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Identification and characterization of a nuclease activity specific for G4 tetrastranded DNA

(G quartet/DNA binding/telomere/meiosis/switch recombination)

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ABSTRACT **We have identified a nuclease activity that is specific for G4 tetrastranded DNA. This activity, found in a partially purified fraction for a yeast telomere-binding protein, binds to DNA molecules with G4 tetrastranded structure, regardless of their nucleotide sequences, and cleaves the DNA in a neighboring single-stranded region 5′ to the G4 structure. Competition with various G4-DNA molecules inhibits the cleavage reaction, suggesting that this nuclease activity is specific for G4 tetrastranded DNA. The existence of this enzymatic activity that reacts with G4 DNAs but not with single-stranded or Watson–Crick duplex DNAs suggests that tetrastranded DNA may have a distinct biological function *in vivo*.**

G4-DNA is a tetrastranded DNA structure in which the four strands are held together by guanine–guanine Hoogsteen base pairing (1). Such structures have a characteristic stack of guanine quartet planes and have the sugar strands in either a parallel or an antiparallel orientation (2, 3). They are formed from G-rich DNA sequences with strings of at least three contiguous guanines. These sequences can be found in evolutionarily and functionally conserved chromosomal regions, such as telomeres and the switch regions of immunoglobulin heavy-chain genes (4, 5). Although the existence of G4-DNAs *in vivo* remains to be established, we describe here an enzymatic activity specific for such G4-DNA. This finding suggests that these structures play a role *in vivo*.

In vitro studies with G-rich oligonucleotides from telomeres and the switch regions have identified four-stranded structures by their different mobilities in nondenaturing gel electrophoresis. G4-DNA generated from intramolecular folding, referred to as G-quartet DNA (3), in which the adjacent strands are in antiparallel orientation, migrates faster than its unfolded precursor. G4-DNA molecules generated from intermolecular interactions, with the adjacent strands in either an antiparallel or parallel configuration, migrate much more slowly than the unfolded oligonucleotides (1, 2). The structures of some of these tetrastranded DNAs were recently elucidated by x-ray crystal diffraction and two-dimensional NMR studies (6, 7). The formation of the four-stranded structure requires Na^+ ions, and the structure, once formed, can be further stabilized by K^+ ions (8).

In a fraction partially purified from a yeast extract, we have identified an enzyme that requires the presence of a G4 structure and cleaves the DNA in a neighboring single-stranded region. Although we do not fully understand the mechanistic details of the cleavage reaction and cofactor effects, we describe here a preliminary characterization of this G4-DNA-specific binding and cleavage reaction.

MATERIALS AND METHODS

Oligonucleotides. All oligonucleotides used in this study were made on an Applied Biosystems DNA synthesizer using phosphoramidite chemistry. The DNA sequences are listed in Fig. 1. Full-length products were isolated from 8 M urea/20% polyacrylamide gels. G4-DNAs were made according to published procedures and purified from a 6% polyacrylamide gel containing 50 mM KCl (8). G4-DNA probes were labeled at the 5′ end with T4 polynucleotide kinase and [γ-^{32}P]ATP, precipitated with 0.3 M sodium acetate in 70% ethanol, and stored in 10 mM Tris, pH 8/1 mM EDTA/50 mM KCl.

Preparation of the Protein Fraction. The yeast extract was prepared and fractionated as described (9). In brief, cells of *Saccharomyces cerevisiae* strain BJ2168 (*MATa pep4-3 prc1-407 prb-1122 ura3-52 trp1 leu2*) were harvested at midlogarithmic phase. The cell paste was frozen in liquid nitrogen, lysed in a Waring blender, and suspended in 100 mM KCl buffer A [50 mM Hepes, pH 7.5/1 mM EDTA, 1 mM dithiothreitol/10% (vol/vol) glycerol]. The crude lysate was cleared by ultracentrifugation and subjected to a 25–50% (percent saturation) ammonium sulfate fractionation. After dialysis, the ammonium sulfate fraction was purified further on a phosphocellulose column and a DNA affinity column, enriching for the DNA-binding activity of telomere-binding factor α (TBFα). The matrix of the DNA column was made from cellulose and a linearized plasmid containing a cloned human telomeric fragment carrying 27 repeats of GGGATT. Yeast proteins bound to the DNA column were eluted with a linear gradient of 0.10–0.60 M KCl in buffer A. After the DNA column, the protein concentration in the fractions was around 10 μg/ml, based on India ink staining on nitrocellulose filter with bovine serum albumin as standard. All the work presented here was done with the material from the DNA column fractions 27–30, as shown in Fig. 2, which we will simply refer to as the protein fraction.

Bandshift Assay. The assay for TBFα binding activity on an agarose gel was performed as described (9). To assay for the G4-DNA-specific binding and cleavage activity, aliquots of protein fractions were mixed with labeled G4-DNA probes (1–5 fmol or as specified) in a final volume of 20 μl containing binding reaction buffer (20 mM Hepes, pH 7.5/50 mM KCl/5 mM $MgCl_2$/5–10% glycerol containing *Escherichia coli* single-stranded DNA at 0.1 μg/ml), incubated at room temperature for 10 min or as specified, and loaded on a 5 or 6% polyacrylamide gel precooled to 4°C. The electrophoresis was carried out in Tris/borate/EDTA buffer containing 50 mM KCl in the cold room at about 7 V/cm, until the bromophenol blue dye had run two-thirds of the full-gel length. The gel was then dried and an autoradiogram was made. For quantitation, the dried gels were directly scanned and analyzed on a blot analyzer.

Analysis of the Cleavage Product. Cleavage reactions were carried out as described above. At the end of the incubation, 20 μl of 10 mM EDTA was added to stop the reaction. Then,

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Abbreviation: TBF, telomere-binding factor.
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P	(49mer):	5′-GGGACCAGACCTAGCAGCTATGGGGGAGCTGGGGAAGGTGGGAATGTGA-3′
TP	(49mer):	5′-TGGACCAGACCTAGCAGCTATGGGGGAGCTGGGGAAGGTGGGAATGTGA-3′
TP-S	(16mer):	5′-TGGACCAGACCTAGCA-3′
CTP-S	(18mer):	5′-GCTGCTAGGTCTGGTCCA-3′
LBG	(35mer):	5′-TAGGGTAGTGTTAGGGTAGTGTTAGGGTGTGGGTG-3′
Oxy-16	(16mer):	5′-TTGGGGTTTTGGGGTT-3′
P55	(55mer):	5′-ATATGATGGACCAGACCTAGCAGCTATGGGGGAGCTGGGGAAGGTGGGAATGTGA-3′
M2	(35mer):	5′-TAGTCCAGGCTGAGCAGGTACGGGGGAGCTGGGGT-3′
M2-S	(21mer):	5′-TAGTCCAGGCTGAGCAGGTAC-3′
HT-G	(29mer):	5′-TCGAGTTAGGGTTAGGGTTAGGGTTAGGG-3′
HT-C	(29mer):	5′-TCGACCGTAACCCTAACCCTAACCCTAAC-3′

FIG. 1. Oligonucleotides used in this work.

1 ml of 1-butanol was added to each reaction mixture in an Eppendorf centrifuge tubes. After brief vortex mixing, DNA was recovered as pellets by centrifugation in an Eppendorf centrifuge for 2 min at full speed. The organic liquid was discarded and the DNA pellets were air-dried for 2–5 min. To open a G4 structure while avoiding nonspecific strand scission, DNA samples were treated in one of the following ways. The DNA pellets were resuspended in 50 μl of 0.1 M NaOH, incubated at 60°C for 10 min, butanol-extracted, suspended in 5 μl of formamide loading dye containing 0.1 M NaOH, and, after being heated in a 90°C water bath for 1 min, loaded on a 20% sequencing gel containing either 8 M urea or 50% formamide. Alternatively, DNA pellets from the cleavage reaction were directly suspended in 5 μl formamide loading dye with 0.1 M NaOH, heated in a 90°C water bath for 5 min, and loaded on a 20% sequencing gel. After electrophoresis, autoradiograms were made from the gels with Kodak XAR-5 film. Sequencing ladders were generated according to the Maxam–Gilbert method (10).

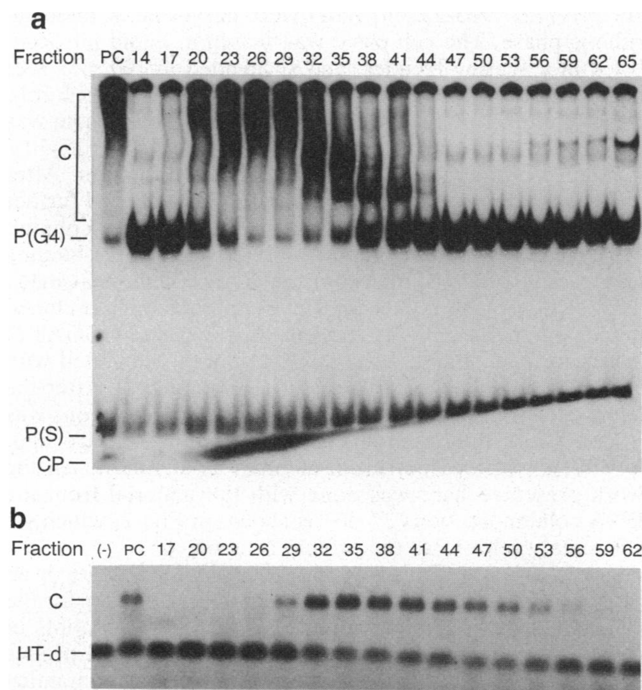

FIG. 2. Column elution profile for activities eluted from a DNA affinity column containing human telomeric DNA cloned in a plasmid. (*a*) G4-DNA binding and cleavage activities. Aliquots of 10 μl from the column fractions were mixed with radioactively labeled P(G4), a G4 tetrastranded DNA, at 0.025 nM. The mixtures were incubated at room temperature for 10 min and then loaded on a 5% polyacrylamide gel buffered with 50 mM KCl in Tris/borate/EDTA buffer. After electrophoresis, the gel was dried and an autoradiogram was made. Labels at left: P(G4), G4-DNA generated from P oligonucleotide (see Fig. 1); P(S), single-stranded P oligonucleotide; C, protein–P(G4) DNA complexes; CP, cleavage product of P(G4). Protein fractions assayed were the partially purified yeast fraction that was loaded onto the column (lane PC) and every third column fractions from 14 to 65. (*b*) Elution of TBFα activity. Aliquots of 10 μl from the same fractions as those assayed in *a* were used in an agarose gel bandshift assay. The probe (2 fmol per assay) was TCGA(CCCTAA)$_4$·TCGA(TTAGGG)$_4$ duplex DNA (HT-d) labeled by filling in with Klenow DNA polymerase.

RESULTS

Identification of a G4-DNA Binding and Cleavage Activity. Liu and Tye (9) previously identified a telomere-binding protein in yeast, TBFα, by means of its specific binding to human telomeric DNA. In exploring further the properties of this protein, we found that the partially purified TBFα fraction also contained a binding activity that is specific for G4-DNA. Fig. 2*b* shows the elution profile of TBFα from a DNA affinity column consisting of a cloned human telomeric DNA, assayed by its binding to a (CCCTAA)$_4$·(TTAGGG)$_4$ duplex. The elution peak of this binding activity centered at fraction 35. While assaying the same column fractions with a G4-DNA probe [P(G4), see Fig. 1 for a list of the oligonucleotides used in this work], we detected a binding activity eluted from the column (Fig. 2*a*, fractions 23–39) at a lower salt concentration. In these same fractions, another migrating species, CP, appears, moving faster than the single-stranded form of the P oligonucleotide. The simplest explanation is that CP is a cleavage product of the P(G4) molecule, since single-stranded probe, P(S), was unaffected. We conjectured that these column fractions contained a nuclease specific for G4-DNA.

Characterization of the Binding and Cleavage Activities. Fig. 3*a* shows that both the binding and cleavage activities require a protein component. Preincubation of the protein fraction at 65°C for 15 min completely inactivated the cleavage activities and partially inactivated the G4-DNA binding activity (lane 3). Treatment with a detergent (lane 4) or digestion with proteinase K (lane 5) inactivated both the binding and the cleavage activities. Furthermore, the inactivation caused by proteinase K was blocked by a serine-protease inhibitor (lanes 6). The single-stranded TP oligonucleotide exhibited no binding and cleavage (compare lane 8 with lane 2). These results suggest that the binding to and cleavage of TP(G4) are probably linked events and that the G4 structure present in the TP(G4) DNA plays a fundamental role in these reactions.

Fig. 3*b* shows a time course of the cleavage reaction analyzed in a 6% nondenaturing gel. Binding to G4-DNA probe seems to precede the cleavage event, since at an early time point (lane 3) most of the TP(G4) material is in the bound

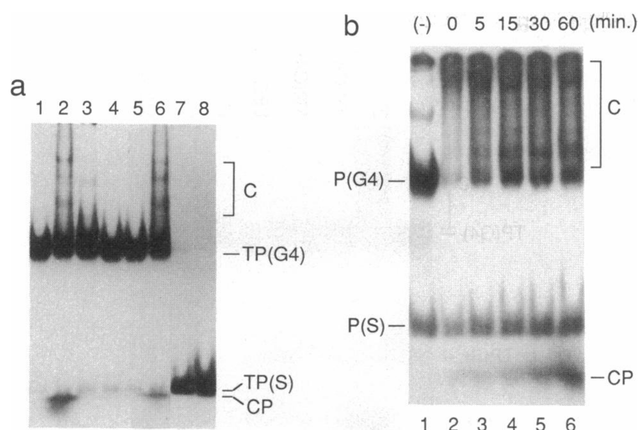

FIG. 3. Characterization of the G4-DNA binding and cleavage activities. (*a*) Inactivation of the cleavage activity. The G4-DNA probe used in the reactions shown in lanes 1–6 was generated from the TP oligonucleotide. The radioactively labeled DNA probe used in reactions shown in lanes 7 and 8 was a single-stranded TP oligonucleotide. TP(S), single-stranded TP oligonucleotide; TP(G4), G4-DNA generated from TP; C, protein–TP(G4) DNA complexes; CP, cleavage product. The assay was the same as described for Fig. 2*a* except that 5-μl aliquots from the DNA column fraction 29 were pretreated for 15 min as follows: lane 2, no treatment; lane 3, heating in a 65°C water bath; lane 4, incubation in the presence of 1% SDS at room temperature; lane 5, digestion with 0.5 μg of proteinase K at room temperature; lane 6, the same as lane 5 except that phenylmethanesulfonyl fluoride was added to a final concentration of 0.1 mM in the reaction mixture. No protein was added in reactions shown in lanes 1 and 7, while the same amount of protein was used in lanes 2 and 8. (*b*) Time course of the cleavage reaction. P(G4) probe, radioactively labeled at the 5′ end, was incubated with 5 μl of the protein fraction at room temperature for the time specified at the top, and then loaded on a 5% polyacrylamide gel and treated as described for Fig. 2*a*. No protein was added in the reaction shown in lane 1, and bands migrating more slowly than P(G4) were probably due to higher-order structure formed between P(G4) molecules (11).

complex and yet very little cleavage product has been produced, which suggests that the binding to the substrate is rapid whereas the ensuing cleavage is slow. The amount of cleavage product increases linearly with the reaction time over the first 30 min (data not shown).

The Cleavage Products. We analyzed the products of the cleavage reaction on sequencing gels. Fig. 4*a* shows that when the probe is TP(G4) labeled at the 5′ end, there is only one major product, which migrates like a dinucleotide with a 3′-OH end. The time course shown in lanes 4–9 suggests that the dinucleotide is either the initial cleavage product or the only product of the reaction, since we detect no other larger fragments that would correspond to cleavages more distal from the 5′ end. Fig. 4*b* shows the cleavage sequences for this and two other G4-DNA substrates, P55(G4) and M2(G4). The cleavage site shifts on P55(G4), compared with TP(G4), even though this molecule is essentially the same as TP(G4) except for a six-nucleotide extension at the 5′ end. There is no sequence consensus or invariant distance that serves as the signal for the cleavage reaction.

Specificity of the Cleavage Reaction. Since the cleavage reaction occurs in a single-stranded region, is the G4 structure required for the reaction? Fig. 5 shows that TP oligonucleotide in single-stranded form cannot be cleaved, in contrast to its G4 form (lane 4 and lane 2, respectively). Furthermore, a single-stranded and truncated TP oligonucleotide, TP-S, corresponding to the 17 nucleotides on the 5′ end of TP, is not cleaved (lanes 3 and 10), consistent with the notion that G4 structure is required for the cleavage reaction. We also converted this truncated TP into a duplex form by annealing it to its complementary strand, CTP-S. Fig. 5, lanes

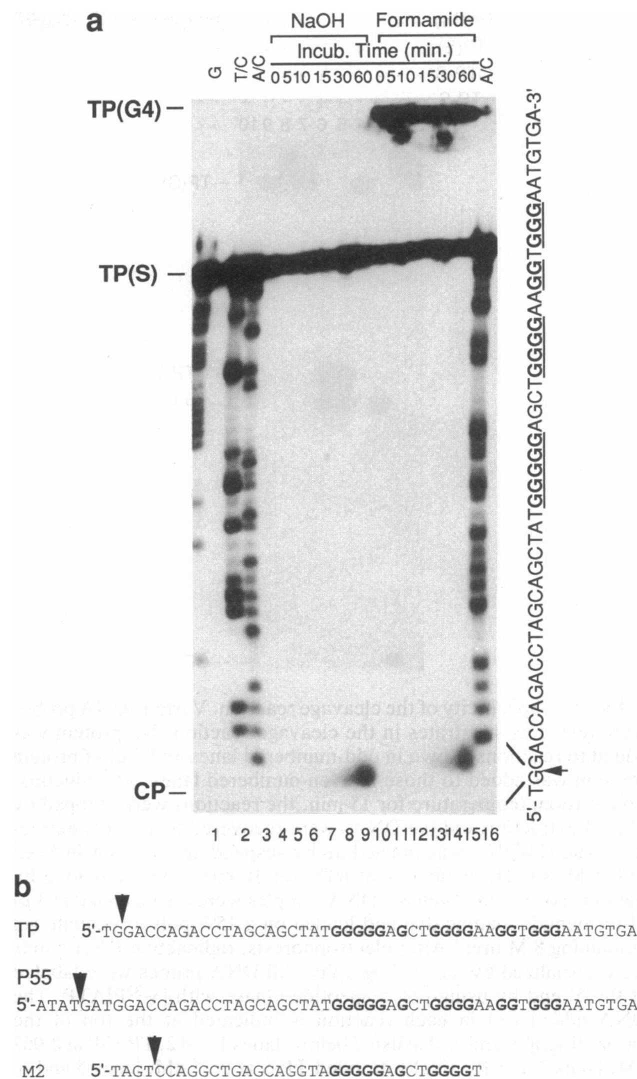

FIG. 4. Analysis of the 5′-end cleavage products on G4-DNAs generated from three different oligonucleotides. (*a*) Cleavage product on TP(G4). Aliquots of 2.5 μl of the protein fraction were incubated with 0.125 nM 5′-end-labeled TP(G4) for various lengths of time, as indicated at the top of the autoradiograph, and then phenol-extracted once. For each sample, the aqueous phase was split in half and the labeled DNA was recovered by butanol extraction. Half of the sample was directly suspended in 3 μl of formamide loading dye. The other half was treated with 0.1 M NaOH as described for Fig. 5, extracted with butanol to dried pellets, and suspended in 3 μl of formamide loading dye. All samples were heated in a 90°C water bath for 2 min, quickly cooled on ice, and then loaded on a 20% polyacrylamide gel containing 50% formamide as follows: lanes 4–9, alkaline-treated samples; lanes 10–14, samples not subjected to alkaline treatment. The incubation time for a particular reaction is indicated at the top. No protein was added in the reaction shown in lanes 4 and 10. Lanes 1, 2, 3, and 16 show Maxam–Gilbert sequencing ladder of 5′-end-labeled TP oligonucleotide. The actual sequence of TP is shown at right, in which the underlined G residues were involved in guanine-quartet formation. Arrow marks the cleavage site. (*b*) The 5′-end cleavage site observed on three different G4-DNAs. The oligonucleotide sequences are aligned based on their G-rich motifs that are engaged in four-stranded DNA formation, as indicated by the G residues in bold type. Arrowheads mark the deduced cleavage sites that give rise to the observed 5′-end cleavage products.

5 and 6, shows that this duplex DNA cannot be cleaved either. Finally, we annealed CTP-S to TP(G4), which converts the 5′ single-stranded region to a Watson–Crick duplex form but leaves the G4 structure intact. This hybrid shows no

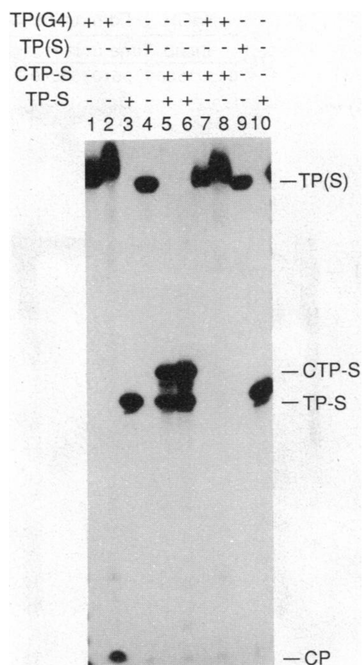

FIG. 5. Specificity of the cleavage reaction. Various DNA probes were tested as substrates in the cleavage reaction. No protein was added to reactions shown in odd-numbered lanes and 5 μl of protein fraction was added to those in even-numbered lanes. After incubation at room temperature for 15 min, the reactions were stopped by phenol extraction and the DNAs were recovered by butanol extraction. The G4-DNA was opened up by suspending the DNA in 50 μl of 0.1 M NaOH, incubating at 60°C for 10 min, and recovering by butanol extraction. Then the DNA samples were resuspended in 3 μl of formamide loading dye and loaded on a 15% polyacrylamide gel containing 8 M urea. After electrophoresis, radioactive DNA bands were visualized by autoradiography. All DNA probes were labeled at the 5′ end by using polynucleotide kinase with [γ-^{32}P]ATP. The DNA added (+) in each reaction is indicated at the top of the autoradiograph and is also listed below: lanes 1 and 2, TP(G4) at 0.063 nM; lanes 3 and 10, single-stranded TP-S at 0.25 nM; lanes 5 and 6, duplex DNA formed between CTP-S and TP-S at 0.25 nM (both oligonucleotides were labeled at the 5′ end); lanes 7 and 8, unlabeled CTP-S annealed to 5′-end-labeled TP(G4) at a ratio of 0.25 nM/0.063 nM; lanes 4 and 9, single-stranded TP oligonucleotide at 0.25 nM.

cleavage (lanes 7 and 8). These experiments demonstrate that both the G4 structure and the single-stranded region are required for the cleavage reaction; furthermore, a Watson–Crick duplex or single-stranded DNA alone is not an effective substrate.

Saturation Competition of Both the Binding and the Cleavage Activity by Various DNAs. What is the role of the G4 structure in the cleavage reaction? If binding to G4 were required for the cleavage reaction, one would expect that competition for the binding should correlate with an inhibition in the cleavage reaction. We reasoned that a competition in this enzymatic recognition could be achieved only at a high substrate concentration where the cleavage reaction proceeds at V_{max} and the addition of more substrate to the reaction, such as a binding competitor, will no longer simply increase the reaction rate. The cleavage reaction can be saturated by a G4-DNA concentration of 10 nM (data not shown). Then the further addition of a 50-fold molar excess of unlabeled competitor DNA produces both a competition for the binding and an inhibition of the cleavage only when G4 structure is present in the competitor molecules (Fig. 6*a*, lanes 3–6). The four-stranded DNA molecules generated from TP and M2 oligonucleotides share similar G4 structure but differ in their single-stranded regions, suggesting that the sequences in the single-stranded region do not affect the

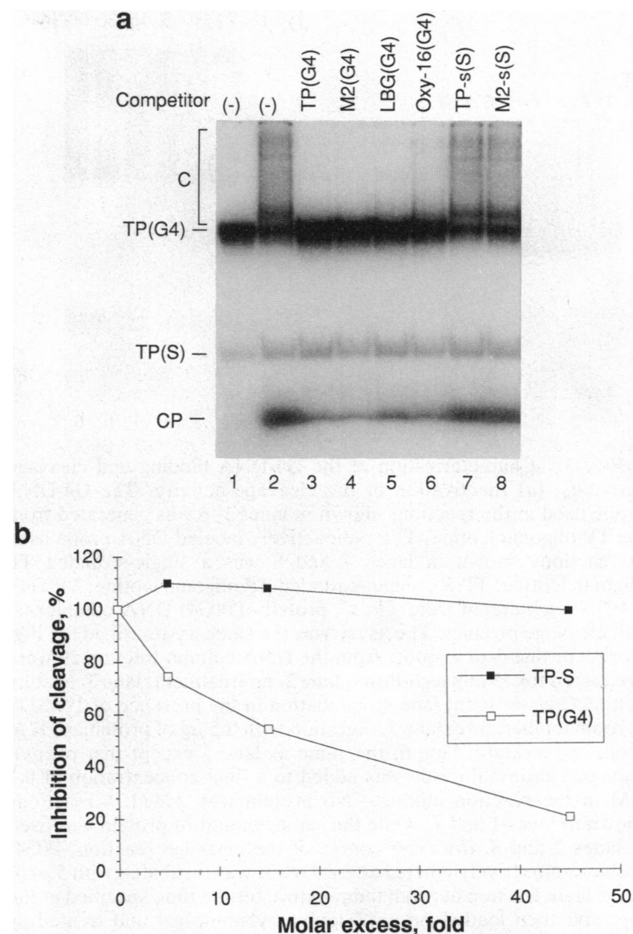

FIG. 6. Saturation competition of the binding and cleavage reactions on 5′-end-labeled TP(G4). (*a*) Each reaction mixture contained 10 nM labeled TP(G4) probe and 500 nM nonradioactive G4-DNAs or 2.0 μM nonradioactive single-stranded DNAs as indicated at the top. No specific competitor was added in reactions shown in lanes 1 and 2 except single-stranded *E. coli* DNA present in the reaction buffer at 5 μg/ml, and no protein was added to the reaction shown in lane 1. For lanes 2–8, aliquots (5 μl) of the protein fraction were mixed with the DNA probes in the reaction buffer, incubated at room temperature for 20 min, and loaded on a 6% polyacrylamide gel. TP(S), single-stranded TP oligonucleotide; TP(G4), G4-DNA generated from TP; CP, cleavage product. In this set of experiments, the final concentration of glycerol was 2.5%, and that of KCl was about 60 mM. (*b*) Inhibition of the cleavage activity by competition with TP-S and TP(G4) oligonucleotides. Cleavage reactions were carried out as described in *a*. The molar excess of TP-S was calculated on the basis of its ratio to the 5′ single-stranded region of TP(G4) probe in the reaction mixture. The percentage of cleavage activity was derived by dividing the amount of CP generated in reaction mixtures containing competitor with that from the no-competitor reaction mixture.

binding and that a 3′ single-stranded region on the G4 structure is not required for the binding and the cleavage reaction. G4-DNAs generated from LBG and Oxy-16, oligonucleotides derived from telomeric G-rich strands from yeast (*S. cerevisiae*) and ciliated protozoans (*Oxytricha*) respectively, have G4 structures different from that of TP(G4). They effectively competed with the TP(G4) probe for binding and inhibited the cleavage activity.

Two single-stranded oligonucleotides, corresponding to the truncated 5′ single-stranded regions of TP and M2 respectively, did not affect the binding or cleavage at a 50-fold molar excess (Fig. 6*a*, lanes 7 and 8). Fig. 6*b* shows that, over a range up to a 45-fold molar excess relative to the TP(G4) probe, TP-S did not affect the cleavage reaction, whereas

TP(G4) consistently inhibited the reaction. Thus, we conclude that protein binding to the G4 structure is required for the cleavage reaction and that the observed cleavage activity is specific for G4 tetrastranded DNA.

DISCUSSION

We describe here a nuclease activity that is specific for G4-DNAs and makes a cut in a single-stranded region 5′ to the G4 domain. The requirement for G4-DNA was established by examining the action of this enzyme on single-stranded and G4-DNA substrates and by saturation competition. In each case, we observed cleavage only when there was binding to a G4-DNA probe; with a single-stranded sequence corresponding to the cleavage substrate, we saw no binding and no cleavage. This line of interpretation is further supported by the results of saturation competition experiments, where we observed competition for binding and cleavage by several tetrastranded DNAs with different G4 motifs but no competition by the single-stranded region of the substrate. So far, we do not know the specificity of the cleavage reaction except that it occurs in a single-stranded region within a certain distance from the G4 structure, with no apparent sequence specificity. The nuclease activity described here could well be part of a general enzymatic machinery that interacts with G4 tetrastranded DNA.

This nuclease activity was found in the course of an investigation of proteins that bound to duplex telomeric DNA. In this search, Liu and Tye (9) identified a yeast protein, TBFα, that can bind to duplex telomeric DNA from yeast, human, and *Tetrahymena*. We conjecture that TBFα is involved in regenerating the telomere *in vivo*, because its general telomere-binding specificity correlates with the observation that heterologous telomeres can serve as substrates for terminal addition in yeast (12, 13). The coincidence that the G4-DNA binding and cleavage activity described here was found in a partially purified TBFα fraction suggests that this activity and G4-DNA might be involved in telomere regeneration. It is not clear how the G4-DNA binding and cleavage activity was retained on the human telomeric DNA column, although a tempting explanation is that TBFα is actually a part of this G4-DNA binding complex, which was disrupted during the elution before TBFα was dissociated from the telomeric DNA.

One possible function for this enzyme could be to remove the fold-back structures, or G-quartets, that might appear at the ends of a linear chromosome, in order to allow the extension of the G-rich strand by telomere terminal transferase. The physical ends of linear chromosomes are G-rich 3′ overhangs, synthesized by a telomerase. However, a telomeric G-rich oligonucleotide from *Oxytricha* readily forms, under physiological conditions, an intramolecularly folded structure that cannot be utilized by *Oxytricha* telomerase (14). This would inhibit telomere replication *in vivo*. The cleavage activity described here could, in principle, provide a release from such inhibition by G-quartet DNA, by removing this stable structure to make chromosomal ends accessible to telomere terminal transferase.

Another role for a nucleolytic enzyme specific for G4-DNA could be envisioned in the context of telomere–telomere recombination or switch recombination. Telomere–telomere recombination occurs between the heterologous telomeric ends of linear plasmids in yeast (15, 16). The recombined telomeric DNA segments share little sequence similarity except their G-richness. This is reminiscent of switch recombination of immunoglobulin heavy-chain genes: the switch regions where recombination occurs are invariably G-rich. Given the highly conserved G-richness in telomeres and switch regions, we hypothesize that these nonhomologous recombination events are mediated by a tetrastranded DNA structure.

G4-DNA has been postulated to play a role in meiotic chromosome pairing (1). This G4-DNA dependent nuclease activity may be part of a complex that permits the DNA to untangle. Overall, the existence of a nucleolytic activity specific for a G4-DNA region suggests that this unusual DNA structure may play a role *in vivo*.

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