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Visualization of Long Human Telomere Mimics by Single-Molecule Fluorescence Imaging

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Abstract

Study of long single-stranded telomeric DNA is important for a variety of basic science and biotechnological applications, yet few methods exist for synthesis and visualization of single copies of this DNA in solution at biologically relevant length scales necessary for assessment of heterogeneity in its structure and behavior. We have synthesized kilobase-long single-stranded human telomere mimics *in situ* by rolling circle replication (RCR) on a microscope coverslip surface and visualized individual strands by staining with SYBR Gold. Under buffer flow, differential extensibility and varying morphology of these long telomere-mimicking DNA sequences were observed at the single-molecule level in real time. Using this procedure, we detected striking differences in the extensibility of individual RCR products based on the human G-rich telomeric sequence in the presence and absence of short, complementary single-stranded oligonucleotides. We also apply this new mode of single-stranded DNA characterization to probe the interaction of kilobase-length telomere mimics with the small-molecule G-quadruplex-binding agent TMPyP4.

Over the past decade, significant effort has been dedicated to characterizing the higher-order structures in repeating guanine-rich DNA sequences, or telomeres, present at the ends of eukaryotic chromosomes.[1] Given the important role of telomeres in cellular aging and the proliferation of cancer cells,[2] a more detailed understanding of the structure and function of telomeric DNA holds promise as a means for researchers to regulate these critical cellular processes; accordingly, the G-quadruplex motif in human telomeric DNA has recently been highlighted as an attractive target for new anticancer therapeutics.[3]

In human somatic cells, telomeres are typically 5 to 8 kb in length, with a 3′ single-stranded overhang of a few hundred bases.[4] There is little consensus as to the precise structure of telomeres in vivo;[5]however, most in vitro experiments have been performed with short (<100 nt) single-stranded DNA (ssDNA) telomere mimics that display high conformational heterogeneity.[6] Current telomere structural models would benefit from refinement via the study of longer sequences that more accurately mimic those found in nature. Indeed, long-range structure is widely believed to be important in telomere function.[7] More generally, long-range interactions in DNA are increasingly relevant to biology, as seen in studies of viral packaging of DNA[8] and the exploration of a nucleosome code for gene expression,[9] both of which employ long-distance information about DNA conformation and subsequent interactions in cells.

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Supporting Information **Available:** Experimental procedures, analyses, spectroscopic data, and DNA stretching videos. This material is available free of charge via the Internet at http://pubs.acs.org.

We present here the proof-of-principle of a method for the preparation of surface-immobilized kilobase-scale telomere-mimicking ssDNAs and their subsequent visualization by single-molecule (SM) fluorescence imaging in the presence of applied buffer flow (Figure 1). Rolling circle replication (RCR) offers a means of rapidly producing long (>1000 nt) ssDNA strands from a small synthetic circular template, which can be designed to encode the $(TTAGGG)_n$ human telomeric sequence.[10] With the use of surface-tethered DNA primers, we have prepared telomere-mimicking RCR products on a solid substrate without the need for extensive sample handling or manipulation before analysis, factors that have complicated the study of long ssDNA in the past.[11] This allows us to address the inherent conformational heterogeneity of the human telomeric sequence by using SM fluorescence techniques to visualize the DNA, thereby avoiding information loss from ensemble averaging that can occur in traditional assays such as gel electrophoresis, thermal melting, circular dichroism, or bulk fluorescence. A small number of single-molecule G-quadruplex studies have been reported,[6,12] but to our knowledge, our RCR-produced strands are the longest telomere-like sequences studied at the single-molecule level.

In order to synthesize kilobase-long telomere mimics for single-molecule observation, rolling circle replication was performed in flow cells using biotinylated primers attached to PEG-coated coverslips.[13] Bacteriophage phi29 DNA polymerase was added to flow cells containing 1 or 5 nM primer-template and incubated at room temperature for 30 min. SYBR Gold dye (Invitrogen) was then added to the sample chamber by syringe pump at a dilution of 1:10 000 to give a final concentration of 2 μM. Applied buffer flow was used to stretch the DNA[8] (illustrated in Figure 1B) in order to visualize the distribution of strand lengths in a given sample. Single molecules were imaged using a total-internal-reflection wide-field microscope with laser excitation at 532 nm, and fluorescence images were captured on a cooled EMCCD with 50 ms time resolution. Two different sequence repeats were produced by RCR, one based on the human telomeric sequence (HT54), and one control sequence consisting of the same base composition as HT54 but in scrambled order (SCR). The HT54 repeating sequence is identical to the human telomere repeat $(TTAGGG)_n$ except that five of the G quadruplex (TTA) loop sequences out of nine were altered to (TCA) and (TAT) to simplify circular DNA synthesis.[14] All GGG repeats are intact and occupy the same positions as in human telomeric DNA.

Observing these tethered repeating DNAs under buffer flow, we noted that, for both HT54 and SCR repeats, strand extensibility was far greater when short ssDNA complements were added to the sample chamber before imaging (Figure 2; video in Supporting Information).

Sets of short single-stranded DNA complements were designed to bind to the rolling circle replication products as diagrammed in Figure 2B; these were added to RCR products at a concentration of 50 μM and incubated for 10 min at room temperature prior to the addition of SYBR Gold. In general, the mean detected length of HT54 strands after complement addition was greater than that for SCR strands, even though the strand lengths for both sequences in the absence of complement were often nearly identical (Figure 2C). We believe this is due to the repetitive sequence of HT54 itself, which allows for more extensive conversion to dsDNA in the presence of a single complement sequence.

The fact that both HT54 and SCR sequences exhibited micron-scale changes in extensibility in the flowing buffer is not surprising, as prior to hybridization, the long RCR product ssDNA presumably possesses a multitude of intrastrand secondary structures that limit the extension of the biopolymer. Upon complement hybridization, many short regions of the DNA undergo a substantial increase in persistence length (from ~3 nt for ssDNA to ~150 bp for dsDNA).[15] Intrastrand interactions are likely disrupted as complement binding rigidifies segments of the DNA, and we observed sequence-specific elongation of the hybrid ssDNA/dsDNA strands.

This marked change in effective length facilitates the collection of SM strand length distributions and real-time viewing of strand dynamics. Various buffer flow rates were tested, and under our experimental conditions it was found that an input rate of 4 mL/min was sufficient to stretch the labeled DNA to its maximum observable length within 0.5 s after manually activating the pump. As shown in Figure 3, this maximum length remained constant over the duration of the imaging period under conditions selected to minimize DNA photodamage.

The observed extended lengths are consistent with repeating DNAs that are at least 1500 nt in length (see Supporting Information for length estimation details), similar in size to native human telomeres.

We next asked whether aspects of folded structure in long, single-stranded telomere mimics could be interrogated by flow-stretching in the presence of 5,10,15,20-tetra-(N-methyl-4-pyridyl)porphine (TMPyP4), a small-molecule G-quadruplex-binding agent.[16] Circular dichroism measurements in solution were used to verify that one discrete segment of the HT54 sequence displayed spectroscopic signatures diagnostic of quadruplex formation under the buffer conditions used (data in Supporting Information). In the single-molecule extension studies, when 100 nM TMPyP4 (Figure 4A) was added to the RCR reaction mixture, followed by SYBR Gold and 18 nt complement, the result was a 4-fold decrease in the mean apparent strand length in telomere-mimicking samples, whereas the mean detected length of scrambled control strands remained largely unchanged (Figure 4B). This is consistent with extensive intramolecular folded structure that is GGG-repeat-dependent and stabilized by the known quadruplex-binding molecule. Moreover, there is precedent for this magnitude of length change in biological DNA sequences in the presence of added stabilizing agents: dramatic compaction of 48 500 bp λ-DNA has been observed in single-molecule and scanning force microscopy (SFM) assays, where the addition of histone proteins produced between 7- to 17-fold changes in detected DNA length, and higher-order structure in this system was manifested as clusters in SFM images of individual strands.[17] It should be noted that some G-quadruplex-stabilizing agents are known to be capable of arresting DNA synthesis,[18] and so the observation of shorter RCR product strands synthesized in the presence of TMPyP4 might simply be due to inhibition of phi29 DNA polymerase by the small molecule. Even if this effect is present to some extent, our assay still reports large differences in the sequence specificity of the interaction of TMPyP4 with long, repeating ssDNA sequences. In addition to acquiring static length measurements, we also observed the altered morphology and dynamic stretching behavior of telomere mimic strands in the presence of TMPyP4 (Figure 4C,D; video in Supporting Information). Instead of homogeneously labeled strands, we detected heterogeneous clusters of fluorescence that migrated at varied rates in the direction of buffer flow, also consistent with extensive folded structure induced by TMPyP4. Further experiments are necessary to determine the nature of these clusters, including further optimization of RCR reaction conditions for the production of specific strand lengths as a function of reaction time, thereby facilitating more precise structural studies with our flow-stretching procedure. Nevertheless, these preliminary data clearly demonstrate the utility of our single-molecule approach for acquiring long-distance morphological and dynamic information for telomeric DNA that would otherwise be unattainable with conventional biochemical methods.

In summary, we have visualized kilobase-length DNA strand mimics of human telomeres by a novel SM detection scheme combining RCR preparation of long telomere-like ssDNA repeats and flow stretching of hybrid ssDNA/dsDNA telomere repeat constructs. Using this procedure, we have directly observed striking changes in the extensibility of individual RCR products upon addition of short complementary ssDNA. Other research groups have used length change at the single-molecule level as a readout of DNA structure and interaction with other biomolecules,[17,19,20] but to our knowledge ours is the first reported use of flow stretching to study ssDNA structure without the use of attached beads and optical or magnetic tweezers.

Although this diminishes our ability to exert precise and rapidly tunable forces on the DNA, it greatly simplifies experimental implementation. We have also applied this new mode of DNA characterization to explore the intramolecular structure and interaction of telomere mimics with a small-molecule G-quadruplex-binding agent. These initial experiments have allowed us to qualitatively characterize a naturally derived DNA repeat at previously unexplored length scales while demonstrating the potential for interrogating real-time DNA dynamics such as strand breakage/disappearance and relaxation rates that are difficult or impossible to access via conventional methods of probing surface-immobilized RCR products, such as bulk fluorescence[21] or AFM.[10,22] Our visualization scheme can also be easily adapted to the study of other repeating DNA sequences of biological import, such as centromeres[23] and triplet repeat expansions,[24] thereby adding to the toolboxes of researchers in disciplines ranging from polymer physics to bioanalytical chemistry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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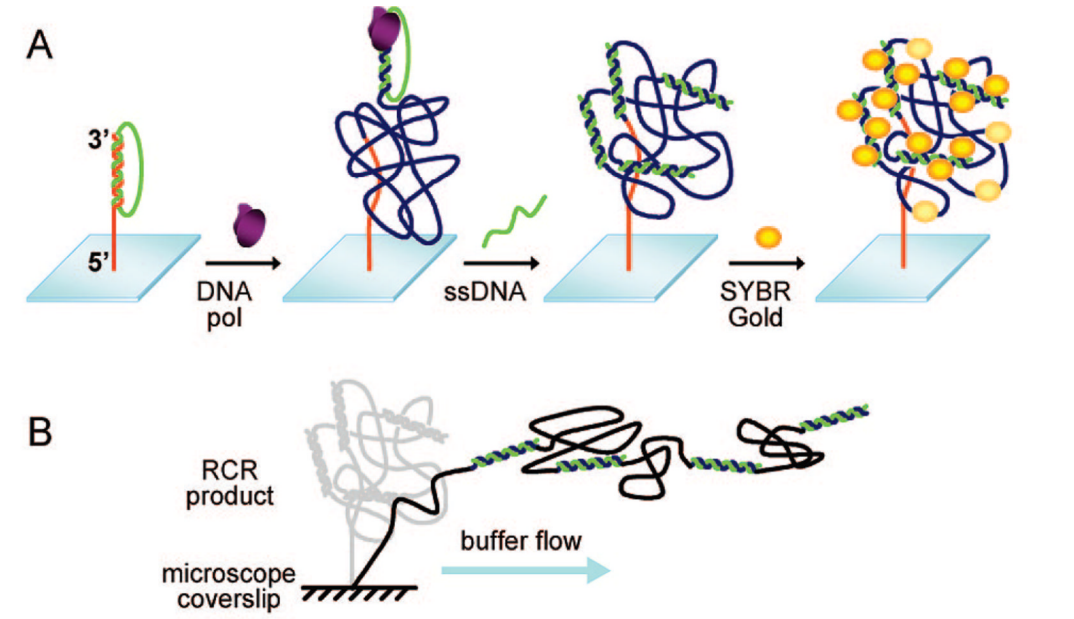

Figure 1.

(A) Schematic diagram of synthesis of long telomere mimics by rolling circle replication (RCR). DNA polymerase is incubated with a surface-immobilized primer and small circular template; after elongation, the repeating RCR product is partially converted to double-stranded DNA (dsDNA) with the addition of short ssDNA complements, and SYBR Gold dye is used for fluorescence detection. (B) Schematized extension of end-tethered RCR product strand with applied buffer flow (dyes are omitted here for clarity). Also shown are the sequences of human telomere mimic HT54 and scrambled control SCR strands investigated in this study (one 54 nt repeat shown).

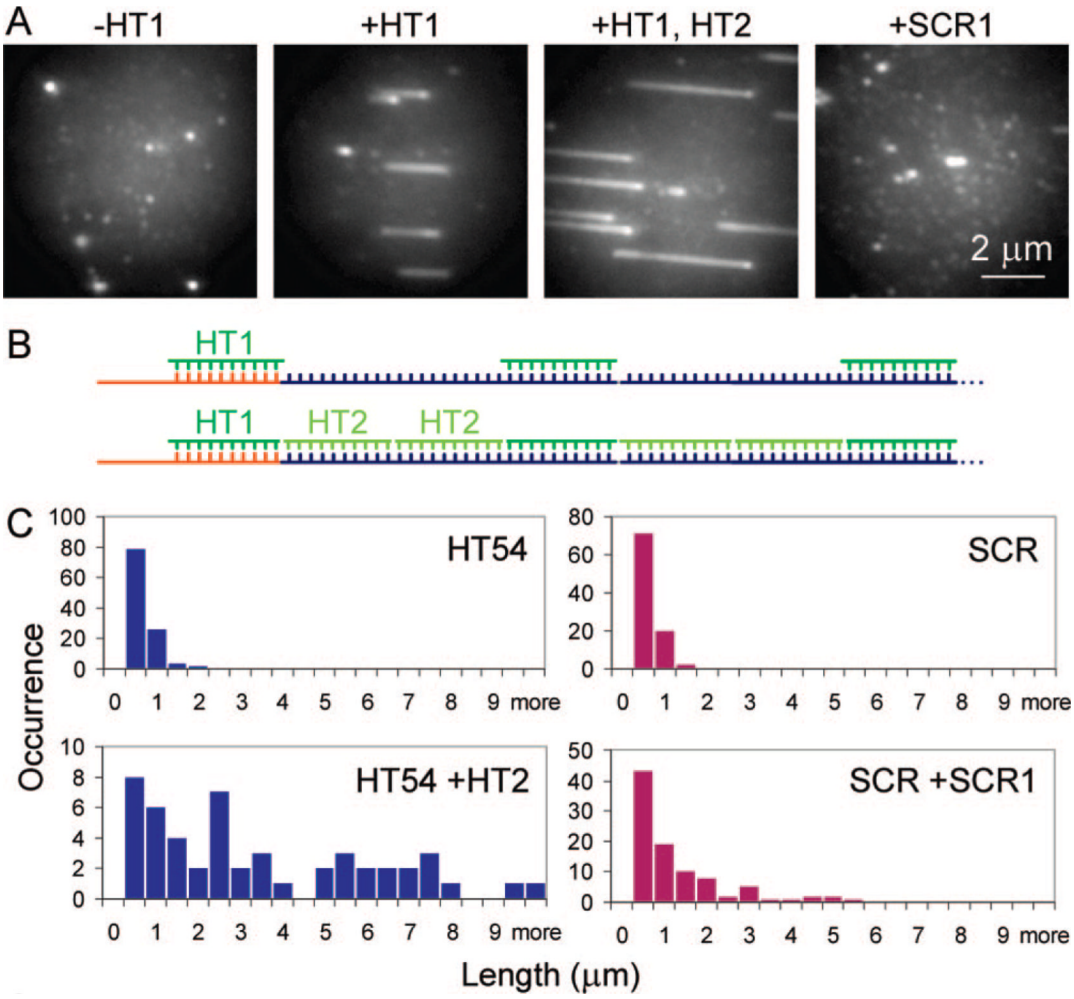

Figure 2.

(A) Representative single-molecule fluorescence images (150 × 150 pixels, 50 ms integration, 4 mL/min applied buffer flow) of flow-extended human telomere mimics (original primer-template concentration: 1 nM) showing sequence-specific response to the addition of 50 μM complementary ssDNA HT1 and HT2 and noncomplementary SCR1. (B) Schematic diagram of hybridization pattern of HT1 and HT2 complements to the telomeric repeats in HT54 (orange = primer, blue = RCR product). (C) Single-molecule length histograms for flow-extended HT54 and SCR strands before and after addition of HT2 and SCR1, respectively. Mean strand lengths before complement addition: 0.47 μm (HT54; N = 111), 0.45 μm (SCR; N = 94). Mean strand lengths after complement addition: 3.22 μm (HT54; N = 50); 1.11 μm (SCR; N = 94) (see Supporting Information for length estimation details).

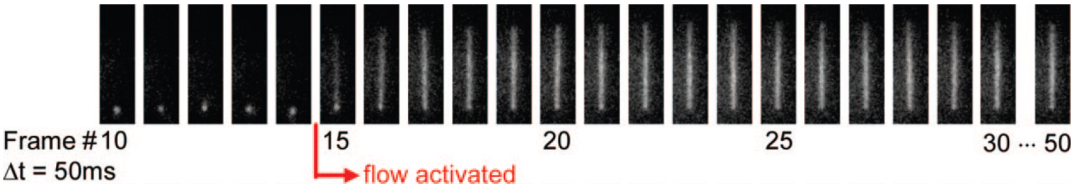

Figure 3.
Flow extension of a single DNA strand (HT54 telomere mimic) in the presence of HT1, HT2, and SYBR Gold (vertical scale = 4 μm).

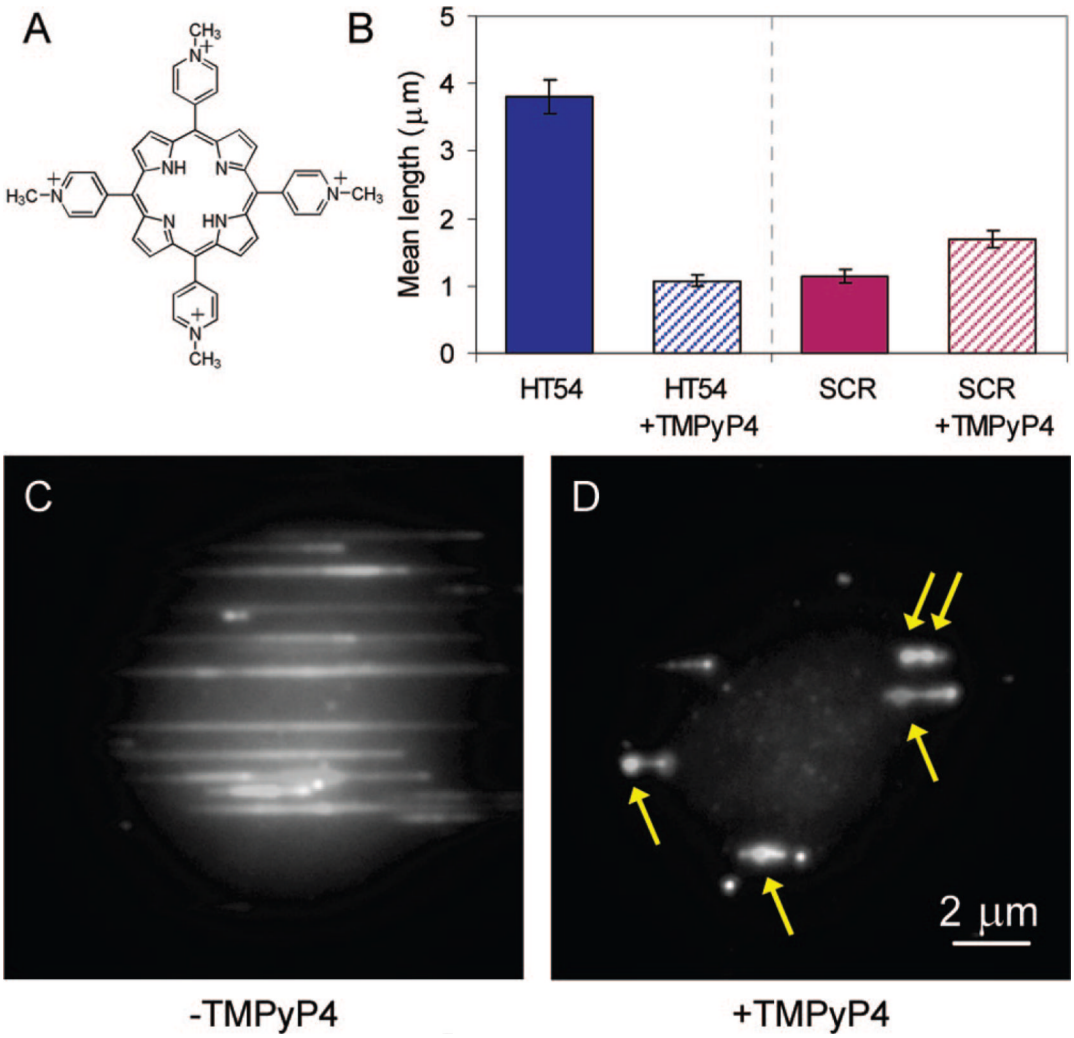

Figure 4.

(A) Structure of TMPyP4. (B) Mean length histograms derived from measurements of individual molecules of HT54 and SCR DNA in samples containing no TMPyP4 (solid bars; N = 182,193) or 100 nM TMPyP4 (dashed bars; N = 184,247). Strand lengths were measured in the presence of HT2 complement (for HT54 samples) or SCR1 (for SCR samples). (C,D) Single-molecule images of flow-extended telomere mimics in the presence of HT2 and SYBR Gold (see video in Supporting Information). Yellow arrows indicate fluorescent clusters, absent in samples where TMPyP4 was not added.