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Letter to the Editor

Superstructural self-assembly of the G-quadruplex structure formed by the homopurine strand in a DNA tract of human telomerase gene promoter

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1. Introduction

G-quartet is a planar structure built up by guanine bases via Hoogsteen hydrogen bonds. G-quartets, in guanine-rich nucleic acid sequences, can stack forming four-stranded either inter or intramolecular structures, named G-quadruplex [1].

In most biological systems, telomeric DNAs consist of short repeats of guanine-rich sequences. This feature makes the telomeres the most-studied source of quadruplex [2].

Furthermore, in the last few years, non telomeric G-rich regions, inherently capable of forming G-quadruplex in the human genome, have arisen interest. Putative quadruplexes, having runs of guanine bases separated by short loop sequences (1–6 nucleotides), have been identified in a number of non telomeric genomic sequences [3,4].

Recently we studied the structural features of a homopurine/homopyrimidine DNA tract, close to the transcription initiation site of the gene coding for the human telomerase reverse transcriptase (hTERT) and belonging to an open chromatin organization domain [5]. During this investigation, we became aware that the G-rich strand of this DNA tract (HTP in the text) is able to form linear superstructures, probably due to G-quadruplex self-assembly.

Previously, it was shown that two guanine-rich oligonucleotides ($G_4T_2G_4$ and $A_{15}G_{15}$) can assemble in super-molecular structures called G-wires or frayed wires respectively [6,7]; it was proposed a model, where overlapping slipped G-quadruplex structures are responsible for the formation of G-wires [6]. Recently, G-quadruplex is becoming of large interest in nanotechnology because of its high rigidity [8], thus the study of HTP self-assembly seems to be of interest in increasing the type of polypurine sequences, which could be functional from the nanotechnological point of view. Moreover, intramolecular and intermolecular G-quadruplex structures, formed by G-rich DNA tracts in biological significant locations, seem to be connected to basic biological processes.

The present paper investigates the structural and superstructural features of HTP self-assembly using Atomic Force Microscopy (AFM) imaging, Circular Dichroism (CD) spectroscopy, polyacrylamide gel electrophoresis (PAGE) and methylation interference assay.

2. Results

Fig. 1a and b illustrate the AFM images of HTP (5'-$G_2A_3G_2A_2G_4AG_4$-3') deposited from the samples prepared in TK buffer (20 mM Tris–HCl pH 7.4, 50 mM KCl) or TN buffer (20 mM Tris–HCl pH 7.4, 50 mM NaCl). In both cases, HTP self-assembles into "dashed" linear structures of different lengths, suggesting the presence of repeated elements (REs). This assembly takes place less efficiently in TK buffer (Fig. 1a), since the RE number along each superstructure appears to be smaller than that observed in TN buffer (Fig. 1b and d). Despite the difference in their lengths, the self-assembled structures appear constituted by the same RE organization. In fact the histograms in Fig. 1e, reporting the distributions of "inter-RE" distances, show the same trend with a maximum of about 15 nm both in NaCl and KCl solutions. Moreover, the determination of lengths and heights of single RE within the arrangements, resulting quite similar in the presence of the two monovalent cations, seems to be another evidence that the visualized assemblies are made of the same building block in both experimental conditions. In fact, the mean values obtained in TN buffer are 13.5±2.2 nm and 1.8±0.2 nm, while in TK buffer the mean values are 14.4±2.7 nm and 1.5±0.3 nm, corresponding to length and height respectively (the errors are expressed as standard deviation, SD). With regard to the real lengths of REs it should be taken into account the broadening effect of the AFM tip radius. Using the formula (1) (see Supplementary materials) it is possible to evaluate the actual size of REs that corresponds to about 5 nm.

The RE height was compared with that of a double stranded DNA co-adsorbed onto mica as internal standard. The topographic comparison is reported in Fig. 1c, while Fig. 1f illustrates a typical cross-section analysis used to collect data, in which the line profile shows that the apparent mean height of REs (~1.5 nm) is about twice larger than that of dsDNA (~0.6 nm), both in the presence of NaCl or KCl. The obtained value is in good agreement with the height of G-quadruplex, reported in previous AFM studies on DNA self-assembly [6,8]. On the basis of these findings, it is reasonable to suppose that the imaged HTP linear superstructures should be constituted by G-quadruplex repetitive elements (REs). It is worth remembering that the height reduction obtained by AFM imaging with respect to the nominal value of the crystal structures is due to the pressure exerted by the AFM tip on molecules [9]. The 40% height reduction of G4-DNA in comparison to dsDNA (60% height reduction) is an index of major resistance of the first structure to deformation.

It is interesting to note that the size of the gap between REs increases with the amount of water used in the AFM rinsing step, suggesting that salt removing gives rise to a partial desorption of HTP from the mica (see Supplementary materials Fig. S1). We have also derived the distributions of "inter-RE" distances, under two different washing conditions (1 ml and 3 ml of water, Fig. S2), finding an increase of about 3 nm in the mean value.

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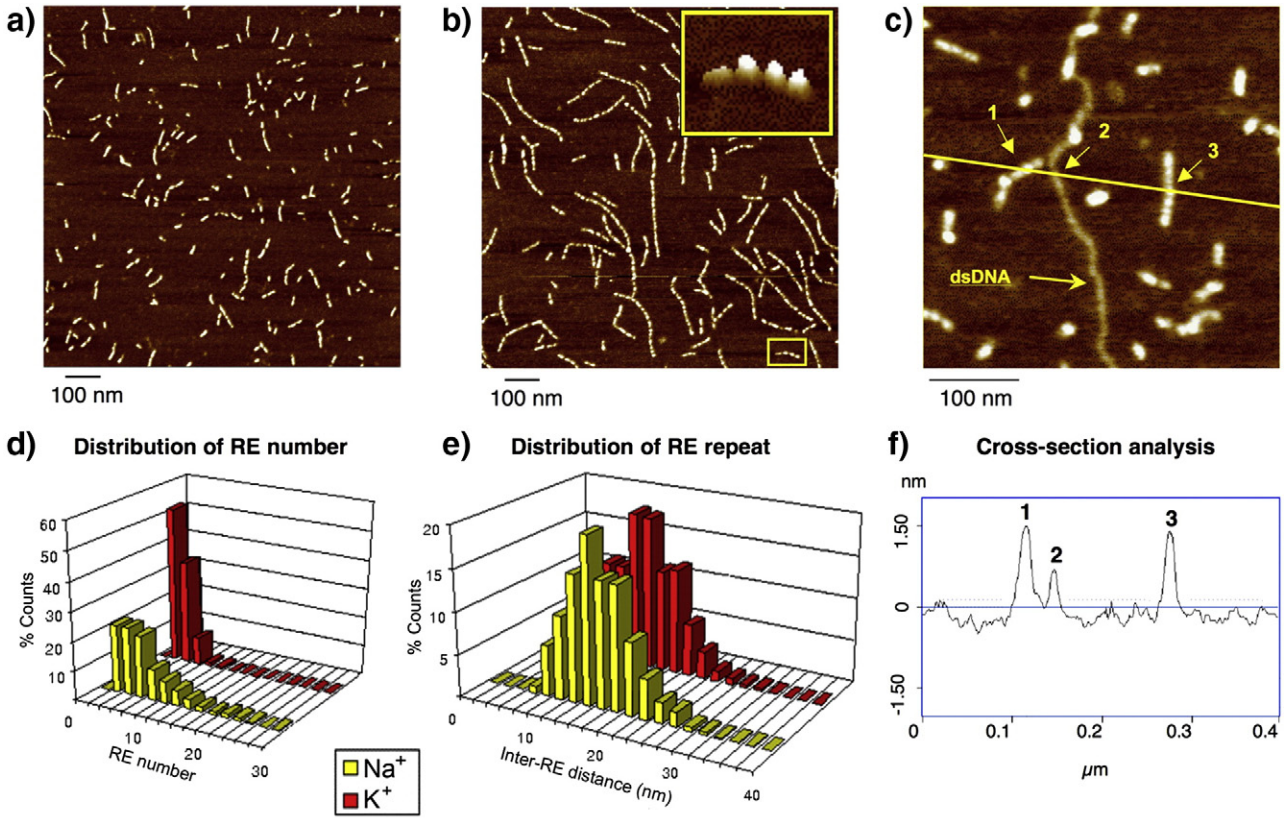

Fig. 1. Typical AFM image of HTP linear superstructures: a) in TK buffer (20 mM Tris–HCl pH 7.4, 50 mM KCl) with 5 mM $MgCl_2$; b) in TN buffer (20 mM Tris–HCl pH 7.4, 50 mM NaCl) with 5 mM $MgCl_2$. The insert is an enlargement of the molecule in the yellow box. c) Comparison between HTP and duplex (ds) DNA structures: AFM topographic image of co-adsorbed HTP linear superstructures and plasmidic DNA onto mica surface in TK buffer. d) Distribution of repeated element number (RE number) on each HTP linear superstructure. e) Histograms of the distribution of the inter-RE distances. This parameter corresponds to the centre to centre distance between two adjacent REs. The mean inter-RE distance is 16.31 ± 4.41 nm, and 14.96 ± 4.36 nm in TK buffer (errors expressed as SD). In both histograms, obtained from at least three independent experiments, yellow is for Na^+, red for K^+. f) Height profile along the segment shown in Fig. 1c. In these images the particles labeled as 1 and 3 correspond to RE, while label 2 corresponds to dsDNA. The height mean value of RE is 1.53 ± 0.26 nm in TK buffer and 1.56 ± 0.19 nm in TN buffer (errors expressed as SD).

Fig. 2 shows CD spectra of HTP in three different buffer compositions (TK, TN and TN with 5 mM $MgCl_2$). All CD spectra, in the wavelength range of 300–200 nm, show similar profiles characterized by a strong positive band around 262 nm and a much lower negative band around 240 nm. CD spectroscopy detects the associated conformations of glycosidic bond [2,10] and the illustrated features, are generally accepted as typical of parallel G-quadruplex topology, characterized by anti conformation of all glycosidic bonds.

The thermal stability of HTP G-quadruplex structure was measured by CD spectra as a function of temperature from 10 °C up to 80 °C. The results reported in Fig. 2 clearly show that the stability in the two conditions is strongly different; in TK buffer (Fig. 2a), the G-quadruplex is stable, since the ellipticity only slightly decreases up to 80 °C. On the contrary, in TN buffer (Fig. 2b), the G-quadruplex structure appears collapsed at 80 °C, as shown by the comparison with the features of the spectra corresponding to unstructured HTP oligonucleotide (Fig. S3a). The presence of 5 mM $MgCl_2$ appears to partially stabilize the G-quadruplex structure in TN buffer (Fig. 2c), while in TK buffer and 5 mM $MgCl_2$ the CD spectrum is almost unmodified with increasing temperature (data not shown).

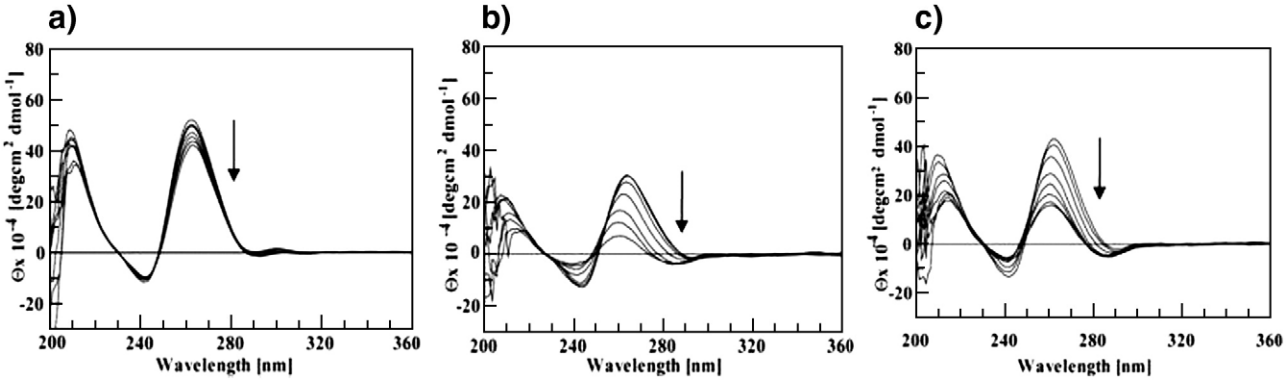

Fig. 2. CD spectra of 20 μM HTP in different experimental conditions: a) TK buffer; b) TN buffer; c) TN buffer with 5 mM $MgCl_2$. Arrows indicate increasing temperature from 10 °C to 80 °C at 10 °C steps. Each spectrum was acquired after the sample has been equilibrated for 1 h at the corresponding temperature.

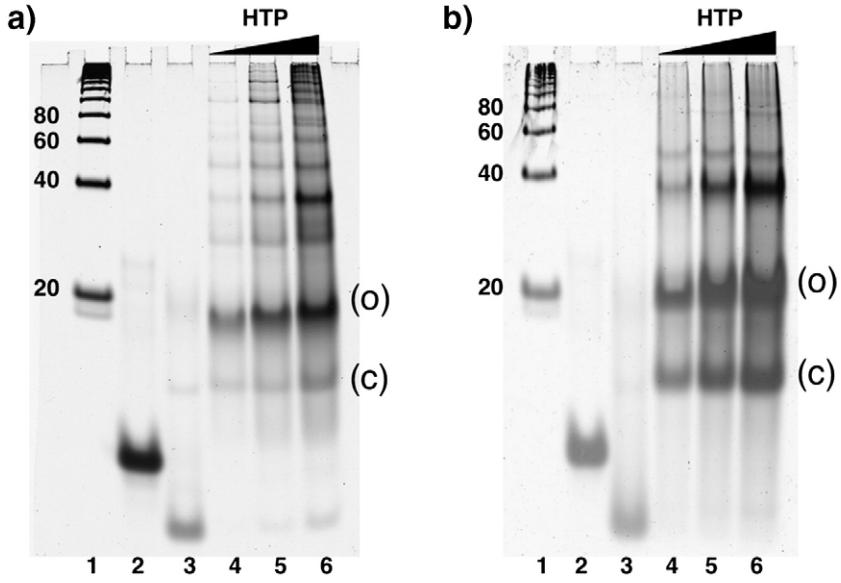

Fig. 3. Non-denaturing 20% PAGE of US, HT and HTP in different salt conditions: a) TN buffer; b) TK buffer. In each panel lanes 1, 2 and 3 correspond to 20-bp dsDNA ladder, 10 μM US, 10 μM HT, respectively; lanes 4, 5 and 6 correspond to HTP at increasing concentration: 10, 25, and 50 μM. The 20-bp dsDNA ladder has been reported to allow the comparison between PAGE different experiments. For more details about (o) and (c), open and closed form of HTP G-quadruplex structure respectively, see the text.

Fig. 3 reports the non-denaturing polyacrylamide gel electrophoretic patterns (PAGE) of HTP in TK or TN buffer. Two oligonucleotides are used as internal standards, US (5'-T_4GTGATGCTCG_3-3') and HT (5'-$G_3[T_2AG_3]_3$-3'), with a similar length to that of HTP: US (lane 2) is used as a standard for unstructured single strand oligonucleotide, whereas HT (lane 3), containing about four repetitions of human telomeric sequence, forms a canonical intramolecular G-quadruplex structure [11]. The latter oligonucleotide is characterized by the highest electrophoretic mobility, due to its compact structure.

The panels in Fig. 3 show band ladders (lanes 4–6) in which the HTP band with the highest mobility is slower than the band corresponding to US oligonucleotide. On the other hand, HTP prepared in 20 mM Tris–HCl pH 7.4 shows a single band with the same electrophoretic mobility of US (Fig. S4). On the basis of these findings it is reasonable to propose that HTP, in suitable conditions (TN or TK buffer) assumes a G-quadruplex intermolecular structure, whose basic unit is a tetramer with parallel topology, as suggested by CD spectra.

It is worth noting that bands with lower electrophoretic mobility, corresponding to longer oligomers, are less represented in PAGE carried out in TK buffer (Fig. 3b), than in TN buffer (Fig. 3a). This finding supports AFM data that show shorter HTP superstructures in the case of TK with respect to those in TN buffer (Fig. 1a and b respectively). Similar differences in G-wires length were previously found in the case of $G_4T_2G_4$ between solution containing Na^+ or K^+ [6]. This behaviour is not surprising, since it is well known that the two cations significantly influence the structure, the topology and the stability of G-quadruplex [2,12]. On the contrary, the presence of $MgCl_2$ is not significant, since the electrophoretic pattern remains practically equal either in TN and in TK buffer with 5 mM $MgCl_2$ (data not shown).

In order to provide a direct evidence of the HTP G-quartets arrangement, we carried out the methylation interference assay that allows us to establish whether the guanine-N7 is engaged in Hoogsteen hydrogen bond [13]. This assay requires the oligonucleotide 5'-end labeling. HTP, when 5'-end phosphorylated, becomes unable to form G-quartets at the 5'-end and cannot oligomerize as demonstrated by its electrophoretic pattern, which shows only one band (Fig. S5). The methylation pattern (Fig. S6) allows us to establish that only the six guanines from G11 up to G17 are inaccessible to methylation in TK buffer and appear in TN buffer less methylated than the control. The details of interference methylation are reported in the text of Supplementary materials and Fig. S6. These results are well supported by CD thermal analysis of 5'-end phosphorylated HTP (Fig. S7), showing a reduction of ellipticity values and a lower thermal stability for 5'-end phosphorylated HTP respect to the CD thermal analysis reported in Fig. 2.

3. Experimental

The experimental section is thoroughly described in Supplementary materials.

4. Discussion

The ability of some intermolecular G-quadruplex structure to self-assemble has long been known [1]. The term "G-wires" was coined by Marsh and Henderson to describe the continuous parallel-stranded DNA superstructures, formed by $G_4T_2G_4$ self-assembly as a consequence of the slippage of chains, visualized by AFM imaging [6]. In the present study, the peculiar features of the HTP linear self-assembly, visualized by AFM in the presence of either NaCl or KCl (Fig. 1a and b), require a more complex explanation. In fact, it is necessary to involve the existence of assembling modules, characterized by different stability and probably by differential interactions with the mica surface. On the basis of CD spectra, PAGE and methylation interference analysis, the intermolecular parallel G-quadruplex structure of each HTP monomer seems consistently established. According to these findings, we propose that HTP may form an arrangement in which the stacking of G-quartets and associated cation complexes are maximized; in this structure the A-tracts loop out (schematic structure on the left in Fig. 4a), with the exception of the adenine connecting the two G_4 blocks, that should participate in the G-quadruplex structure [14]. The presence of extruded A-loops in our model is in good agreement with the evaluated actual length of REs (about 5 nm) corresponding to 13 stacked G-quartets. Taking into account the methylation interference assay, we suggest that the monomer structure is characterized by two regions with different stability (labeled as * and ** in Fig. 4a), which can give rise to two forms: a closed unit (c) and an open one (o) as shown in Fig. 4a. These two forms are distinguishable on PAGE (Fig. 3) because of their different electrophoretic mobility, slower for the form (o) which is more flexible and faster for the form (c) which is more rigid.

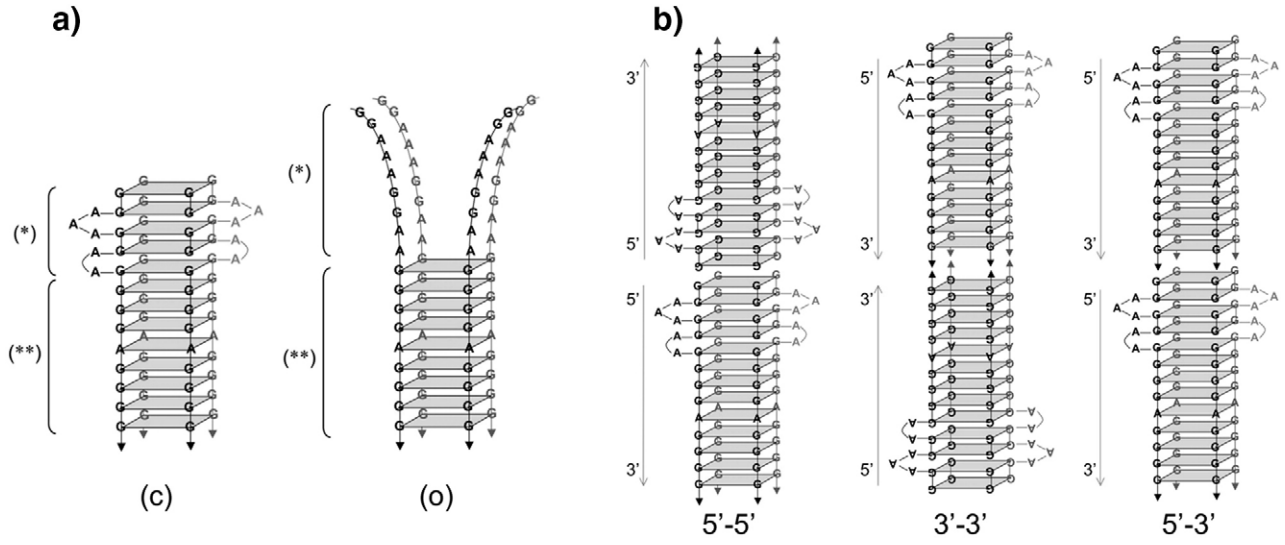

Fig. 4. a) Schematic representation of the proposed HTP structure. The more stable region is marked with $^{(**)}$, the less stable one with $^{(*)}$. On the left, (c) represents the closed monomer, in which the stacking of G-quartets is maximized; on the right, (o) is the open one. b) Pictorial drawing of 5'-5', 3'-3' and 5'-3' interconnections between two HTP intermolecular parallel G-quadruplexes. Only the A-loops on the opposite strands of G-quadruplex are reported.

Because of the electrostatic repulsion of the phosphate ends, the 5'-end phosphorylation of HTP moves the equilibrium toward the form (o), that for this reason is the only one identifiable by methylation interference assay. This interpretation is further supported by the lower thermal stability of 5'-end phosphorylated HTP, as shown by CD spectra (Fig. S7). As regard to the formation of "wires", we propose that the closed structure can be stabilized by end-to-end stacking, producing a G-wire-like assembly, that because of their appearance in AFM imaging we named "dashed-wire". The oligomerization reaction of (c) monomers can be characterized by 5'-5', 3'-3' and 5'-3' interconnections. In Fig. 4b a schematic drawing of the dimers, according to the three interconnections, is reported. For the oligomer lengthening, it could be relevant which moiety (* or **) of the two interacting monomers is involved because of their different stability: the 5'-5' interconnection seems to be the least favored, while the 3'-3', which should correspond to the most stable dimer, could act as the nucleation step in the oligomerization process, that can then occur indefinitely by the 5'-3' assembly of subsequent units.

AFM data allow us to suggest that the interaction of such arrays with the mica surface is not equivalent along the G-quadruplex self-assembly, because of both the presence of recurrent A-loops and the possible RE opening at 5'-terminal. This hypothesis is supported by the fact that the increase of the water amount in the AFM washing step causes an increase of the dashed features of the superstructures (Figs. S1 and S2); in fact the presence of (o) forms should raise on account of the ionic strength decrease. Although the opening of the RE at 5'-end should occur randomly, the interactions of A-loops with the mica should be regulated by the superstructural features of the "dashed wires" (helical twist within the G-quartets of about 30° [15] and strand permutation), recurring with the same features with a period of three closed units. This occurrence could explain the apparent periodicity of "dashed" elements in the AFM images.

Even though further investigations are surely necessary to obtain a deeper insight in the oligomerization of HTP G-quadruplex structure, the reported study indicates that oligonucleotide sequences, able to form G-wires-like nanostructures, can be significantly expanded with respect to those so far reported in the literature [6,7].

Finally, it is interesting to note that in the case of HTP, in contrast with the previous G-wire model, the driving force to self-assembly seems to be connected to the end-to-end stacking of G-quartets. The same force has been recently shown to give rise to liquid crystal for short DNA duplex in very crowded conditions [16]. As suggested by these authors, this short duplex DNA arrangement could be important in prebiotic organization. Since G-wires-like superstructures are possible at relatively low concentrations, it seems intriguing to speculate that also the G-quartets ability to self-assemble by end-to-end stacking might have had a role in prebiotic organization.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bpc.2008.04.009.

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