N constructs were concatenated and/or degraded (experiment 1 of N–N), in other cases, N–N constructs were joined into circular forms similar to *in vitro* reactions (experiment 2 of N–N). vT_{700}–vT_{700} constructs were consistently stable in several injection experiments. MW, λ/*Hin*dIII marker; remaining lanes labeled with time of incubation in extract. multi, multimers; d, dimer; rc, relaxed circle; m, linear monomer; ccc, covalently closed circle; de, degradation product.

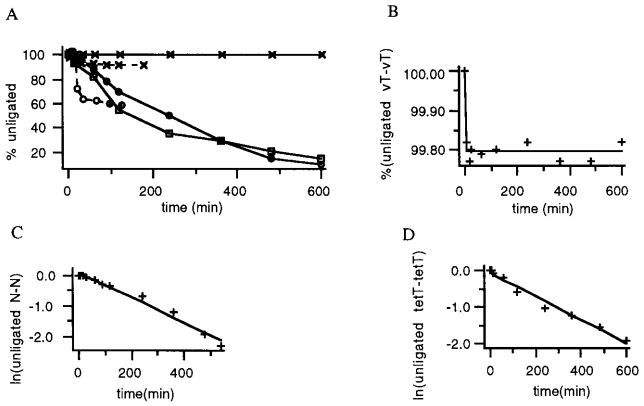

Figure 4. Kinetic analysis of the stability and specificity of telomere protection in *Xenopus* extract. Autoradiograms were quantified with a Molecular Dynamics PhosphorImager. (**A**) Linear plot of vT_{700}–vT_{700} (×), N–N (○) and $tetT_{120}$–$tetT_{120}$ (❑) reactions, showing almost complete stabilization of vT_{700}–vT_{700} constructs and ligation of N–N constructs at 12 pM (solid lines), whereas only partial protection and ligation occurred at 120 pM (dotted lines), accelerated by concatenation. vT_{258}–vT_{258} and vT_{70}–vT_{70} constructs gave identical results. (**B**) An expanded plot of the vT_{700}–vT_{700} reaction, showing the initial reaction and subsequent stability. At 12 pM concentration, vT_{700}–vT_{700}, vT_{258}–vT_{258} and vT_{78}–vT_{78} constructs were 99.8% protected from ligation. (**C** and **D**) Semi-log plots of reactions of N–N and $tetT_{120}$–$tetT_{120}$ constructs showing first order kinetics with $t_{½}$ of 244 ± 12 and 238 ± 10 min respectively.

investigated. Thus the two protective functions of natural telomeres can be recapitulated *in vivo* and the protective activities of the extract are probably physiologically relevant to the protection of the ends of chromosomes from fusion.

Kinetic analyses of the end-joining reactions demonstrate the extreme stability of vertebrate telomere ends and finite capacity of the extract activities

Figure 4 shows the kinetics of end-joining. At low concentrations (24–28 pM ends), non-telomeric and telomere-like constructs reacted to near completion at similar rates (Fig. 4A), whereas vT_{700}–vT_{700} constructs were only joined to a small extent (0.2%) during the first 5 min and were completely stable thereafter (Fig. 4B). Figure 4A also shows that incubation of constructs at high concentrations (240–280 pM ends) gave faster initial reactions due to intermolecular end-joining. The N–N constructs were only partially joined, apparently due to saturation of the ligation activity of the extracts. The vT_{700}–vT_{700} constructs reacted long after 5 min, apparently due to exhaustion of the telomere-protective capacity of the extract.

Kinetic analyses of the reactions at low concentration show that the reactions of the N–N and tetT–tetT constructs could be expressed as an irreversible first order reaction A → B with a rate equation $d[A]/dt = -k[A]$, where $k \approx 4.8 \times 10^{-5}$/s (Fig. 4C and D). Thus *Tetrahymena* telomeric ends were not afforded even partial protection from reaction. The reactions initiated without a noticeable lag phase for assembly of a putative ligation complex and had a fixed rate constant throughout the reaction, showing that the extracts did not lose activity during at least 10 h and that the rate of ligation is independent of the presence of nucleosomes, which accumulate in 4 h (45). The fact that the N–N and tetT–tetT constructs underwent change at the same rates but formed products that were covalently attached and primarily covalently unattached, respectively, suggests that the reaction rate was not limited by ligation but at an earlier step.

We can estimate the rate constants for the assembly and fusion of the protected telomeres. Because the telomeres are fully protected by 5 min, an apparent first order rate constant for assembly of the protected ends is $>7 \times 10^{-3}$/s. Assuming that the telomeric ends have become fully protected by 5 min, a linear regression analysis of the data from 5 min to 10 h in Figure 4B indicates an apparent rate constant for fusion of the protected vT–vT constructs of $3.3 \times 10^{-10} \pm 6 \times 10^{-9}$/s. Thus the best estimate for the apparent half-life of the vertebrate telomere ends is 66 years. However, given the large standard deviation of the measurements, we prefer the more conservative limit to the half-life of >3.5 years. This is >8000 times more stable than the non-telomeric ends. The rates and extents of the reactions of telomeric, telomere-like and non-telomeric constructs were highly reproducible using the same batch of extract and very similar using extracts that did not have strong exonuclease activities. On the basis of the specific and quantitative protection of the vertebrate telomere ends from end-joining, which recapitulates the primary function *in vivo* of protecting ends of chromosomes from fusion, we describe the stabilized telomeric constructs as 'model telomeres'.

Model telomeres are not protected by covalent processing of the DNA ends

There are two possible ways to protect the termini of nucleic acids: (i) covalent modification, used at the ends of mRNA and certain linear viral DNA; (ii) protein binding, used to protect ciliate telomeres; (iii) a combination of the two. Figure 5 illustrates these protective schemes, including four specific hypotheses about possible covalent modifications: (i) cleavage of

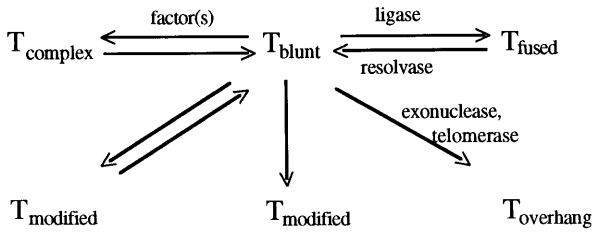

Figure 5. Schematic representation of the *Xenopus* extract reactions that might protect blunt telomeric ends from fusion. Clockwise: resolution of fused telomere junctions at a much faster rate than the ligation reaction, leading to a low steady-state level of fused ends; exonucleolytic creation (and/or telomerase addition) of single-strand overhangs that are inherently unligatable or able to bind to single-strand factors; irreversible modification to the DNA; reversible modification to the DNA; reversible binding of factors to the blunt ends.

telomere–telomere junctions formed by end-joining; (ii) formation of a 3′ single-strand overhang able to form G quartet secondary structure or bind specific proteins; (iii) irreversible covalent blockage of the telomeric DNA ends; (iv) reversible covalent blockage of the telomeric DNA ends. In principle, any of these processes could be necessary and/or sufficient to protect telomeres from fusion. We tested the four hypotheses of covalent modification and found them inadequate to explain telomere protection in *Xenopus* extracts.

If the presence of 0.2% circular vT–vT molecules in our experiments reflects a steady-state between the known end-joining activity and a putative resolvase activity, the expected half-life for resolution would be 5000 times less than that for ligation, namely 3 s. Thus this hypothesis predicts that telomere–telomere junctions would be rapidly resolved in the extracts. To test this hypothesis the -$vT_{258}vT_{258}$- fusion construct was incubated in *Xenopus* egg extract for 4 h, isolated and analyzed by electrophoresis (Fig. 6A). The inverted telomeric junction was stable, showing that cleavage of telomeric junctions cannot be responsible for the stability of telomeric ends in the extract.

In principle, telomeres could be sequestered from the end-joining machinery by creation of G-rich single-strand overhangs, which might form unusual secondary structures, such as quadruplex, triplex and duplex structures stabilized by G:G hydrogen bonding (reviewed in 50) and/or bind specific proteins. Oocytes (and potentially eggs) contain strong 5′→3′ exonuclease activities (49) and it has been speculated that such an exonuclease might produce the G-rich telomere overhangs found in *Saccharomyces cerevisiae* and human cells (6,51). In addition, telomerase activity is found in *Xenopus* extracts (52) and is capable of extending or degrading 3′ telomeric overhangs *in vitro* (53,54). Therefore, it is important to test whether the ends of our constructs have been degraded or extended.

Figure 6B shows the lengths of the C-rich and G-rich strands of the vT_{78}–vT_{78} constructs before and after 10 h incubation in the extract. The C-rich and G-rich telomeric fragments are expected to have lengths of 100 and 104 bases, respectively, and the C-rich strands have anomalously high mobility in this and other constructs (not shown). Almost 20% of the C-rich strands were ~3 bases shorter than expected, perhaps caused by the mung bean digestion. The identical length distributions of both strands before and after incubation in the extract indicate a lack of net processing by nuclease or polymerase. The lack of overhang on

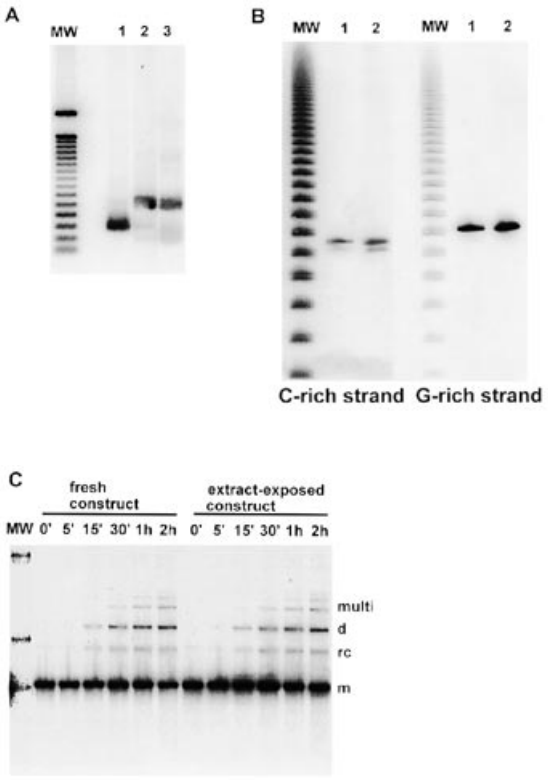

Figure 6. There is no covalent processing of telomeric DNA ends in *Xenopus* egg extract. (**A**) Autoradiogram showing that telomere–telomere junctions are stable in extract. MW, 100 bp ladder; lane 1, monomeric 278 bp *Nla*IV–*Pst*I fragment of pHuR93 with one 258 bp telomeric end; lane 2, 556 bp fusion construct, -$vT_{258}vV_{258}$-; lane 3, -$vT_{258}vV_{258}$- sample after 4 h extract incubation, showing lack of cleavage at the telomere–telomere junction to produce monomers. (Note that the original fusion construct contained ~6% shorter fragments.) (**B**) The lengths of the G-rich and C-rich telomeric ends are not altered in extract. The vT_{78}–vT_{78} construct was incubated for 10 h in extract, extracted, cleaved with *Eco*RI, electrophoresed in 10% denaturing acrylamide and electrotransferred to a filter. (Left) Filter hybridized with random primed marker and kinase-labeled $(TTAGGG)_4$; (right) same filter hybridized with labeled marker and $(CCCTAA)_4$. Lane MW, 10 bp ladder; lane 1, construct without incubation; lane 2, construct after incubation. (**C**) Reincubation experiment showing that fresh and extract-exposed telomeric ends are partially protected and partially ligated to the same extents at high concentration (120 pM). MW, λ/*Hin*dIII marker; remaining lanes are labeled with extract incubation times. multi, multimers; d, dimer; rc, relaxed circle; m, linear monomer.

protected ends suggests that single-stranded G-rich DNA is not necessary for protection of telomere ends. Using the same tests, we did not find degradation of either end of the non-telomeric constructs (data not shown), showing that exposure of a single-strand is not required for the end-joining reaction.

To detect any irreversible protection of the DNA from ligation, we tested whether telomeric ends that were protected during one extract incubation were automatically protected during a subsequent extract experiment. To prepare completely protected telomere ends, vT_{700}–vT_{700} constructs were incubated in the egg extract at low concentration (24 pM ends, as in Figs 2C and 4B). The model telomeres were then digested with proteinase K/SDS, phenol extracted and separated by electrophoresis. These once-protected linear molecules and fresh vT_{700}–vT_{700} constructs were separately incubated at high concentration (240 pM ends) in the egg extract. As expected at high concentration (see Fig. 4A),

the fresh constructs were partially ligated and partially protected (results shown in Fig. 6C). The phenol-extracted once-protected DNA was ligated to the same extent as the fresh DNA, showing that the telomeric ends had no memory of the protected state, although the reincubation experiment does not rule out the possibility that the DNA was reversibly modified by the extract.

As a final test, sensitive to both irreversible and reversible modifications to the DNA in the extract, the inherent ligatability of the extract-treated telomere ends was tested outside the extract using purified components. T4 ligase was used to join DNA isolated from the fresh and once-protected vT_{700}–vT_{700} constructs. The ligase was able to join both types of constructs to the same extent (not shown). Thus the constructs were inherently ligatable before and after incubation in the extract, indicating the retention of blunt ends with accessible 3′-OH and 5′-P termini.

Because all four hypotheses about covalent processing tested false, we conclude that covalent modification of the blunt telomeric ends is not necessary for protection of telomeric ends from the extract ligation activities.

Model telomere protection is saturable and end specific

Exclusion of all the possible mechanisms whereby the model telomeres could be protected by covalent modifications leads us to test the last hypothesis in Figure 5, that factor binding is responsible for protection. If terminus binding factors are responsible for telomere protection there should be a limit to the number of ends that can be protected, with little competition from excess amounts of double-stranded telomere DNA.

Figure 7A shows that stepwise addition of vT_{258}–vT_{258} and vT_{700}–vT_{700} constructs saturated the protective activities, suggesting a limiting amount of protective factor. The constructs were ~99.8% stable to a concentration of 50 pM construct (100 pM ends). At higher concentrations, the telomeres were only partially protected. The extract was able to ligate non-telomeric ends at the same rate throughout the entire concentration range of vT–vT constructs, confirming that the telomeric ends did not compromise ligase activity (data not shown). These results suggest that it is unlikely that protection is merely due to formation of unusual DNA secondary structure at the ends and suggest a requirement for factors that bind to the end. Assuming a volume of 1 µl extract/egg, the saturation results suggest that one egg has the capacity to tightly protect at least 1.7×10^7 telomeric ends. This is more than sufficient to protect all the chromosomes even at the mid-blastula transition, when the embryos are expected to have ~4×10^5 telomeres. Some batches of extract have a protective capacity about twice as large.

To test the ability of larger amounts of bulk double-stranded telomeric DNA to compete for the protective factors, extracts were preincubated for 1 h at 16°C with 1 nM intact Sty11 plasmid before adding 12 pM vT_{700}–vT_{700}. This amount of bulk telomeric DNA was 20 times more than the maximum amounts protected in Figure 7A and 83 times more than the amount of vT_{700}–vT_{700} in the reaction. Figure 7B shows that the telomeric ends were totally protected from ligation. The same result was found for vT_{258}–vT_{258} (data not shown). Addition of competitor reduced the efficiency of ligation of the N–N construct 2- to 3-fold (data not shown). Addition of a 330 times excess of Sty11 competitor enabled partial ligation of vT_{700}–vT_{700} as well as exonucleolytic degradation of both the N–N and vT_{700}–vT_{700} constructs (data not shown). These data suggest that telomere protection is limited by end binding factors, although great excesses of double-stranded telomeric DNA interfere with telomere protection from ligation and nucleases. Thus we cannot rule out participation of double-strand binding factors present in greater excess or factors that bind to double-stranded $(TTAGGG)_n$ more weakly than to telomere ends. Taken together with the fact that double-stranded telomeric tracts as small as 78 bp are stringently protected, these results suggest that end-specific factors are required for protection of the telomeres in the *Xenopus* egg extract.

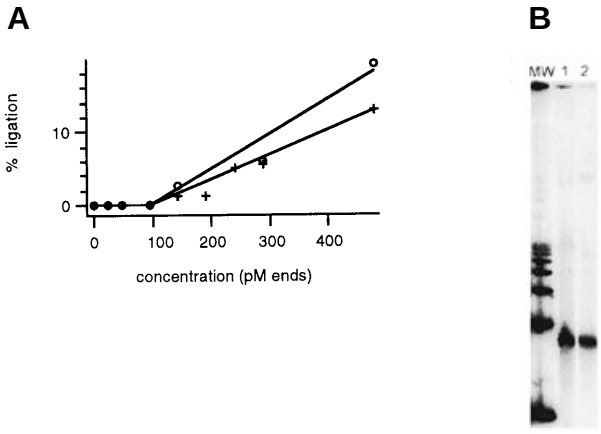

Figure 7. The telomere protective activity of the extracts is saturable and end specific. (**A**) Stepwise addition of vT_{700}–vT_{700} (+) or vT_{258}–vT_{258} (○) constructs to 24, 48, 96, 144, 192, 288 and 480 pM ends saturated the protective activities at the same molarity of telomere ends (~100 pM) independent of length. Constructs were added stepwise to the extracts at 15 min intervals and aliquots removed and incubated for an additional 9 h at 16°C. Non-telomeric ligation activity was shown to be constant in each aliquot, by adding 12 pM N–N to each 9 h incubation (not shown). (**B**) Competition of bulk double-stranded telomeric DNA for protective factors. Ligation reactions were done on 12 pM construct, without and with addition of Sty11 plasmid competitor before the reaction. Lane 1, vT_{700}–vT_{700} without competitor; lane 2, vT_{700}–vT_{700} with 1 nM competitor.

DISCUSSION

This report is the first documented recapitulation of vertebrate telomere protection *in vitro*. It is difficult to compare most earlier telomere studies in *Xenopus* with our results, because the high construct concentrations (0.6–320 nM) (38–40) were probably beyond the end-joining capacities (280 pM ends in our extracts) and well beyond the capacity to protect vertebrate telomeric ends (100 pM ends in our extracts). The high concentrations of constructs could explain previous observations of partially protected yeast telomeres, poorly ligated non-telomeric ends and DNA degradation. The single exception is the study by Berg and Gall (48), showing that *Tetrahymena* telomeres at a low concentration (15–45 pM) readily fuse, in agreement with our results. Our results are consistent with the successes of transfection experiments using constructs with one vertebrate telomeric end to seed the formation of new human telomeres and failure using heterologous ends (32).

The inherent advantages of the *Xenopus* egg and egg extract systems are that: (i) the chromosome ends can be assembled and studied under conditions with excess amounts of stoichiometric

and catalytic factors; (ii) the two protective telomere functions can be studied independent of the broader roles of telomeres in replication, cell cycle control and chromosome localization; (iii) the structure of the DNA constructs can be manipulated to study the role of DNA in telomere function; (iv) the molecular events of DNA processing, nucleoprotein assembly and protection can be studied with high spatial and temporal resolution. The use of well-defined DNA substrates circumvents a major problem in studying vertebrate telomeres, namely the inherently long and heterogeneous length. However, there are also limitations to the interpretation of our results, which are shared by studies of replication, repair, transcription and chromatin assembly in extracts. First, the extracts represent a unique stage in the cell cycle of a gamete and might not reflect activities at other stages in the cell cycle or in somatic cells. Second, the nucleoproteins have been assembled on pre-existing DNA rather than during replication. Third, different batches of extract can have different properties. Fourth, the primary or secondary structure of the DNA introduced into the extract might be different than that present *in vivo*. The first two limitations are difficult to address, because there are insufficient numbers of frog nuclei to determine the structure or function of the egg telomeres and no efficient means to replicate the constructs in the extracts. The third limitation is addressed in Materials and Methods.

The fourth limitation is relevant, because the primary and secondary structures of the DNA at the ends of vertebrate chromosomes are not completely known. If end replication is only incomplete during lagging strand synthesis, then 50% of the termini should be blunt in cells lacking telomerase activity. Yeast have long 3′ overhangs at the end of S phase, which later heal to form blunt ends or very short overhangs (5). We have found that >80% of human telomeres have G-rich overhangs, averaging 100–200 nt (6), although others report overhangs on only half of the ends (8). Even if most vertebrate telomere ends have 3′ overhangs, the protection of blunt ends might have physiological relevance in those cases where a few ends are blunt, at certain stages in the cell cycle when many ends are blunt or as a back-up mechanism to protect telomeres that have undergone incomplete processing, recombination or breakage. It is also possible that our model telomeres became single-stranded in the extract due to binding of telomere-specific single-strand binding proteins or helicases (e.g. Ku or a homolog of PIF1, which is implicated in maintenance of yeast telomeres) (55,56).

In contrast to the uncertainty about the native structure of telomere DNA and whether our constructs have adopted that structure, we are confident that a primary function of telomeres is to protect chromosome ends from fusion and that we have recapitulated that function in *Xenopus* eggs and extracts. In eggs the telomeric ends are also protected from unknown activities that sometimes degrade non-telomeric ends. The characteristics of the protection from fusion agree with reasonable expectations of natural telomeres. The protection is very rapid (<5 min), complete (99.8%) and persistent ($t_{½}$ > 3.5 years). The protection is not afforded to the telomere-like sequence $(TTGGGG)_n$, which has been shown to be non-functional in human cells (32). More than 10^7 telomeric ends can be protected in the extract from one egg, which agrees with the expectation that the eggs store 10^4- to 10^6-fold excesses of factors for early development. While the rates and extents of the fusion and protection reactions in the extracts cannot be extrapolated to the situation *in vivo*, we feel it is justified to call the nucleoproteins that are assembled model telomeres. To the extent that the behavior of the model telomeres resembles the activities of telomeres in chromosomes, our results can be used to test hypotheses about the roles of telomere length, sequence, covalent and non-covalent DNA structure and cellular factors in protection of chromosomes from fusion.

The extracts were used to test a number of hypotheses about the role of covalent processing in protection of vertebrate telomeres. The inability of the extract to cleave telomere–telomere junctions suggests that vertebrate cells do not have a mechanism to resolve such junctions once formed. The inability to detect any covalent modifications to the telomeric ends that inhibit telomere joining by the permissive endogenous *Xenopus* end-joining activity, or by the stringent T4 ligase activity, suggests that telomere protection does not require a covalent 'cap' at the end of vertebrate telomere DNA. The demonstrations that a reversible or irreversible covalent cap is not formed in the *Xenopus* egg extracts and that the protective activity is saturated by excess telomere ends suggest that protection is mediated by factors bound to the termini.

The factors that protect telomeres from recombination are still uncertain. The findings that the protective capacity of the extracts extends to telomeric tracts as short as 78 bp suggests that the factors can form small end complexes and thus do not depend upon the telomere-specific nucleosome arrays. Because it is reasonable that telomere protection involves blocking both the 3′- and 5′-termini from ligation, we expect that the protective factor or factors will be able to bind to double-stranded telomeric sequences. TRF1 and TRF2 are two double-strand telomere proteins potentially involved in protecting the ends of chromosomes from fusion. By itself, TRF1 has no apparent preference for a terminus, whereas the limiting factor in the extracts seems to bind to bulk telomeric tracts less strongly than termini. Expression of a dominant negative mutant of TRF1 does not lead to noticeable chromosome instability, suggesting that TRF1 is not required for protection of chromosome ends from fusion (57). Recently a dominant negative mutant of TRF2 has been found to arrest growth and cause chromosome fusion, indicating a direct or indirect role in chromosome capping (58).

Our finding that $(TTGGGG)_n$ ends are unable to resist fusion in *Xenopus* extract suggests that vertebrate chromosome function is very specific for the vertebrate telomeric sequence. Because the critical length for vertebrate telomere protection is <78 bp, even short tracts of non-homologous telomere sequences, as found embedded in the centromere proximal portion of human telomeres (59,60), might interfere with telomere function. We suggest that exposure of such sequences during aging followed by end-joining could be key mechanistic steps in age-related chromosome instabilities, such as dicentric chromosomes (61), which recent speculation has linked with carcinogenesis and senescence (30). These results suggest a plausible explanation for the observation that proliferative senescence and crisis occur in tissue culture cells still possessing 1–4 kb of telomeric DNA on the ends of chromosomes and that terminal telomere sequence might be more important than telomere length in protecting chromosomes from fusion.

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