

A *Xenopus* egg factor with DNA-binding properties characteristic of terminus-specific telomeric proteins

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We have identified a *Xenopus laevis* protein factor that specifically recognizes vertebrate telomeric repeats at DNA ends. This factor, called *Xenopus* telomere end factor (XTEF), is detected predominantly in extracts of *Xenopus* eggs and ovaries, which are estimated to contain sufficient XTEF to bind $\sim 3 \times 10^7$ DNA ends. In contrast, XTEF is much less abundant (~ 90 per cell) in extracts of somatic cell nuclei. Mobility retardation analysis of the XTEF activity in egg extracts indicates that this factor binds the vertebrate telomeric repeat sequence (TTAGGG)₂ when present in a single-stranded 3' overhang. Single-stranded 3' extensions of (TTTGGG)₂, (AAAGGG)₂, (TTACCC)₂, or a nonrepetitive sequence fail to bind XTEF efficiently, whereas changes in the double-stranded sequence 5' to the TTAGGG repeat tail are tolerated. TTAGGG repeats are not recognized at internal positions, at a 5' protruding end, or in double-stranded DNA. In addition, the factor does not bind RNA with single-stranded UUAGGG repeats at a 3' end. XTEF-DNA complexes form and are stable in high salt. The DNA-binding properties of XTEF resemble the characteristics of a class of terminus-specific telomere proteins identified previously in hypotrichous ciliates.

[Key Words: *Xenopus* egg; single-stranded DNA-binding activity; telomeric DNA; vertebrate telomeres]

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Telomeres have at least two activities important for the stability of eukaryotic chromosomes (for review, see Zakian 1989; Blackburn 1991). First, telomeres must provide a special strategy for the complete duplication of linear genomes, because replication of linear DNA by conventional DNA polymerases will result in progressive loss of terminal sequences (Watson 1972). Such gradual decline is directly manifested by the ends of broken chromosomes in *Drosophila* (Biessman and Mason 1988; Levis 1989). In contrast, natural chromosome ends are usually stable. Second, telomeres protect chromosome ends from irreversible ligation. Unlike intact telomeres, broken chromosome ends have a strong tendency to fuse (McClintock 1941), perhaps because uncapped DNA ends are subject to DNA repair.

The telomeres of divergent eukaryotes, such as protozoa, plants, fungi, and vertebrates contain similar short tandem DNA repeats, which usually have clusters of guanine residues in the 3' strand. Telomeric repeats are synthesized at least in part by telomerase, a ribonucleoprotein enzyme with a short RNA template that directs the addition of G-rich repeats to the 3' ends of chromosomes (for review, see Blackburn 1992). This elongation reaction presumably balances the gradual loss of termi-

nal sequences that accompanies chromosomal DNA replication.

Telomeric DNA is thought to associate with telomere-specific factors to form a protective nucleoprotein complex. The chromatin structure of chromosome ends in ciliates and *Saccharomyces cerevisiae* bears evidence of a large telomeric nucleoprotein complex (Blackburn and Chiou 1981; Gottschling and Cech 1984; Wright et al. 1992), and a disruption of the telomeric nucleoprotein complex has been proposed to be responsible for the deleterious phenotype of mutated telomeric repeats in *Tetrahymena* (Yu et al. 1990). At present, two classes of telomere-binding proteins have been identified. The first class, exemplified by RAP1 protein in budding yeast, consists of factors that bind double-stranded telomeric repeats and do not require the proximity of a DNA end (Berman et al. 1986; Buchman et al. 1988; Longtine et al. 1989; Conrad et al. 1990; Lustig et al. 1990; Sussel and Shore 1991; Kyriou et al. 1992). Additional candidates for this class of telomere proteins have been identified in slime molds and mammals (Coren et al. 1991; Zhong et al. 1992).

There is a second class of telomere proteins, whose members specifically bind telomere termini. In the hypotrichous ciliates *Oxytricha* and *Euplotes*, such factors encapsulate the abundant telomeres in macronuclear DNA and protect DNA ends from exonuclease action (Lipps et al. 1982; Gottschling and Cech 1984;

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Gottschling and Zakian 1986; Price 1990). These telomeric proteins associate with the single-stranded telomeric TTTTGGGG repeats that protrude 3' from the chromosome ends in these ciliates, resulting in a tenacious noncovalent complex that is resistant to high salt (Klobutcher et al. 1981; Gottschling and Zakian 1986; Price and Cech 1987; Raghuraman et al. 1989; Price 1990). Although this terminal complex appears to be a conserved feature of telomere termini in hypotrichous ciliates (Price 1990; Fang and Cech 1991; Wang et al. 1992), telomere capping factors have not been demonstrated in other eukaryotes.

The telomeres of all vertebrate animals, including mammals, reptiles, amphibia, birds, and fish, contain long arrays of tandem TTAGGG repeats in the DNA strand, which runs 3' to the chromosome end (Moyzis et al. 1988; Meyne et al. 1989; Brown et al. 1990; de Lange et al. 1990). These repeats are considered sufficient for telomere function in vertebrate cells on the basis of their involvement in telomere synthesis in mammalian cells (Morin 1989, 1991; Wilkie et al. 1990; Farr et al. 1991) and on the basis of the conservation of telomeric DNA. Although the protein components of vertebrate telomeres are as yet undetermined, preliminary observations point to the presence of a specialized nucleoprotein complex. In human interphase nuclei, the telomeric TTAGGG repeats are attached to the nuclear matrix, suggesting an interaction with nonhistone proteins (de Lange 1992). In addition, we have identified a nuclear factor (TRF) in mammalian cell lines that specifically binds long arrays of double-stranded TTAGGG repeats independent of the proximity of a DNA end (Zhong et al. 1992). Here, we extend our search for vertebrate telomere proteins to factors that interact with telomere ter-

mini. We describe a factor from *Xenopus laevis* eggs that binds in vitro to terminal TTAGGG repeats in a 3' overhang.

Results

Identification of XTEF in *Xenopus* egg extracts

On the assumption that the terminal structure of vertebrate telomeres features a 3' extension, we synthesized a DNA probe [putative telomere end (PTE); see Table 1] with two double-stranded TTAGGG repeats and a 3' protrusion of the sequence (TTAGGG)₂. At the other end, PTE contains a non-TTAGGG duplex region for proper alignment and a 3' gap to allow labeling with Klenow enzyme. Using PTE as a probe and S-100 extract from *Xenopus* eggs as a source of DNA-binding factors, we detect a specific electrophoretic mobility retardation complex that is competed by a 50-fold molar excess of unlabeled PTE (Fig. 1). We refer to this activity as *Xenopus* telomere end factor (XTEF). The XTEF complex is not diminished by the addition of 5 µg of several types of nonspecific competitor DNAs, including double-stranded and single-stranded *Escherichia coli* DNA, HeLa cytoplasmic RNA, and yeast tRNA (data not shown). No PTE-binding activity is detected when the reactions are treated with proteinase K prior to gel electrophoresis (data not shown), indicating that XTEF contains a proteinaceous component.

In addition to a specific complex, PTE and other probes used in this study form several complexes that result from nonspecific interactions. These additional complexes are not affected by the addition of a 50-fold molar excess of unlabeled PTE (Fig. 1), and their appearance is

Table 1. XTEF binds DNAs with TTAGGG repeats in a 3' overhang

	DNA ^a	Complex ^b	Competition ^c
Putative telomere end	AAAAC TCGACTTAGGGTTAGGGTTAGGGTTAGGG 3' * * * TGAGCTGAATCCCAATCCC	+	+
Random duplex	AAAAC TCGACTAGTGCATCGACTTAGGGTTAGGG 3' * * * TGAGCTGATCACGTAGCTG	+	+
(GGGTTA) ₂ tail	AAAAC TCGACTTAGGGTTAGGGGGTTAGGGTTA 3' * * * TGAGCTGAATCCCAATCCC	+	+
(AAAGGG) ₂ tail	AAAAC TCGACTTAGGGTTAGGGAAAGGGAAAGGG 3' * * * TGAGCTGAATCCCAATCCC	±	±
(TTGGG) ₂ tail	AAAAC TCGACTTAGGGTTAGGGTTTGGGTTTGGG 3' * * * TGAGCTGAATCCCAATCCC	±	±
(TTACCC) ₂ tail	AAAAC TCGACTTAGGGTTAGGGTTACCCTTACCC 3' * * * TGAGCTGAATCCCAATCCC	-	-
Random tail	AAAAC TCGACTTAGGGTTAGGGTAGTGCATCGAC 3' * * * TGAGCTGAATCCCAATCCC	-	-
Duplex (TTAGGG) ₄	AAAAC TCGACTTAGGGTTAGGGTTAGGGTTAGGG 3' * * * TGAGCTGAATCCCAATCCCAATCCCAATCCC	-	-

^a(*) Positions of radiolabeled thymidine residues in DNA probes.

^bDNA probes were tested for complex formation with XTEF by electrophoretic mobility shift assay (see Figs. 2 and 3). (+) Efficient XTEF complex formation; (±) severely reduced complex formation with XTEF; (-) no detectable complex formed.

^cFor competition analysis, unlabeled DNAs were tested for their ability to compete with the putative telomere end probe for complex formation with XTEF. (+) <5% residual complex at 50-fold molar ratio competitor/probe; (±) 20–50% residual complex at 50-fold molar ratio competitor/probe; (-) >50% residual complex at 50-fold molar ratio competitor/probe.

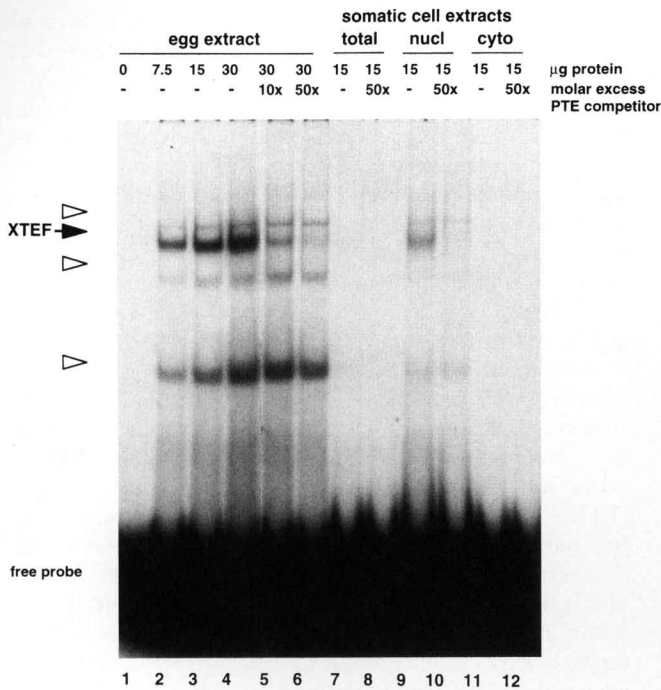


Figure 1. Identification of XTEF in *Xenopus* egg extracts. Electrophoretic mobility retardation assays (see Materials and methods) with labeled putative telomere end DNA (PTE; see Table 1) and increasing amounts of *Xenopus* egg extract (S-100, lanes 2–6) and total (lanes 7,8), nuclear (lanes 9,10), and cytoplasmic (S-100, lanes 11,12) extracts of somatic (whole blood) cells. The amounts of protein assayed are indicated above the lanes. Lanes 5, 6, 8, 10, and 12 contain reactions in the presence of unlabeled PTE DNA in molar excess to the probe as indicated above the lanes. Lanes 2–4, 7, 9, and 11 contain reactions without added unlabeled PTE DNA. The position of the free probe, nonspecific complexes (open arrowheads), and XTEF (arrow) are indicated.

variable and dependent on the amount and nature of the nonspecific competitor DNA included in the assay.

Because *Xenopus* oocytes contain significant amounts of nuclease activity (Maryon and Carroll 1989), we were concerned about the nature of the DNA bound by XTEF. To examine this question, DNA-binding reactions were performed with a PTE probe that was 5'-end-labeled in both strands. When the DNA from the XTEF complex was extracted and analyzed by denaturing gel electrophoresis, both the C-rich and G-rich strands were present in about equal amounts (data not shown). This indicates that XTEF does bind intact PTE DNA bearing the putative telomere end structure.

Although readily detected in extracts from *Xenopus* eggs (Fig. 1) and ovaries (data not shown), XTEF activity is not apparent in whole-cell or cytoplasmic (S-100) extracts from a variety of somatic frog tissues, including heart, kidney, and blood (Fig. 1; data not shown). Weak XTEF activity, however, can be demonstrated in the nuclear protein fraction from blood, heart, and kidney cells (Fig. 1; data not shown). The specific activity of XTEF is approximately fivefold lower in nuclear extracts of somatic cells as compared with egg extracts.

We estimate that one *Xenopus* egg yields ~3.5 µg of protein in our extraction procedure. On the basis of quantitation of the experiment shown in Figure 1 and other similar experiments, we deduce that the XTEF activity in 3.5 µg of egg extract protein is sufficient to bind 0.05% of 0.1 pmole of probe. Thus, one egg yields sufficient XTEF to bind at least 3×10^7 probe molecules. The actual abundance of XTEF may be much higher, depending on the efficiency of extraction of the factor and its activity in the electrophoretic mobility retardation assay. In contrast with the egg, whole blood cell nuclei

yield only ~35 pg of protein each when extracted under the relatively mild conditions employed (see Materials and methods). From our quantitative measurements, we estimate that 35 pg of protein of a whole blood nuclear extract (derived from at least one nucleus) contains sufficient XTEF to bind ~90 PTE molecules. According to these data, eggs yield 10^5 - to 10^6 -fold more XTEF than somatic cell nuclei. Therefore, egg extracts were employed as a source for further characterization of XTEF.

XTEF recognizes single-stranded TTAGGG repeats at a 3' end

We examined which of the features of the putative telomere end probe are recognized by XTEF. Initially two DNA probes were employed in which either the tail or the duplex contains the sequence $(TTAGGG)_2$ while the remainder of the DNA bears no resemblance to telomeric repeats (random duplex and random tail; see Table 1). The random duplex probe is bound by XTEF to form a complex that is competed by PTE (Fig. 2). In addition, this DNA effectively competes for the binding of XTEF to PTE (Fig. 2; Table 1). In the experiment in Figure 2 and in other experiments, XTEF binds the random duplex probe somewhat better than PTE. This difference could be attributable to unfavorable annealing products (e.g., hybridization of a second C-strand oligonucleotide to the 3' tail) that can occur with PTE but not with the random duplex probe. In contrast to the random duplex probe, the random tail probe does not associate with XTEF (Fig. 2). The lack of interaction between random tail DNA and XTEF is also apparent from competition experiments in which this DNA fails to compete for XTEF binding to PTE (Fig. 2; Table 1). These results illustrate

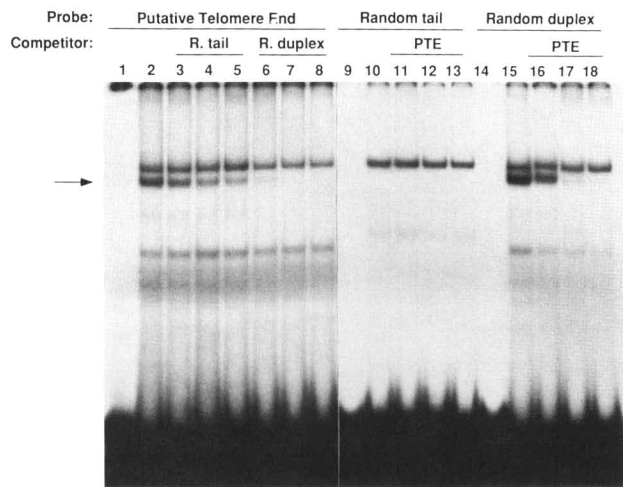


Figure 2. XTEF requires TTAGGG repeats in the 3' tail. Putative telomere end (PTE, lanes 1–8), random tail (R. tail, lanes 9–13), and random duplex (R. duplex, lanes 14–18) probes were tested for binding to XTEF (see Table 1 for the sequence of the probes). Each probe was incubated in the absence (lanes 1,9,14) or in the presence (lanes 2–8,10–13,15–18) of 30 μ g of egg extract. For competition experiments, unlabeled random tail (lanes 3–5), random duplex (lanes 6–8), or PTE (lanes 11–13,16–18) DNAs were added in the following molar excess: 10-fold (lanes 3,6,11,16), 20-fold (lanes 4,7,12,17), 100-fold (lanes 5,8,13,18). The arrow marks the position of XTEF complex.

that whereas the sequence of the duplex is not essential, some feature of the single-stranded 3' (TTAGGG)₂ tail is critical for recognition and binding by XTEF. In agreement with this notion, XTEF fails to recognize TTAGGG repeats at the 3' end of fully double-stranded DNA [see data with duplex (TTAGGG)₄ summarized in Table 1].

To define the specificity of XTEF for (TTAGGG)₂ tails, a set of telomere end probes was tested in which the (TTAGGG)₂ tail was changed to (TTTGGG)₂, (AAAGGG)₂, or (TTACCC)₂ (see Table 1). Each sequence alteration strongly reduces the ability of XTEF to form a complex with the DNA (Fig. 3). Furthermore, the ability of these altered DNAs to compete for XTEF is severely impaired compared with PTE or random duplex DNAs (summarized in Table 1). In contrast to the DNAs with A \rightarrow T, T \rightarrow A, or G \rightarrow C changes in the 3' tail, a binding substrate with a permuted (GGGTTA)₂ tail forms complex with XTEF and performs well as an XTEF competitor (Fig. 3; Table 1). These results show that XTEF has a sequence-specific interaction with the tail moiety of the DNA probes and that the binding site conforms or closely resembles telomeric TTAGGG repeats.

The (TTACCC)₂ tail probe forms a new complex that is specifically competed by PTE DNA (Fig. 3). However, because (TTACCC)₂ tail DNA does not compete for XTEF complex formation with PTE (Table 1) and because the (TTACCC)₂ tail complex migrates to a different position in the gel, this complex is not attributable to XTEF. Therefore, the nature of this new complex with (TTACCC)₂ tail DNA was not analyzed further.

In the preceding experiments, XTEF was shown to bind (TTAGGG)₂ sites at a 3' terminus. To test whether this 3' end is important for XTEF binding, we analyzed the interaction of XTEF with DNAs in which two single-stranded TTAGGG repeats occupy alternate positions (Fig. 4). In the random duplex probe the telomeric repeats are immediately adjacent to a 3' end, whereas in two additional probes the single-stranded (TTAGGG)₂ stretch is followed by a 15-nucleotide single-stranded segment or by a duplex region of 15 bp (r.duplex + 3'ss and r.duplex + 3'ds, respectively). A fourth DNA molecule contains two single-stranded TTAGGG repeats in a 5' extension. When used as labeled probes in mobility retardation assays, only DNAs with TTAGGG repeats immediately adjacent to a free 3' end efficiently form complexes with XTEF (Fig. 4). XTEF does bind DNA with an extra 15-nucleotide segment 3' of the (TTAGGG)₂ tail, but complex formation is nearly 10-fold reduced as compared with the random duplex probe. Furthermore, competition experiments show that whereas the random duplex is an effective competitor at 5-fold molar excess, none of the other three DNAs strongly affect XTEF complex formation at 40-fold molar excess (Fig. 4). These experiments suggest that XTEF requires the close proximity of a free 3' end for optimal interaction with (TTAGGG)₂ tails.

XTEF binds single-stranded telomeric DNA but not UUAGGG repeats in RNA

To test whether XTEF binds (TTAGGG)₂ at the 3' end of

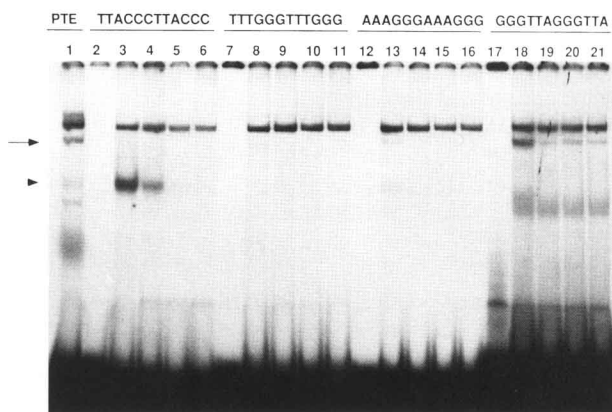
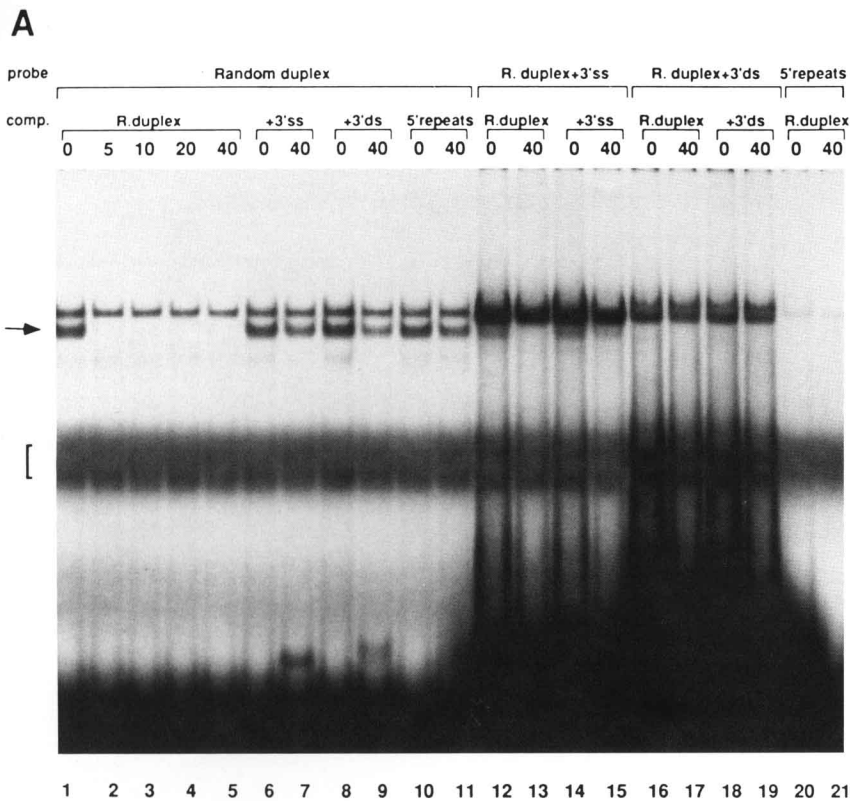


Figure 3. Effect of changes in the 3'-tail sequence. Four DNA probes with altered 3' tail sequences (as indicated above the lanes) were tested for complex formation with XTEF. (Lanes 2–6) (TTACCC)₂ tail; (lanes 7–11) (TTTGGG)₂ tail; (lanes 12–16) (AAAGGG)₂ tail; (lanes 17–21) (GGGTTA)₂ tail. The structure of these probes is given in Table 1. Lane 1 shows a control reaction with PTE resulting in XTEF complex (arrow). Lanes 2, 7, 12, and 17 show incubations without extract. The remaining lanes contain incubations with extract and various amounts of PTE competitor DNA: no competitor DNA (lanes 3,8,13,18), 10-fold molar excess (lanes 4,9,14,19), 50-fold molar excess (lanes 5,10,15,20), 100-fold molar excess (lanes 6,11,16,21). The arrowhead indicates a new complex formed with the (TTACCC)₂ tail probe.



		COMPLEX	COMPETITION
Random duplex	AAACTCGACCAGTACAGCTACTTAGGGTTAGGG 3' ***GAGCTGGTCATGTCGATG	+	+
R.duplex+3'ss	AAACTCGACCAGTACAGCTACTTAGGGTTAGGGCAGCTGAGATCTCCC 3' ***GAGCTGGTCATGTCGATG	±	-
R.duplex+3'ds	AAACTCGACCAGTACAGCTACTTAGGGTTAGGGCAGCTGAGATCTCCC 3' ***GAGCTGGTCATGTCGATG GTCGACTCTAGAGGGAA	-	-
5'repeats	5' TTAGGGTTAGGGCAGCTGAGATCTCCC**3' GTCGACTCTAGAGGGAA	-	-

residual XTEF-random duplex complex remaining at 40-fold molar excess of competitor. (-) >30% residual XTEF complex remains at 40-fold molar excess of competitor.

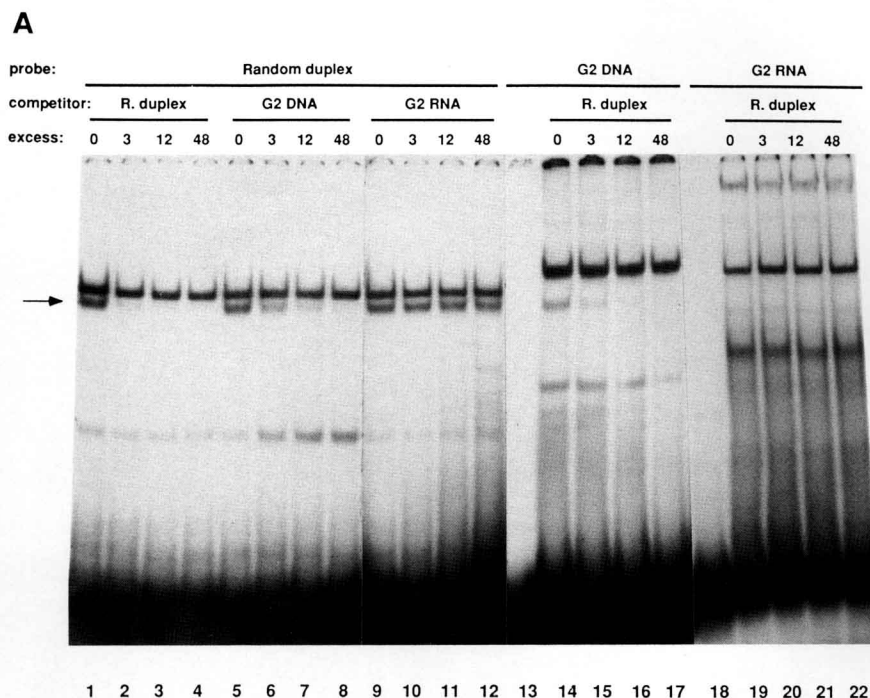
a completely single-stranded molecule, we employed modified assay conditions that reduce the binding of nonspecific factors to single-stranded probes but do not inhibit XTEF (see Materials and methods). Under these conditions XTEF binds to a single-stranded 34-mer with (TTAGGG)₂ at its 3' end (G2 DNA, the G-strand of random duplex) (Fig. 5). In addition, G2 DNA competes for the binding of XTEF to random duplex probe (Fig. 5). However, both the abundance of the XTEF complex and the competition efficiency of G2 DNA are reduced as compared with its partially double-stranded counterpart (random duplex). The reduced activity of XTEF with G2 DNA is not attributable to degradation of the probe. Denaturing gel electrophoresis showed that >99% of the G2 DNA probe remained intact during the mobility retardation assay (data not shown). Whether the dimin-

Figure 4. XTEF requires single-stranded TTAGGG repeats near a 3' end. XTEF binding was assayed by mobility retardation analysis using four probes in which the sequence (TTAGGG)₂ occurs at alternate positions. (A) Random duplex (R.duplex) with (TTAGGG)₂ at a 3' end is employed as a mobility retardation probe in lanes 1-11 and as competitor in lanes 2-5, 13, 17, 21. The molar excess of competitor DNA is indicated above the lanes. R.duplex+3'ss in which the (TTAGGG)₂ site is flanked 3' by 15 nucleotides is tested for XTEF complex formation in lanes 12-15 and is employed as a competitor at 40-fold molar excess in lanes 7 and 15. R.duplex+3'ds in which the (TTAGGG)₂ site is flanked by 15 bp is tested for XTEF complex formation in lanes 16-19 and is employed as a competitor at 40-fold molar excess in lanes 9 and 19. The DNA 5' repeats, in which (TTAGGG)₂ is at a 5' end are used as a probe in the reactions in lanes 20 and 21 and as a competitor at 40-fold molar excess in lane 11. The XTEF complex formed on random duplex is indicated by the arrow. The bracket indicates an artifactual band with no relevance to XTEF. (B) The sequence of the probes used in the experiment shown in A is given, and the ability of XTEF to associate with these DNAs is summarized. Complex formation was scored by mobility retardation analysis with labeled probes as in A: (+) efficient complex formation; (±) a minor amount of XTEF complex is detected; (-) no XTEF complex is detected. Competition efficiencies of the DNAs were determined by adding various amounts of unlabeled DNA to XTEF-binding reactions with the random duplex probe and the residual complex, quantitated by PhosphorImager: (+) <5%

ished activity with G2 DNA reflects actual characteristics of XTEF will have to be explored further with biochemically pure factor. No XTEF complex formation was found with single-stranded DNAs lacking TTAGGG repeats (e.g., see C4 DNA in Fig. 5B).

We note that the probes used in this study have the potential to form G-quartets (Williamson et al. 1989). Although most XTEF substrates, such as the random duplex and G2 DNA, lack the four TTAGGG repeats required for intramolecular G-quartet formation, these probes may form a G-quartet by intermolecular interactions (Sundquist and Klug 1989; Kang et al. 1992; Smith and Feigon 1992). We have not been able to detect such dimers under the conditions used for XTEF binding (data not shown). Two additional observations argue against a preference of XTEF for G-quartets. First, a probe with

Figure 5. XTEF interaction with single-stranded DNA- and RNA-binding substrates. (A) Mobility retardation analysis of XTEF binding to random duplex, the G-strand of random duplex (G2 DNA), and an RNA oligonucleotide with the same sequence as the G-strand of random duplex (G2 RNA). The structure of the probes is shown in B. In lanes 1–12 random duplex is used as a probe with variable amounts of the following competitors: random duplex DNA (lanes 1–4), G2 DNA (lanes 5–8), and G2 RNA (lanes 9–12). The molar ratio of unlabeled competitor molecules to the probe is indicated above each lane. In lanes 14–17, G2 DNA is used as a probe in the presence (lanes 15–17) or absence (lane 14) of random duplex competitor DNA as indicated above the lanes. In lanes 19–22, G2 RNA is used as a probe in the presence (lanes 20–22) or absence (lane 19) of random duplex competitor DNA as indicated above the lanes. Lanes 13 and 18 show G2 DNA and G2 RNA probes incubated without egg extract. Incubation conditions are described in the Materials and methods as specified for single-stranded probes. Overexposure of lanes 18–22 fails to show XTEF complex formation with G2 RNA. The arrow indicates the XTEF complex. (B) Summary of the structure and binding activity of single-stranded XTEF substrates. Complex formation was analyzed by mobility retardation assay as described for A: (+) efficient complex formation; (\pm) reduced XTEF complex formation; (–) XTEF complex is not detected. Competition efficiencies of the DNAs were determined by adding various amounts of unlabeled DNA to XTEF-binding reactions with the random duplex probe, and the residual complex was quantitated by PhosphorImager: (+) <10% XTEF–random duplex complex remaining at 12-fold molar excess of competitor; (\pm) <10% XTEF–random duplex complex remaining at 48-fold molar excess of competitor; (–) >30% residual XTEF complex detected at 48-fold molar excess of competitor.

**B**

		COMPLEX	COMPETITION
Random duplex	AAAACCTCGACTAGTCATCCGACTTAGGGTTAGGG 3' ***TGAGCTGATCAGCTAGCTG	+	+
G2 DNA	*AAAACCTCGACTAGTCATCCGACTTAGGGTTAGGG 3'	\pm	\pm
G2 RNA	*AAAACUCGACUAGUGCAUCGACUUAAGGGUUAGGG 3'	–	–
G4 DNA	*AAAACCTCGACTTAGGGTTAGGGTTAGGGTTAGGG 3'	–	–
G4 RNA	*UUAGGGUUAGGGUUAGGGUUAGGG 3'	–	–
C4 DNA	*CCCTAACCCCTAACCCCTAACCCCTAAGCTGAGT 3'	–	–

four tandem telomeric repeats (G4 DNA), which should form G-quartets more efficiently than any of the other probes, is not a good substrate for XTEF (Fig. 5B). Second, XTEF complex is efficiently formed with probes that are prepared and assayed in the presence of 100 mM Li⁺ (instead of K⁺) (see Materials and methods), which is known to interfere with G-quartet formation (Williamson et al. 1989). Although these observations argue against a requirement of XTEF for G-quartets, we cannot exclude the possibility that XTEF interacts with some other G–G base-paired structure.

We were concerned that XTEF might be an RNA-binding protein with fortuitous specificity for single-stranded TTAGGG repeats at a 3' end. To address this question, we synthesized an RNA oligonucleotide (G2 RNA) that has the same sequence as G2 DNA. Although G2 DNA forms a complex with XTEF, no such complex is de-

tected with end-labeled G2 RNA (Fig. 5). Denaturing gel electrophoresis showed that the RNA probe is not degraded during the assay (data not shown). The lack of XTEF interaction with G2 RNA is corroborated by the competition data in Figure 5, which show that G2 RNA fails to compete for the binding of XTEF to random duplex probe even at 48-fold molar excess. Similarly, an RNA oligonucleotide with four UUAGGG repeats failed to show evidence for interactions with XTEF (Fig. 5B). These results demonstrate the specificity of XTEF for deoxyribonucleic acids with 3' single-stranded TTAGGG repeats.

Stability and salt resistance of XTEF–DNA complexes

The stability of the XTEF–DNA complexes was determined by challenging preformed random duplex com-

plex with a 100-fold excess of unlabeled DNA. At successive time points after the addition of competitor DNA, samples were loaded on a running gel and the disappearance of the XTEF complex was quantitated. An example of such an off-rate experiment is shown in Figure 6. In this and other experiments, ~50% of the XTEF complex has disappeared in 30 min. In a parallel reaction without competitor DNA, the XTEF activity increases during this time period (Fig. 6), indicating that XTEF is not inactivated during the experiment. Therefore, the gradual reduction to 50% complex over a period of ~30 min is likely to reflect the rate at which XTEF releases the labeled DNA substrate, that is, the off-rate of XTEF. In contrast, the disappearance of the nonspecific complex that migrates slightly slower than XTEF appears to be attributable to factor inactivation because this activity is also decreased after prolonged incubation without competitor DNA.

We then asked whether the activity and stability of XTEF is altered by high concentrations of salt. In these assays XTEF was allowed to associate with the random duplex probe in binding reactions containing between 0.1 and 2 M potassium acetate. Binding was allowed to equilibrate for 1 hr and was subsequently diluted 20-fold while the final potassium acetate concentration was adjusted to 0.1 M in each case. The diluted samples were immediately loaded on a running gel, and the resulting XTEF activity was quantitated. To ensure that the ob-

served XTEF complexes were formed before the dilution step, a large (500-fold) molar excess of unlabeled random duplex DNA was included in each dilution mixture (for details, see Materials and methods). As an additional control, reactions were carried out in which the addition of either the extract or the probe was delayed until the time of dilution. In these controls, no XTEF complex was observed (Fig. 7; data not shown). The results in Figure 7 show that XTEF is relatively refractive to high salt conditions, resulting in robust activity at 1.5 M potassium acetate and 14–23% residual XTEF activity at 2 M salt. The competition experiments in Figure 7 show that the XTEF complex displays the expected specificity for DNA substrates, irrespective of the salt concentration in the binding reaction. XTEF activity can also be detected in the presence of 2 M KCl or NaCl and the resistance of XTEF to high salt is observed both with the random duplex probe and PTE (data not shown).

In contrast with XTEF, one of the nonspecific DNA-binding activities is highly sensitive to elevated salt concentrations and completely inactivated at 1 M potassium acetate (Fig. 7). Remarkably, a second nonspecific complex that migrates slightly slower than XTEF is strongly enhanced at 0.5–1.0 M potassium acetate (but not at 0.5 M KCl; data not shown) and fairly resistant to further increases in the salt concentration.

Although XTEF is quite resistant to 1.5 and 2 M potassium acetate, the activity is more potent at 0.1–1.0 M

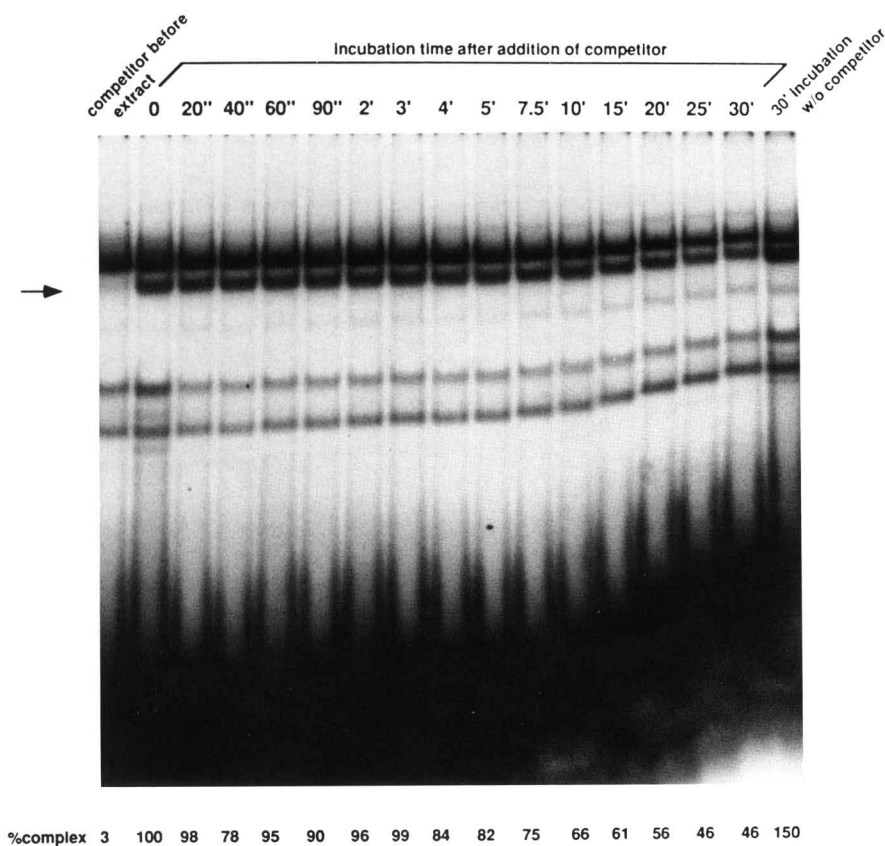


Figure 6. Determination of the stability of XTEF-random duplex complex. XTEF complex was allowed to form for 5 min at 25°C with random duplex probe (lane 0) and challenged with a 100-fold molar excess of unlabeled random duplex DNA. At the indicated time points (between 20 sec and 30 min) after the challenge, aliquots were loaded on a continuously running native gel. The first lane contains a mobility retardation reaction in which the competitor was added before the extract. The last lane contains a control reaction in which the incubation was allowed to proceed for 30 min without the addition of unlabeled competitor DNA. The numbers below the lanes (% complex) indicate the residual radioactivity in the XTEF complex (as determined by PhosphorImager analysis of the gel) expressed as the percentage of the radioactivity in the XTEF complex in lane 0. The arrow indicates the XTEF complex. Experimental details are given in Materials and methods.

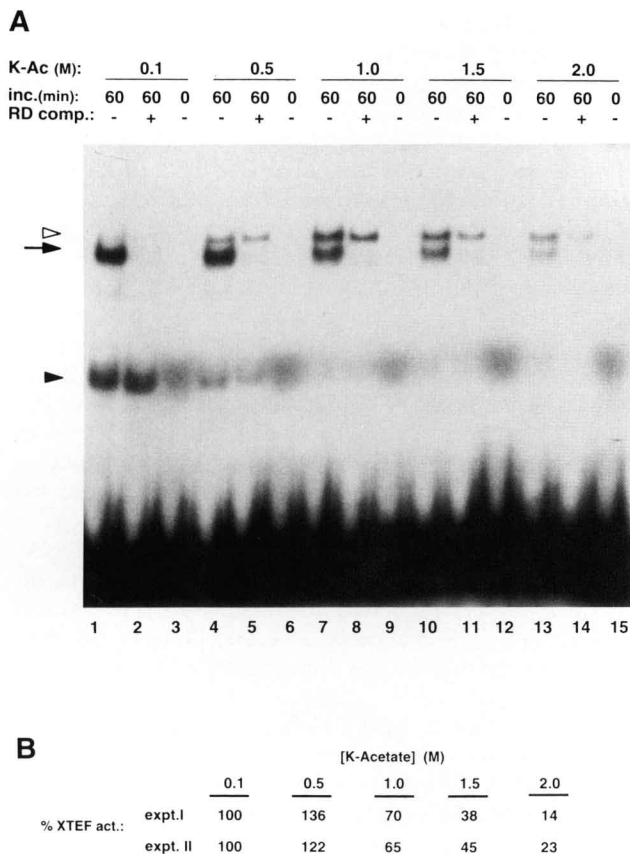


Figure 7. Effects of high salt on XTEF. (A) XTEF binding to random duplex probe assayed in the presence of 0.1 (lanes 1–3), 0.5 (lanes 4–6), 1.0 (lanes 7–9), 1.5 (lanes 10–12), and 2.0 (lanes 13–15) M potassium acetate. Incubations were carried out for 60 min in the presence (lanes 2,5,8,11,14) or absence (all other lanes) of a 50-fold molar excess unlabeled random duplex competitor DNA. Immediately prior to gel electrophoresis, each incubation was diluted 20-fold while the potassium acetate concentration was adjusted to achieve a final salt concentration of 0.1 M. The dilutions were carried out in the presence of 500-fold molar excess competitor DNA. Lanes 3, 6, 9, 12, and 15 represent control reactions in which the addition of probe was delayed until the time of dilution (0-min incubations). Full experimental details are given in Materials and methods. The arrow indicates the position of XTEF. The arrowheads mark two non-specific activities with differential sensitivity to increasing salt concentration. (B) Quantitation of XTEF activity as a function of potassium acetate concentration. The relative XTEF activities in the presence of the indicated potassium acetate concentrations was quantitated with the PhosphorImager using the data from the experiment in A and a second similar experiment.

salt. A similar result was obtained when the incubation time was limited to 10–20 min rather than 1 hr, indicating that this salt effect is not the consequence of XTEF inactivation or an altered rate of substrate binding (data not shown). We also examined whether high salt concentrations affect the off-rate of XTEF. The off-rate is pertinent to the data in Figure 7, because in these experiments preformed complex is diluted rapidly into a mix-

ture with excess competitor DNA; thus, a dramatically elevated off-rate at high salt could result in an apparent reduction of XTEF activity. However, competition challenge experiments similar to the experiment shown in Figure 6 demonstrate that the off-rate is not increased in high salt (data not shown). Therefore, our data indicate that XTEF binds DNA and forms a stable complex in the presence of 1.5 and 2 M potassium acetate. Maximal XTEF activity is observed at salt concentrations between 0.1 and 1.0 M.

Discussion

Telomeric DNA has been characterized in representatives of most eukaryotic phyla, resulting in a catalog of similar repetitive DNA sequences. These repeats are thought to form a nucleoprotein complex that mediates the various activities ascribed to telomeres, including the protection of DNA ends, replication of chromosome termini, modulation of gene expression, and subnuclear localization. With the exception of telomerase, which adds telomeric repeats to the 3' ends of the chromosome, the role of telomere factors in these activities is poorly understood. In addition, apart from the demonstration of telomerase activity in ciliates and mammals, evolutionary conservation of (candidate) telomere factors, as predicted by the similarity of their target DNAs, has not been demonstrated (Greider and Blackburn 1985; Zahler and Prescott 1988; Morin 1989; Shippen-Lentz and Blackburn 1989). The identification of telomere proteins in widely diverged eukaryotes should help to elucidate common themes in the mechanism of telomere function. This report describes a DNA-binding factor from *Xenopus* eggs with strong similarity to a class of telomere terminus-specific proteins identified previously in hypotrichous ciliates.

Our approach to identify candidate telomere terminus factors was based on the assumption that vertebrate telomeres terminate in a 3' single-stranded tail of TTAGGG repeats. Such 3' extensions are the expected products of both conventional DNA replication and telomerase-mediated telomere synthesis (Greider and Blackburn 1985; Morin 1989). Furthermore, 3' overhangs have been demonstrated at telomere termini in several ciliate genera, an a-cellular slime mold, and in *S. cerevisiae*, suggesting that this configuration is a conserved feature of all eukaryotic telomeres (Klobutcher et al. 1981; Pluta et al. 1982; Henderson and Blackburn 1989; Wellinger et al. 1993). We found that *Xenopus* eggs contain a DNA-binding factor (XTEF) with specificity for this presumed arrangement of vertebrate chromosome ends.

In vitro, XTEF displays high specificity for deoxyribonucleic acids with TTAGGG repeats in a 3' single-stranded tail. An RNA version of this kind of nucleic acid is not an effective XTEF-binding substrate. Our data suggest that at least three additional features of our telomeric DNA probes are recognized by XTEF. First, minor alterations in the 3' tail sequence diminish the interactions with XTEF, indicating that a specific sequence

in the TTAGGG repeat tail, rather than, for instance, its G-rich nature, contributes to the XTEF-binding site. At least three different nucleotides within the TTAGGG repeats appear important, because 3' tails with T → A, A → T, or G → C changes all fail to bind XTEF efficiently. The XTEF recognition site is probably not longer than one and one-half repeats (9 nucleotides), because (GGGTTA)₂ and (TTAGGG)₂ tails are equally good substrates for XTEF complex formation. Second, the region 5' of the (TTAGGG)₂ site influences the interaction with XTEF. Although the (TTAGGG)₂ sequence is recognized in a completely single-stranded probe, a configuration with double-stranded DNA 5' of the (TTAGGG)₂ site is favored. It is likely that the structure rather than the sequence of the double-stranded moiety is important because several probes with different double-stranded sequences are effective XTEF-binding substrates. A third feature of the telomeric DNA probes that determines whether they interact with XTEF efficiently is the proximity of the (TTAGGG)₂ sequence to the 3' end. Maximal binding activity occurs with DNAs in which the site is immediately at a 3' end. No XTEF binding occurs when the single-stranded TTAGGG repeats are at a 5' end or when the site is surrounded by double-stranded DNA. Furthermore, a single-stranded 3'-flanking region of 15 nucleotides reduces the interaction of XTEF with the (TTAGGG)₂ site. The preference for a 3'-terminal position of (TTAGGG)₂ sites is probably not attributable to a sequence-specific interaction with the 3' nucleotide, because XTEF binds DNAs ending in either A or G residues.

XTEF activity is found predominantly in frog eggs and oocytes; trace activity is detected in nuclear extracts of several somatic tissues. The low activity of XTEF in extracts from somatic cell nuclei (<100 binding units per nucleus) could be attributable to the predicted low abundance of telomere end factors in *Xenopus* cells if only one such factor is required per telomere. However, in view of our data on the stability and salt resistance of XTEF-DNA complexes, it is also possible that some portion of this factor resists extraction from the nucleus. Regardless, XTEF appears relatively abundant in frog eggs and oocytes. We estimate that the egg yields 10⁵- to 10⁶-fold more XTEF than a somatic cell nucleus. Perhaps excess XTEF is stored in the egg in preparation for the rapid divisions in early *Xenopus* development. Similarly, the egg stockpiles many other nuclear and cytoplasmic components to make cells before the onset of transcription in mid-blastula (Laskey et al. 1979; Newport and Kirshner 1982). In the 14 divisions that precede the mid-blastula transition, ~3 × 10⁵ telomeres (~4000 nuclei with 72 telomeres each) are generated. Although the amount of XTEF per egg cannot be determined precisely at this point, our data suggest that each egg contains sufficient XTEF (~10⁷-10⁸/egg) to keep up with the possible demand for telomere factors in early development.

Recently, two other vertebrate factors with affinity for single-stranded TTAGGG repeats were identified in nuclear extracts of murine and avian cells [single-stranded (TTAGGG)_n binding protein (sTBP) and muscle factor 3

(MF3), respectively] (Gualberto et al. 1992; McKay and Cooke 1992a). The murine sTBP was subsequently identified as the RNA-binding factor heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 (McKay and Cooke 1992b). Because our data fail to show RNA-binding activity for XTEF, we consider it unlikely that XTEF is similar to sTBP. XTEF also seems different from the avian MF3 which, unlike XTEF, displays a high tolerance for sequence alterations in the telomeric repeats, possibly because this factor requires G-G base-paired folded structures rather than a specific sequence (Gualberto et al. 1992).

In terms of its DNA-binding characteristics, XTEF strongly resembles the telomere end factors of *Oxytricha* and *Euplotes*. Each one of these proteins behaves as a single-stranded DNA-binding factor that correctly recognizes the telomeric repeat sequence of its species. For instance, neither XTEF nor the ciliate factors bind altered telomere termini probes with T → A or G → C transversions (Raghuraman et al. 1989; Price et al. 1992; this paper). Furthermore, each of the factors requires only two telomeric repeats in the 3' tail, and the sequence and structure (double- or single-stranded) 5' of this site does not seem of central importance (Gottschling and Zakian 1986; Price et al. 1992; this paper). In contrast, the proximity of a 3' DNA end to the binding site is crucial. Neither XTEF nor the ciliate proteins bind well when the telomeric repeats are internal to the DNA end (Raghuraman et al. 1989; Price et al. 1992; this paper). The *Oxytricha* telomere protein is known to bind poorly to its substrate DNA when it is folded in the G-quartet conformation (Raghuraman and Cech 1990). Similarly, our data on XTEF fail to implicate the G quartets. Finally, XTEF can bind DNA and form a stable complex in high salt. Although it is not known whether the ciliate telomere proteins can bind DNA in high salt, their complex with telomere termini is completely resistant to 2 M NaCl and 6 M CsCl (Gottschling and Zakian 1986; Price and Cech 1988; Price 1990). Thus, XTEF and the ciliate telomere proteins share a number of unusual activities that suggest a common function. Cytological and genetic data on XTEF will be required to further substantiate this tentative functional similarity.

Clearly, XTEF and the ciliate telomere proteins may well be different in certain aspects of their activities. Perhaps a noteworthy difference is that the ciliate telomere proteins remain bound to telomeric DNA when chromatin is treated with high salt (Gottschling and Zakian 1986; Price and Cech 1988; Price 1990), whereas soluble XTEF activity can be extracted from nuclei under relatively mild conditions. However, it is possible that these nuclei retain additional XTEF bound to telomeres. Another possible difference is that whereas the ciliate proteins bind well to substrates with four single-stranded telomeric repeats (Raghuraman et al. 1989; Price et al. 1992), the XTEF activity in the egg extract does not bind such substrates. Whether this difference will also be evident with purified XTEF remains to be determined. In this regard, it has been noted previously that the telom-

ere proteins from *Euplotes* and *Oxytricha* are also dissimilar in some of their DNA-binding characteristics, each appearing to be tailored to its cognate telomeres (Price 1990).

The capping complex of hypotrichous ciliates protects telomere termini from exonuclease digestion *in vitro* and may have the same activity *in vivo* (Gottschling and Zakian 1986; Price 1990). Additional proposed functions include inhibition of telomere fusion, regulation of telomere synthesis by interaction with telomerase or its products, and the localization of telomeres to a specific subnuclear locale. Macronuclei of *Oxytricha* and *Euplotes* contain $>2 \times 10^7$ telomeres as a result of extensive chromosome fragmentation during macronuclear development. These telomeres are identical and extremely short (the G-rich strand is 36 nucleotides in *Oxytricha* and 42 nucleotides in *Euplotes*) (Klobutcher et al. 1981). A priori, one could argue that this unique situation demands special solutions to the problems posed by chromosome ends and that larger nonfragmented chromosomes with less strictly maintained telomeres may not require the same kind of telomere capping complex. In view of these considerations it will be of interest to compare the structure and function of vertebrate telomere terminus factors in detail with the telomere proteins in hypotrichous ciliates.

Materials and methods

Preparation of DNA and RNA oligonucleotides

DNA and RNA oligonucleotides were synthesized with an Applied Biosystems 391 DNA synthesizer and purified by electrophoresis in 15–20% polyacrylamide gels containing 8 M urea. The (UUAGGG)₄ RNA oligonucleotide was generously provided by Thomas Cech (University of Colorado, Boulder).

Oligonucleotides were annealed at 10 pmoles/ μ l in 10 mM Tris-HCl (pH 7.5); 100 mM NaCl; 1 mM EDTA (TNE) by heating to 100°C for 1 min followed by slow cooling to room temperature. Annealing reactions were monitored by nondenaturing polyacrylamide gel electrophoresis of labeled DNAs and, in some cases, were verified by analysis of mung bean nuclease products. Hybridization of the oligonucleotides was typically 85% or greater. Single-stranded DNA and RNA probes were end-labeled with [γ -³²P]ATP and T4-polynucleotide kinase. Double-stranded DNA probes were prepared by filling in the 3'-end gap with [α -³²P]dTTP and Klenow enzyme. Labeled DNAs were isolated by gel filtration (Sephadex G50) and extraction with phenol-chloroform. After precipitation with ethanol in the presence of 5 μ g of *Hae*III-digested *E. coli* DNA, the probes were dissolved at 0.1 pmole/ μ l in TNE. In some cases, probes were annealed and prepared in the absence of Na⁺ by replacing NaCl in the annealing, labeling, and isolation buffers with LiCl.

Cell-free extracts

Xenopus egg and ovary extract was prepared as described by Newport (1987), with the following modifications. The ionophore activation step was eliminated, and eggs were rinsed, packed, and lysed in lysis buffer containing 20 mM HEPES-KOH (pH 7.7), 50 mM KCl, 0.2 mM EDTA-KOH, 250 mM sucrose, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluo-

ride (PMSF), and the following reagents: 5 μ g of cytocholasin B, 1 μ g of pepstatin, 50 μ g of aprotinin, and 50 μ g of leupeptin per milliliter. Following lysis, the cytosol was cleared by ultracentrifugation at 70,000 rpm for 1 hr at 4°C using a TL-100.3 rotor in a TL-100 Beckman ultracentrifuge. The clear supernatant was removed carefully with a needle avoiding the lipid layer at the top and dialyzed against lysis buffer (without sucrose and containing 15% glycerol). The egg extract was stored at -80°C. The protein concentration of this extract as determined by Bradford assay (Bio-Rad) was typically 30–35 mg/ml.

For the isolation of proteins from ovaries and somatic cells, frogs were first anesthetized on ice for 20 min and subsequently decapitated. Ovaries were processed as described above for eggs. Blood was collected in 0.1% sodium citrate by cardiac puncture; other somatic tissues were dissected and homogenized gently with a Brinkman homogenizer, followed by homogenization with a Dounce homogenizer with loose fitting pestle in phosphate-buffered saline (PBS) on ice. Single-cell suspensions were washed twice with PBS at 4°C. Nuclear and S-100 extracts were prepared as described by Dignam et al. (1980). Whole-cell extracts were prepared as described by La Bella and Heintz (1991). All extraction buffers contained PMSF, leupeptin, aprotinin, and pepstatin as protease inhibitors at the same concentrations as indicated above for egg lysis buffer. Before storage at -80°C, extracts were dialyzed to 20 mM HEPES-KOH (pH 7.4), 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 20% glycerol.

Electrophoretic mobility retardation assays

The DNA-binding reactions involving partially double-stranded DNAs included 0.05–0.1 pmole of radiolabeled DNA probe ($\sim 1 \times 10^5$ cpm, Cerenkov), 25–30 μ g of protein from egg extracts, 2 μ g of *Hae*III-cleaved *E. coli* DNA in 20 μ l of DNA-binding buffer containing 20 mM HEPES-KOH (pH 7.7), 100 mM KCl, 5 mM MgCl₂, 0.02% NP-40, 0.5 mM dithiothreitol, and 10% glycerol. XTEF is also active when the KCl in the reaction buffer is replaced by 100 mM LiCl. For DNA-binding reactions with single-stranded DNA and RNA probes, 3–5 μ g of cytoplasmic RNA from HeLa cells and 20 units of RNasin were added to the reactions; MgCl₂ was omitted and 1 mM EDTA and 1 mM EGTA were included. Unless otherwise indicated, the reactions were incubated for 5 min at 37°C (for partially double-stranded probes) or 10–20 min at 25°C (reactions involving single-stranded probes or competitors). After incubation the mixture was loaded onto 6% polyacrylamide gels (5.8% acrylamide/0.2% bisacrylamide) in TBE (89 mM Trizma-base, 89 mM boric acid, 2 mM EDTA). Gels were electrophoresed in TBE at 150 V for 2.5 hr at 25°C. DNA-protein complexes were visualized by autoradiography (usually 20 hr at room temperature) of the dried gels on Kodak XAR-5 films.

For the single-stranded DNA and RNA probes used in this study we determined that >95% of the labeled nucleic acid remained intact during the assay. This was deduced from the comigration of labeled probes before and after incubation with the egg extract on 15% polyacrylamide/7 M urea gels. Some of the partially double-stranded probes were also tested for their intact persistence during the assay by a similar analysis of 5'-end-labeled molecules.

Quantitative measurements

Quantitation of the DNA-protein complexes was performed by scanning dried gels with a PhosphorImager (Molecular Dynamics) or by densitometry of autoradiographs on a Joyce-Loebel microdensitometer.

Off-rate experiments

The exchange rate of XTEF between labeled and unlabeled random duplex DNA was measured over 30 min. XTEF was allowed to bind labeled random duplex DNA in a 400- μ l mixture containing 20 μ l of egg extract (~600 μ g of protein), 2 pmoles of labeled random duplex DNA, 40 μ g of sonicated DNA from *E. coli*, 20 mM HEPES-KOH (pH 7.7), 100 mM KCl, 1 mM DTT, 0.02% NP-40, and 10% glycerol. After a 5-min incubation at room temperature, 20 μ l was loaded on a running gel next to a control reaction with the identical composition except for the presence of 50 \times molar excess unlabeled random duplex DNA. A second 20- μ l sample was set aside for analysis at $t = 30$ min. The off-rate measurements were initiated with the addition of 100 pmoles of unlabeled random duplex DNA to the main incubation. At successive time points, 20- μ l samples were loaded on a running gel. After 30 min, the last sample was taken and loaded next to the control sample that was set aside before the addition of unlabeled DNA. The amount of radiolabeled probe bound by XTEF was quantitated using the PhosphorImager. Three independent experiments gave results similar to that result shown in Figure 6.

Effects of salt concentration on XTEF activity

Egg extract (1 μ l with ~30 μ g of protein) was incubated with 0.1 pmole of labeled random duplex DNA in the presence of 3 μ g *Hae*III-digested DNA from *E. coli* in 10 μ l of 20 mM HEPES-KOH (pH 7.7), 1 mM DTT, EDTA, and EGTA, 0.02% NP-40, 10% glycerol, and 0.1–2.0 M potassium acetate. Parallel control incubations were supplemented with 5 pmoles of unlabeled random duplex DNA. For each potassium acetate concentration, a third reaction was prepared without probe or competitor DNA. After incubation for 1 hr at room temperature, 1.5- μ l aliquots were added to 28.5- μ l dilution mixture with 7.5 pmoles of unlabeled random duplex DNA (500 \times molar excess to the probe), 4 μ g of *Hae*III-cleaved *E. coli* DNA, HEPES buffer, DTT, EDTA, EGTA, NP-40, and glycerol at the concentrations given for the initial incubation, and variable amounts of potassium-acetate to adjust the final potassium acetate concentration to 0.1 M. Immediately after the dilution step, each sample was loaded on a running gel. For the incubations that initially did not contain the probe, the dilution mixture was supplemented with 0.015 pmole of labeled random duplex DNA. Dried gels were exposed for 1–2 days at -80°C with an intensifying screen. Quantitative measurements were performed on 20-hr exposures for the PhosphorImager.

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