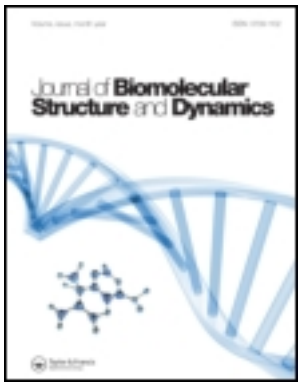

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Fold-Back Tetraplex DNA Species in DNase I-Resistant DNA Isolated from Hela Cells

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Abstract

A DNase I-resistant DNA species has been isolated and purified from HeLa cells by gel electrophoresis. Our studies indicate that the DNase I-resistant DNA species was about 40 -60 bp fragment sizes responding to double-strand DNA marker and has higher guanine content. The image of AFM showed that this species has been assumed to be tetraplex structure according to its apparent width and height. Its CD, UV spectrum also exhibited characteristics similar to some tetraplex structure, which was different from the standard duplex DNA. ^{32}P-labeled probes $(TTAGGG)_4$ and 5′-TGGGGAGGGTGGGGAGGGTGGGGAAGG-3′ could be hybridized to purified DNase I-resistant species. All results suggest that the DNase I-resistant DNA species have at least two components, which adopt an intrastrand fold-back DNA tetraplex. Their sequences were similar to human telomere and human c-*myc* locus (NHE), respectively.

Introduction

The human genome contains a varity of DNA sequences present in multiple copies (1-3). It has been shown that single stranded DNA sequence containing such the long tracts of continuous purine and pyrimidine sequences can form unusual DNA structures stabilized by interactions between the guanine bases (4,5). Of these structures, four stranded DNA complexes have been studied most extensively. In such complexes, groups of four guanine bases, one from each strand, are bound by Hoogsteen hydrogen bonds, thereby forming stacked G-quarts that hold the chains together (6-11).

Recent studies in vitro reported that (1) the telomere of the linear chromosomes of eukarotes are composed of tandem repeats of short DNA sequences (TTAGGG)n, one strand being rich in guanine. Telomeric G-strand DNAs from a variety of organisms adopt compact structures, the most stable of which is explained by the formation of G-quartets (12,13); In recombination, formation of four stranded DNA may account for initial chromosome pairing (6,14-15), At telomeres, the formation of four stranded DNA could inhibit the telomerase, as shown by in vitro assays of the enzyme (16). In both cases, the cells would be expected to possess a mechanism for resolving the four stranded DNA into single strands. (2) A major nuclease–hypersensitive element III (sequence: 5′-TGGGGAGGGTGGGGAGGGTGGGGAAGG-3′) termed NHE, which corresponds to bases 2186-2212 in the sequence of the human *c-myc* locus, has an unusual strand asymmetry: one strand is an almost perfect homopurine tract (17,18). In transcription, it adopts an intrastrand fold-back DNA tetraplex. that is bound by hnRNP AI and/or CNBP. The exposed homopyrimidine strand is then bound by hnRNP K, which attracts the RNA polymerase II transcription machinery and activates c-*myc* (10). Since all these structures readily occur under the physiologically compatible conditions of pH, temperature and ion strength, they have been presumed to play an important functional role in various biological

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processes that involve DNA. Proposals regarding these functions include transcription regulation, gene recombination as well as chromosome organization and packing (19-21). In addition, other studies also indicated that mutations in the sequences that can form these structures might be related to some human genetic disorders such as Huntington's disease, Kennedy's disease and myotonic dystrophy (22). Therefore, a detailed investigation of these structures not only is required for our elucidating their potential functions, but also can provide us an insight into the intrinsic mechanism underlying these genetic diseases. Moreover, such understanding can further help us to design some rational oligonucleotides for specific therapeutic, diagnostic and other biotechnological applications.

To date, a large number of experiments have been carried out to study such fold-back DNA tetraplex structures. Four stranded DNA structures are formed in vitro in buffers containing physiological concentrations of Na^+ and K^+ ions at neutral pH. The same structures may occur in living cells and affect essential cellular processes. But the paper on study of DNA directly from living cells is a little. These led us to examine the isolation and structural chartchtertct of such fold-back DNA tetraplex in cells. Here we report that there were at least two kind of DNA species with the fold-back DNA tetraplex structure in DNA isolated from HeLa cells. Their structural properties were studied in detail by UV, CD, fluorescence, AFM and HPLC determination.

Materials and Methods

Cells and Chemicals

HeLa cell lines were obtained from Beijing Normal University. Cells were cultured in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin, at 37°C in a CO_2 incubator .

Oligonucleotides (purchased from Bio-Red Co.) were purified by polyacrylamide gel electrophoresis by a standard method. And were 5′-end labeled with [γ^{32}P]dATP (purchased from Amersham), using the T4 polynucleotide kinase (purchased from Promega) [23]. proteinase K and RNase were obtained from Sigma. λ DNA/ EcoRI + Hind III marker was obtained from Promega. Pancreatic DNase I and ethidium biomide (EB) were obtained from Fluka Co..

Isolation of DNase I Resistant DNA Species

DNA was isolated from whole HeLa cell samples according to the method described by Marmur (24). The isolated DNA was treated with proteinase K and RNase to remove the protein and RNA. Briefly About 1×10^8 cells in medium were collected by centrifugation, washed 3 times in PBS and then resuspended in 0.35 ml of lysis buffer containing 0.1 mM NaCl, 0.02 M Tris-HCl (PH8.0), 6 mM EDTA, 0.5% SDS and 100 ml of RNase (10 mg/ml) for 6hr at 37°C, 300 μl proteinase K (10 mg/ml). at 37°C for overnight. DNA was extracted by adding quiet vol of phenol/chloroform (1:1) solution, repeated 4 times, The supernatant was collected and then cooled to 0°C. 3 vol of ethanol (at -20°C) was added to precipitate DNA, which was then collected by centrifugation, dried in air, dissolved in 100 μl of TE buffer containing 10 mM Tris-HCl, 1mM EDTA, pH 7.5. The determination of UV spectrum showed that the maximum absorption peak is 257.8 nm. The ratio of A_{260} to A_{280}, was 1.83. The content of proteins in samples was detected by the method of Lowry et al. (25). DNA was determined by a fluorescence method with Hoechst 33258, as described by Cesarone et al. (26).

DNA purified was then incubated with DNase I (3 μg/ml) in the presence of 5 mM Mg^{2+} for 4 - 5 h at 37°C until maximum absorbency at 260 nm and the single- and

double- stranded DNA were removed (27). A DNase I-resistant DNA species was finally obtained and stored in refrigerator at 4°C. The determination of UV spectrum showed that the maximum absorption peak is 260.8 nm.

Gel Electrophoresis:

Digested samples were analyzed by agaross gel electrophoresis on 1.5 % agaross gels, about 4.5 h at 5.0 v/cm gradient, in a buffer containing 0.1 mol of Tris, 0.1 mol borate and 2 mmol of EDTA per liter (pH 8.0, at the room temperature). λ-DNA Hind III was used as control and a standard of molecular weight. After electrophoresis, the gels were stained in a 0.5 μg/ml ethidium bromide solution for 20 min before being viewed under 254 nm UV light and taken a photograph.

Atomic Force Microscopy (AFM):

A 5 μl drop of the solution containing DNA species was deposited onto a freshly cleaved mica surface. About 1 min later, the residual solution on the mica was carefully removed by a slice of filter paper, followed by drying in air. A nanoscope III (Digital Instruments) AFM was used. The microfabricated cantilevers used in this study were tapping-mode specific tips (nanoprobes), and all images were recorded in a tapping-mode operating at height-mode. The scan rate was usually set to 1.0-2,5 Hz. In order to prevent DNA molecules from forming a network of interconnecting structure because of interaction between DNA molecules (28). A lower concentration (0.5 μg/ml) of DNA species A was used in this study.

UV Absorption:

Samples were placed in the quartz cell (1.0 cm), and then measured the absorption spectrum with Hitachi U-3200 spectrophotometer. The sample cell has equipped with a thermostated accessory, the temperature rise was about 1~2°C/min during the determination of temperature-absorbency curve. In this work, the value of temperature, which represents the temperature in sample cells, has been corrected with a standard thermometer.

Circular Dichroism Spectroscopy (CD)

CD spectra were measured with Jasco-500 model CD Spectropolarimeter using a cell of 1mm path length. Sample temperature was controlled by a external circulating water bath. All CD spectra were averaged over 8 acquisitions with a scan rate of 50 nm/min. All CD data were baseline corrected for signals due to the cell and buffer. The temperature was kept 15.0 $\pm$ 1.0°Cduring experiments.

High Performance Liquid Chromatography (HPLC)

To quantify the guanine content in DNA, the DNA samples were hydrolyzed according to the method (29) Briefly, 100 μg of DNA and 1.0 ml of 99% trifluoroacetic acid were combined in a glass vial which was then sealed to the atmosphere. The mixture was heated for lh at 155-160°C, cooled, and dried under reduced pressure prior to analysis. The DNA samples hydrolyzed then analyzed by reversed phase high performance liquid chromatography (HPLC). The HPLC columns used was partied 10 ODS-2 (10 mm) with dimensions 25 cm X 4.6 mm. i.d., mobile phase: 100% water at 1.0ml/min. wavelengths: 254 nm.

Results and Discussion

1. Isolation of DNase I-Resistant DNA Species

A different migration pattern on agarose gel of DNA treated by DNase I compared to

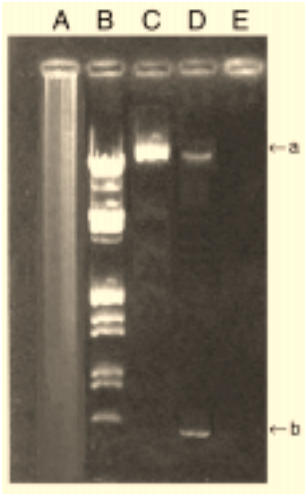

Figure 1: Agaross gel electrophoresis plots of DNase I-resistant DNA species. A. Thermal denatured DNA; B.λ-DNA EcoR I/Hind III; C. purified DNA isolated from HeLa cells; D.DNA by DNase I treatment; E. Thermal denatured DNA by DNase I treatment

the native DNA was obtained as shown Figure 1. The native DNA yields a distinct fluorescence bands (band a in column C). The sample after DNase I treatment a fluorescence band became week and yielded a new fluorescence band, which could not be digested by DNase I. (band b in Figure1, column D). Here, the fragment size of DNA species a and b in column D is estimated to be about 2000 bp and 40-60 bp, according to the molecular weight standard from the well-known duplex DNA (Figure 1, column B). Otherwise, the denatured solution of DNA (0.5 mg/ml) gives a strong fluorescence band with dispersing molecular weights (Figure 1, column A). Above results show that the DNase I –resistant species exist in the cell extracts and the fragment size of DNA species (Figure 1, column D); A decrease of the DNase I–resistant species showed in Figure 1, column E suggests the DNase I–resistant species is a ordered structure. This is because the DNase I –resistant species unwinded was digested by DNase I, the DNase I–resistant species remained in the denatured DNA solution is too small that the band a and b will be observed obviously, but a strong fluorescence is noted in the original point. During experiment, we found that the yield of DNase I-resistant DNA species (band a and b) was lower using the buffer without K^+ during isolation of DNA, the higher concentration of K^+ was advantage for the existence of DNase I-resistant DNA species, the formation of DNase I-resistant DNA species (band a and b) might dependent on their base sequence and the concentration of monovalve cations, such as the role of K^+ ion in stabilizing G-tetraplexes (7). Its formation did not depend strongly on pH, which is different to that of pymotif triplex DNA. such as cytosine could be protonized in the condition of lower pH (30). We think that the information given from the agarose gels is limited, it is only beginning of further study, but it is difficult and impossible to explain their structure chartchtertct only from above results of agarose gels.

2. Direct Observation of AFM Images

AFM images of above native DNA solution showed that there were two different kinds of DNA species (Figure 2-1). In average, one (species A) was 0.8 nm height and 11nm width respectively, corresponding to a typical double stranded DNA tractate (31). Another one (species B) was 2.2 -6.4nm height and 18 nm width respectively, which were significantly bigger than those of double stranded DNA under the same conditions, suggesting they were triple-, four- or mult-stranded DNA. The species A all almost disappeared after DNase I-digestion, (Figure 2-2). The longer molecules, 2.2 -6.4 nm height still can be observed and some smaller molecules can easily be visualized. Figure 2-3 showed a typical AFM images of smaller DNA species isolated from the band b in the agarose gel and purified by Bio-Red gel. The molecular is at average 13.9 nm length, (lengths range from 8 to 20 nm) corresponding to be about 40-60 bp size. The average height is 2.43 ± 0.15 nm, it probably is four-stranded DNA according to the molecular height (32).

3. Sequence Analysis of the DNase I-Resistant DNA Species

Short DNA sequence repeats, in which one strand consists of clusters of 3-8 guanine residues intersprsed with other short sequences, are found in eukaryotic genomes at various chromosomal sits, including telomers (1,2), immunoglobulin switch regions (6,33), the control region of the retinoblastoma susceptibility gene (34), upstream of the insulin gene (35) and other promoters (36,37). For this purpose,

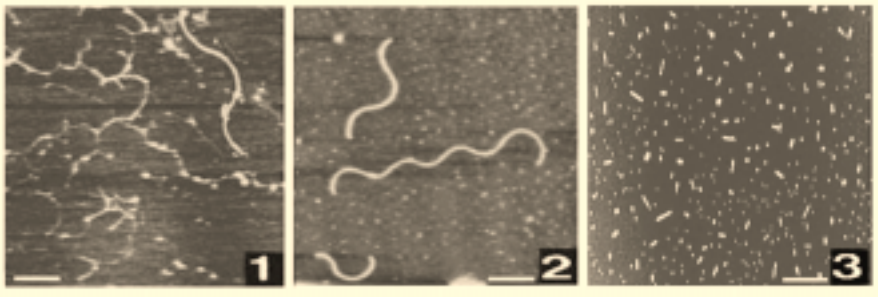

Figure 2: AFM images of DNA. DNA was isolated and purified from HeLa cells was diluted to l ng/ml and adjusted to pH 7.0 before aligning. Imaging was performed in tapping mode in air at 26′C with a Nanoscope III microscope (Digital instruments Inc., Santa Barbara, CA). All images were processed only by flattening. 1. Purified DNA isolated from HeLa cells, Scale bars 200 nm; 2. DNA digested by DNase I, Scale bars 200 nm; 3. Small DNase I - resistant DNA species from band b. Scale bars 50 nm.

we synthesized two different structures probes: A synthetic 27-base-long deoxyoligonucleotide (sequence: 5′- TGGGGAGGGTGGGGAGGGTGGGGAAGG-3′), PU27, complementary to the coding of the c-myc gene targeted to the NHE [11], and a synthetic 24-base-long deoxyoligonucleotide (sequence: 5′- TTAGGGTTAGGGTTAGGGTTAGGG-3′), corespording repetitive DNA sequence of human telomeres. We used the purine-rich PU27 and $(TTAGGG)_4$ as a probs, When DNase I resistant DNA species were pre-incubated in presence of 100 mM potassium ions, and then mixed it with either radiolabelled PU27 or $(TTAGGG)_4$, it gave rise to two bands in native polyacrylamide gels (Figure 3). Suggesting that the PU27 or $(TAAGGG)_4$ co-migrated with the DNA species. Neither of the oligonucleotides co-migrated with the DNase I-resistant DNA species when it had been pre-incubated with sodium ions. It indicates that potassium ions influence the interaction of PU27 or $(TTAGGG)_4$ with the DNase I-resistant DNA species and there were at least two prominent specific sequence, PU27 or $(TAAGGG)_4$, in the DNase I-resistant DNA species.

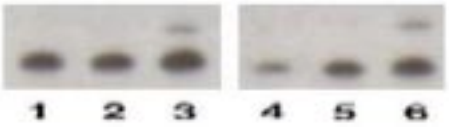

Figure 3: Gel electtophoresis of PU27 or $(TAAGGG)_4$ and DNase I- resistant DNA species in the presence of different cations. Radiolabelled oligonucleotides were mixed with DNase I- resistant DNA species pre-incubated at 37°C for 48 h in the presence of either sodium or potassium ions. Lane l, PU27/ DNA species pre-incubated in 50 mM $MgCI_2$, lane 2, PU27/DNA species pre-incubated in 100 mM NaCI, lane 3 PU27/DNA species pre-incubated in 100 mM KCI, Iane 4, $(TAAGGG)_4$/ DNA species pre-incubated in 100 mM $MgCI_2$, lane 5, $(TAAGGG)_4$/DNA species pre-incubated in 100 mM NaCI, lane 6 $(TAAGGG)_4$/DNA species pre-incubated in 100 mM KCI.

4. Guanine Content in DNA

The interaction of PU27 or $(TTAGGG)_4$ with the DNase I resistant DNA species shown that the DNase I-resistant DNA species might contain such PU27 or $(TAAGGG)_4$ sequence. To support and further quantitate the guanine content in DNA species. The DNA samples hydrolyzed were analyzed by reversed phase high performance liquid chromatograpy (HPLC). Table I gives the guanine content of in the DNase I resistant DNA solution, including both DNA species isolated from band a and b in the agarose gel. Both show that the guanine content of DNase I- resistant DNA species was higher than that in the naitive DNA species, but DNA species from band a shows that the difference is very small, the possible reason is that there are other unusual DNA with DNase I-resistant, which are not composed of tandem repeats of short DNA sequences contenting (TTAGGG) or PU 27. Here we can find that the guanine contant of DNA species isolated from band b was obseriously higher than that in DNase I sensitive DNA species, however, it is lower than guanine contant (50% or 80%) of the DNA species if it is composed of (TTAGGG)n or PU 27 alone, which may be reasonably deduced from the influuence of other unknown DNA species (38,39).

Table I

The base contents of the DNase I-resistant DNA species and the native DNA species.

	Native DNA species		DNase I resistant DNA species			
			band a		band b	
Bases	**M%**	**SD**	**M%**	**SD**	**M%**	**SD**
A	28.68	±0.31	27.65	±0.39	26.66	±0.45
G	22.83	±0.11	23.72*	±0.32	38.37*	±0.72
C	21.09	±0.28	20.38	±0.15	9.32	±0.15
T	27.11	±0.27	28.02	±0.53	25.52	±0.63

*$p < 0.05$

5. Spectrum Characteristics of the DNase I Resistant DNA Species

5.1. CD Spectrum

Circular dichroism (CD) can discriminate between single stranded DNA and tetraplex structures quite readily (40-45). The native DNA species gave a typical CD spectrum of natural B-form DNA (31), characterized by two positive bands at 270 nm and 218 nm respectively, and a negative band at 240 nm (Figure 4, curve a). DNase I-resistant DNA species yielded a different CD spectrum, however, with the decrease of 230-240 nm negative band and the increase of 250-260nm and 280-290nm positive band (Figure 4, curve b), which was similar to the determined results of qudraplexes in previous work (46). For the band b DNA species, its CD characteristics were significantly different from those of various known duplex DNA (47-49), triplex DNA (50,51) and self-assembled structure of single-stranded DNA induced by proton (47), indicating the existence of ordered structure in DNA.

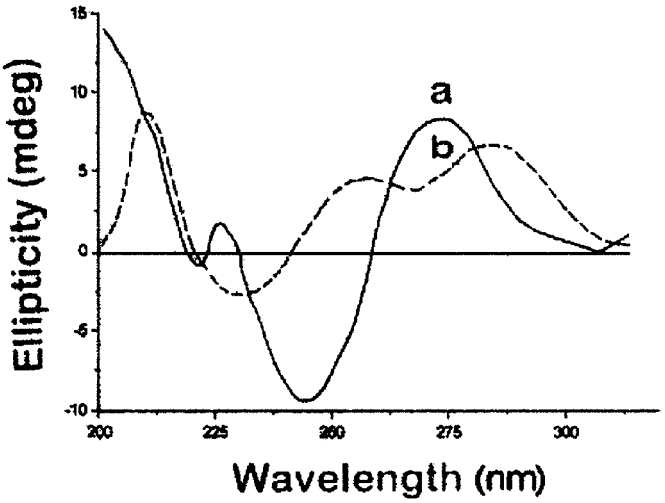

Figure 4: CD spectra of DNase I resistant DNA species in redistilled water, pH 7.0. a. native DNA species without DNase I treatment; b, DNase I-resistant DNA species.

5.2. UV Absorption Spectrum

Compared with their CD spectra, the UV-absorption spectra of two kinds of DNA structures are quite similar. The spectra are not characteristic enough to distinguish obviously these forms. However, if observed carefully, some subtle changes among them can be found. The species B in this range, especially at 260-280 nm differ from both triplex and duplex, showing a little higher absorbency than the species A, their maximum absorbency peak is 260 nm (species A) and 257.8 nm (species B), receptively. In addition to the static spectra, thermal denaturation was observed by monitoring the absorbency change at 260 nm. With the rising of temperature, the absorbency increases gradually and then reach a plateau around 90°C. Hence, a significant UV hypochromism can be also recorded. As shown in Figure 5 that the denaturation profiles of the DNase-resistant sample is different from native DNA species. There is only one temperature-jump melting curve for native DNA without the DNase I treatment with Tm being 83.5±0.5°C and 38% of hypochromism. On contrary, there are two obvious unwinding processes for the DNase-resistant DNA species with Tm being 66.4±0.5°C and 88.5±0.5°C respectively and 30% of hypochromism. On other hand, the ratio of A_{280} to A_{260} for species B was high than native double helix DNA. Above results also indicate that under the similar conditions, the native DNA species is more stable than the DNase-resistant DNA species, possible reason is that the energy required to rupture the base pairs of GC and AT will be more than that for the base pairs of GG and AA (52). This thermal melting behavior shows also that the DNase-resistant DNA species is a ordered DNA molecule.

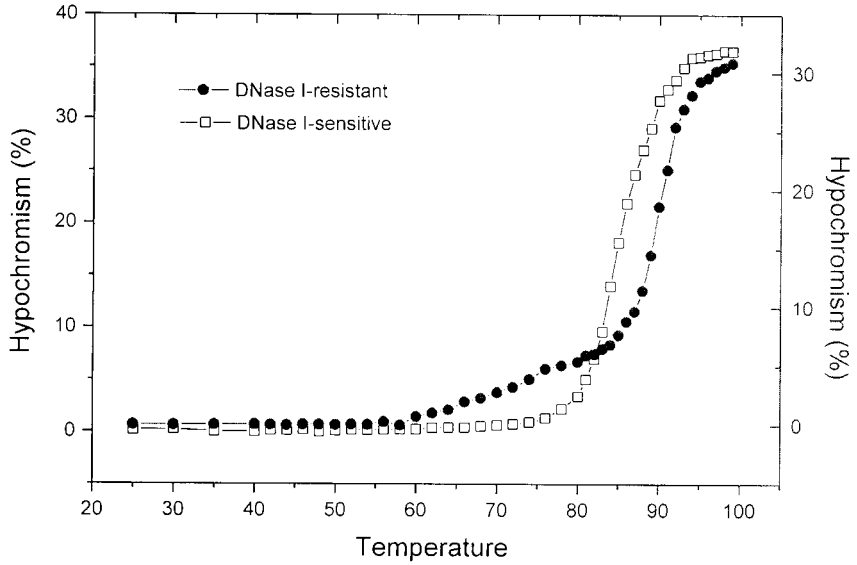

Figure 5: Thermal denaturation of DNase I -resistant DNA specie and naitive DNA species without DNase I treatment. Thermal denaturation of DNA specie was monitored by the Absorbency (A) at 260 nm. The hypochromism presenets (A96-A20)/A20 X 100.

Sum up, agarose gel electrophoresis showed that DNA species isolated from HeLa cells have a DNase I -resistant species, which were about 40-60 bp fragment sizes responding to double-strand DNA marker by gel electrophoresis. The image of AFM showed that this species was assumed to be tetraplex structure according to its apparent width and height, which was observeously different from the standard duplex DNA. HPLC analysis showed it has higher G content. Its UV, CD spectrum also exhibited characteristics different from the standard duplex DNA. A synthetic 27-base-long oligonucleotide (5′-TGGGGAGGGTGGGGAGGGTGGGGA AGG-3′) or the human telomere sequence $(TTAGGG)_4$ could be hybridized to isolated DNase I-resistant DNA species. All results suggest that the DNase I -resistant DNA specie b isolated from HeLa cell has at least two components which adopt probably an intrastrand fold-back DNA tetraplex. Their sequences were similar to human telomere or NHE of the human *c-myc* oncogene, respectively.

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