Telomeres are an essential nucleoprotein structure at the ends of all eukaryotic chromosomes. Studies on the structure and function of telomeres have received particular attention owing to their possible significance to ageing and cancer. In this work, human telomere from HeLa cells were isolated and purification by bio-gel P-2 column. Large duplex loops with a tail were first observed directly in the purified telomeric DNA species by atomic force microscopy (AFM). The lengths of the loop and tail are \( \sim 2.5 \pm 0.5 \) and \( 2.0 \pm 1.5 \) kb, respectively. The AFM images also showed that the circular portion of loops is assumed to be a double-stranded structure according to its apparent height, but to be a triplex or tetraplex structure for the loop–tail junction. These results indicate that telomeres in human cells may end in large loops, which is an advantage in further studies on the structure and function of telomeres in cells and provides a new procedure for the study of telomere structure.

**INTRODUCTION**

Telomeres are an essential nucleoprotein structure at the ends of all eukaryotic chromosomes.\(^1\) Telomeres impart stability on linear eukaryotic chromosomes by acting as caps, protecting the chromosome ends from fusion and degradation.\(^2\) In human somatic cells, telomeres have 500–3000 repeats of TTAGGG, which gradually shorten with age in vivo and in vitro. In contrast, the telomeres of germ line cells do not shorten, consistent with the behaviour of immortality.\(^1\) Maintenance of the telomeric TTAGGG repeats at human chromosome ends, either by telomerase or an alternative mechanism (ALT—Alternative Lengthening of Telomeres) is essential for long-term replicate survival of cells in vitro.\(^3–5\) Studies on the structure and function of telomeres have received particular attention owing to their possible significance to ageing and cancer.

The telomeric DNA usually consists of a simple repeated sequence with one G-rich strand. This G-strand extends to the 3′-end of the chromosome and protrudes to form a single-stranded overhang.\(^6\) We know that of the five nucleosides a guanine quartet.\(^7\) Because guanine is rich in the single-stranded portion of the telomere, there may be an existing hairpin or parallel structure containing G-G base pairs. Some investigators have shown that *in vitro* single-stranded G-rich telomere DNA can form a variety of non-canonical structures, including G quartets, triple helices and G-G base pairing.\(^1,8,9\) We showed that an intrastrand fold-back DNA tetraplex with telomere sequences presented in a DNase I-resistant DNA species isolated from HeLa cells.\(^10\) Until now, there has been no evidence in support of G-G base-paired structures at telomere termini in vivo. Recently, Griffith and Lange using electron microscopy showed that the single-stranded ends of human chromosomes seem to loop back on themselves in a unique structure termed the T-loop. They suggested that the long stretches of the double-strand telomere DNA are looped around and the single-stranded telomere is tucked back inside the double-stranded DNA.\(^11,12\) But unfortunately these techniques provide no more than average properties of telomere structure and permit only limited and indirect interpretation (they cannot resolve the details of structure), so these results force us to research the fine structure of telomeres by atomic force microscopy (AFM).

Atomic force microscopy has advantages not only in observing the real morphology with high resolution but also in the rapid sample preparation. Processing with AFM enables the study of transition states to be possible. In this paper, we firstly observed directly the purified telomeric DNA species by tapping mode AFM. A circular loop structure in telomeres was observed by AFM, and a ‘D-loop–T-loop–G-quartet’ model was proposed according to the heights of the loop and the loop–tail junction. Observations of telomeres using high-resolution AFM are expected to be helpful for understanding the structure and function of telomeres.
MATERIAL AND METHODS

Cell culture
HeLa cells were obtained from Beijing Medical University (Beijing, China). The cells were maintained in RMPI 1640 medium, with 10% fetal bovine serum, 100 IU ml⁻¹ penicillin and 100 IU ml⁻¹ streptomycin, and in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. After seeding and culture for 72 h, a total of 3 × 10⁸ cells were gently scraped from the dishes and washed twice in cold pH 7.4 phosphate-buffered saline (PBS). The pellets were collected by centrifugation and resuspended in 3 ml of PBS.

Preparation of nuclei
Isolation of HeLa cell nuclei was performed according to the method described by Griffith et al.² 2–3 × 10⁶ HeLa cells were suspended in 10–20 ml of homogenization buffer (10 mM TRIS-HCl, 1 mM EDTA, 0.1 mM EGTA, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 0.2% sodium dodecyl sulphate SDS, and 5% sucrose, pH 7.4) for 10 min on ice followed by centrifugation for 15 min at 1300 g, then washed in homogenization buffer and suspended in 3 ml of cross-linking buffer (15 mM TRIS-HCl, 15 mM NaCl, 60 mM KCl, 1 mM EDTA, 0.1 mM EGTA and 0.25 M sucrose, pH 7.4).

Isolation and purification of telomere DNA
Psoralen (10 mg ml⁻¹ stock dissolved in DMSO) was added to >3 ml of nucleus suspension; final concentration was 250 µg ml⁻¹. The mixture was spread on a 100 mm plastic petri dish on ice and stirred for 30 min while exposed to a 365 nm UV light bulb at a distance of 2 cm. Then, nuclear suspensions were treated with proteinase K (20 mg ml⁻¹) and cleaved with RsaI (1250 units) and HinFl (1250 units) in a buffer of 10 mM TRIS, 10 mM MgCl₂, 30 mM NaCl, 1 mM DTT and 100 µg ml⁻¹ of bovine serum albumin for 12 h at 37 °C. During the final hour, RNase (Pharmacia) was added to 20 µg ml⁻¹. The sample was then extracted once with phenol–chloroform–isoamylalcohol (50 : 49 : 1), precipitated with ethanol and suspended in 3 ml of TE. The sample was purified by 2.5 × 20 cm bio-gel P-2 column and eluted by water at a flow rate of 0.2 ml min⁻¹. Fractions of 0.6 ml were taken and the optical density (OD₂₆₀) of each was determined. The first peak (fraction 5) was identified to contain nearly all of the telomere species by 3²P-labelled probes (TTAGGG)₄ and fluorescent labelled probes using the EB method and the second peak included the digested DNA species. The characteristics of the determined DNA are listed in Table 1.

Atomic force microscopy
An ~2 µl solution with a UV absorbency of 0.7360 at 260 nm was distributed on freshly cleaved mica and dried immediately under an infrared lamp prior to AFM observation. Tapping mode images were obtained on a Nanoscope III with a multimedia microscope using nanoprobe silicon tips.

Table 1. Analysis of the telomere DNA species

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Psoralen no. (TTAGGG)₄</th>
<th>Absorption intensity</th>
<th>Fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+ + + +</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>0.72</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>5</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>31</td>
<td>1.75</td>
</tr>
</tbody>
</table>

Electron microscopy
Telomeres were diluted at room temperature to 1 µg µl⁻¹. Then 3 µl of the diluted solution was spread on carbon-coated grids and left to adsorb for 2 min, the excess solution on the grid was wicked away with a tissue from the edge of the grid. The grids were washed in distilled deionized water and then dried in 90% ethanol. The grids were rotary shadowed with platinum at an angle of 10°. Samples were examined in a Siemens Elmiskop 1 A electron microscope operating at 100 kV. The microscope magnification was calibrated by means of a carbon grating replica grid from Agar Aids (Stanstead, UK).

RESULTS AND DISCUSSION

Visualization of telomeric DNA loops by AFM
The high resolution and cross-sectioning measurement function of AFM make it convenient for us to observe the characteristics of the determined DNA in cells by AFM. The DNA from HeLa cells was isolated by a bio-gel P-2 column following psoralen–UVA treatment of nuclei, deproteinization and restriction cleavage and diluted to 1 ng ml⁻¹ and adjusted to pH 7.0 before aligning. All cells were treated with psoralen cross-linking except the cells in (d). (a, b) Typical loop structure of telomeric DNA (scale bar = 250 nm and 300 nm). (c) Telomeric DNA loop and degraded DNA species (scale bar = 1000 nm). (d) The DNA species of cells without psoralen cross-linking (scale bar = 1000 nm).
telomere morphology and analysis of its fine structure. Figure 1 shows typical AFM images of telomeric DNA isolated by the above method. The images show that there is a loop with a tail in the telomeric DNA. The lengths of the loop and tail were 2.5 ± 0.5 and 2.0 ± 1.5 kb, respectively, which are shorter than those values measured by electron microscopy. This may be related to the cell types, which are different from each other. The length of the telomere from human somatic cells is also related directly to the mitotic history of the cells, with an average shortening of ~100 base pairs per division.\(^\text{15}\) On the other hand, AFM images (Fig. 1) show that the height of the circle portion of the loop is uneven, which may result from psoralen cross-linking the T-residues of opposite DNA strands randomly.

The T-loop is a large loop-back structure most likely formed through invasion of the single-stranded telomeric 3' overhang into the duplex telomeric repeat array,\(^\text{16}\) and the single-stranded protrusion of TTAGGG repeats found at all mammalian telomeres is lost upon inhibition of TRF2 (telomeric repeat-binding factors).\(^\text{7}\) There is not enough direct evidence for the tail-loop junction being a triplex structure. Results of AFM showed that the average value of the height of the circle portion of the loop is ~0.426 ± 0.172 nm (n = 41). This value could illustrate that the loop circle portion of the loop is duplex DNA. The average height of the tail–loop junction area is ~0.851 ± 0.293 nm. Owing to the height of the triplex structure being twice that of the duplex structure, we conclude that the structure of the junction area is triplex.\(^\text{17,18}\) For example, in Fig. 2(a) the heights of the circle and junction portions are 0.289 and 0.536 nm, respectively. But the heights of some tail–loop junctions are above average height, i.e. 1.286 ± 0.051 nm (n = 4), suggesting that a tetra-stranded structure may exist [see Fig. 2(d)]. In Fig. 2(b), the heights of the circle and junction portions are 0.463 and 0.872 nm, respectively and 1.463 nm for a crossed telomere structure of two duplex DNA (not tetraplex) structures.

On the other hand, the 3'G strand extension invades the duplex telomeric repeats and may form a D-loop (displacement loop). The resulting displacement loop at the junction is

![Figure 2](image-url)

**Figure 2.** Typical loop structures of telomeric DNA and its corresponding height measurements.
Human telomeric DNA

Double telomeric repeats sequence

(TTAGGG)n

(AATCCC)n

G-strand overhang

HinfI/RsaI endonuclease

Non-telomeric DNA

D-loop- T-loop model

Duplex DNA binding protein (TRF1)

End-specific telomeric protein (TRF2)

T-Loop

D-Loop

ss DNA

D-Loop- T-Loop –G quartet model

pottassium

T-Loop

D-Loop

ss DNA

G quartet

Psoralen-binding site

Psoralen

3′

G quartet

D-Loop

TTAGGG

AATCCC

Figure 3. Telomeric DNA structure and psoralen-binding site.
expected to contain up to 300 nucleotides of single-stranded TTAGGG repeats. This assumption is dependent on the following: when the T-loops were incubated with SSB (single strand binding) protein, 35% of the T-loops had one or several SSB protein complexes at the loop–tail junction; the SSB-bearing sites contain a single-stranded segment of possibly 75–200 nucleotides, and a specialized telomere-binding protein, for example TRF2 protein, binds the D-loop at the junction of the loop, which may play a role in stabilizing or allowing the formation of the D-loop, as shown in Fig. 2(c). The exists of the D-loop were observed and the related heights are the 0.571 (TS—Triple Strand), 0.173 (SS—Single Strand) and 0.315 (DS—Double Strand). The height of the tail–loop junction is about 1.9 times that of the T-loop circle portion and about 3.4 times that of the D-loop circle portion, respectively, so they should be triple, double and single DNA. However, the telomeric DNA usually consists of a simple repeated sequence with one G-rich strand. In vitro, single-stranded G-rich telomere DNA can form a variety of non-canonical structures, including G quadrets, triple helices and G-G base pairing. As shown in Fig. 2(d), the related heights are 1.356 nm (junction area) and 0.333 nm (loop area), respectively. Thus the D-loop–T-loop DNA model and D-loop–T-loop–G-quartet telomeric DNA model was proposed (Fig. 3).

According to x-ray analysis the height of duplex DNA is 2.0 nm, but owing to the effort of the AFM tip the DNA strand is flattened. Thus, the height measured by AFM is less than that in theory. Generally, the height of the DNA duplex is ~0.36–0.76 nm using AFM. In this work, the average height of duplex DNA is 0.426 nm (the maximal is 0.768 nm, and the minimal is 0.235 nm). There will be different values under the different measurement conditions, such as the tip, humidity of the environment, etc. The difference in the height of the duplex loop may be due to the different humidity of air. Thus we should compare the height of a different DNA loop in the same images to eliminate these influences. Our attempts to address this by directly visualizing the intermediates using AFM but without drying have been unsuccessful to date. Also, during the AFM sample preparation process, the rinse procedure after binding the DNA to the surface of mica made the effect of drying negligible. Additionally, the effects of capillary forces on the DNA adsorbed or attached to solid supports are typically quite dramatic, producing highly stretched molecules. Hence, because there are no obvious effects from drying we feel that it is unlikely to have contributed significantly to the structures shown.

Electron microscopy

We also observed the telomeric DNA by electron microscopy, as shown in Fig. 4. In the electron microscopy images the same T-loop is observed as in the AFM images; and furthermore, the length of the T-loop using electron microscopy is similar to that using AFM. The electron microscopy technique is particularly suitable for the analysis of T-loops, because the strong surface tension at the airbuffer interface forces DNA molecules to spread out so that the strands seldom cross over themselves. But, compared with AFM, electron microscopy could not quantitate the width and height of the telomeric DNA.

Figure 4. Electron microscopy visualization of telomeric DNA

Psoralen cross-linking plays a major role in preserving the T-loop

Telomeres consist of a repetitive DNA sequence and protein complexes. During the extraction of a telomere, the possible isolation of protein and telomere DNA could result in an unstable telomere structure. Such an observed DNA structure might be formed during preparation. To overcome this difficulty, we use a photosensitive probe called a psoralen, which could intercalate into a duplex or triple DNA and, upon UVA irradiation, preferentially cross-link T-residues of opposite strands. Thus psoralen cross-linking of the DNA strands is predicted to preserve the T-loops after removal of protein. According to the above method, no loop with a tail was observed in the samples without treatment using psoralen and UVA. Only a few looped DNA stands (it is unstable) can be observed in crude cell DNA extract without any treatments such as deproteinization, enzyme digestion and purification. Hence, the looped DNA in the AFM could not be an artifact of DNA folding back on itself during preparation. Psoralen cross-linking could stabilize T-loops that might exist in vivo before removing proteins from the DNA. Sometimes, some small circles in cells without treatment with psoralen and UVA were found in DNA purified by bio-gel P-2; these are different to T-loops, as shown in Fig. 1(d). Such DNA circles comprise several hundred base pairs, formed by the short DNA strand after HinfI and Rsal digestion. It may be an artifact product during preparation.

The elution profile of the cell DNA showed the T-loop telomere DNA obviously much richer in the psoralen-treated cells, and DNA rarely existed for the cross-linking without psoralen because it had been digested (see Table 1). The result of electrophoresis also indicated that there is no telomere DNA for photocross-linking, without psoralen, related to the lower DNA product and the very small DNA molecular structure (<40 base pairs). As mentioned above, psoralen cross-linking plays a major role in preserving the
T-loop after removal of protein, which is mainly TRF2. Possibly the presence of psoralen works in the same way as TRF2.11,12

SUMMARY
The main result presented here is that we observed the T-loop of telomeric DNA in human cells by AFM. Meanwhile, these results not only provided further evidence that telomeric DNA in human cells has a D-loop–T-loop structure, as observed by electron microscopy recently, but also quantified the length and height of the T-loop, indicating that the DNA is a double-stranded structure at the loop portion, according to its height. The structure of the junction site of loop and tail, may be a triple- or tetra-stranded structure. Observation of telomeres using high-resolution AFM is expected to be helpful for understanding the structure and function of telomeres.

Acknowledgements
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REFERENCE