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STABILIZATION OF TETRAHELICAL DNA BY THE QUADRUPLEX DNA BINDING PROTEIN *QUAD*

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The 57-kDa hepatic nuclear protein *QUAD* binds tightly and specifically a parallel tetrahelical form of the IgG switch region DNA (Weisman-Shomer, P. and Fry, M. (1993) *J. Biol. Chem.* **268,** 3306-3312). Here we show that *QUAD* is a heat-stable protein, maintaining ~90% of its tetrahelix binding activity after 10 min at 100 °C and becoming fully inactivated only after 30 min at 100 °C. To demonstrate that *QUAD* protects bound quadruplex DNA, naked and *QUAD*-bound tetrahelices were boiled, the protein residue in the complex was digested with trypsin and quadruplex and single-strand forms of the DNA component were resolved by electrophoresis. Whereas naked quadruplex DNA became fully denatured after 2 min at 100 °C, 55% of the *QUAD*-bound DNA was conserved as a tetrahelix after 6 min at 100 °C. These findings support the proposal that *QUAD* may act *in vivo* to stabilize tetrahelical DNA.

Clusters of contiguous guanine residues appear at various locations along the genome such as in telomers (1-3), in gene promoters (4-6), and in immunoglobulin switch region recombination sites (7,8). Guanine-rich single stranded DNA fragments from such tracts are capable of forming tetrahelical structures that are stabilized by Hoogsteen-bonded guanine quartets. Oligomers that contain multiple runs of contiguous guanine residues can fold back in the presence of physiological concentrations of KCl or NaCl to generate mono-molecular (9-10) or bimolecular (10-12) antiparallel quadruplex DNA. Single stranded DNA fragments that contain one or several guanine clusters form in highly concentrated solutions (13-16), or under desiccation (17), a four stranded structure, designated G4 DNA, in which the strands have a parallel orientation (18). Generation of quadruplex DNA under physiological conditions invoked the suggestion that these structure may exist *in vivo*. The reported tight binding of quadruplex DNA to proteins such as Myo D (19), scavenger receptor (20)and hepatic *QUAD* protein (17), as well as the identification of a G4 DNA-specific nuclease in yeast (21,22), strengthen the notion that quadruplex DNA has a physiological significance. It was suggested that parallel, Hoogsteen-bonded tetrahelices link *in vivo* guanine-rich tracts in meiotic homologous chromosome to align them correctly and promote DNA recombination (13,16). In view of the high specificity and affinity at which rabbit liver

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QUAD protein binds parallel G4 tetrahelix, we proposed that this protein may serve to stabilize the weakly bonded short stretches of tetrahelical DNA at synapses between homologous chromatids (17). In line with this proposal we demonstrate here that *QUAD* protects G4 DNA against heat denaturation.

MATERIALS AND METHODS

Materials and Enzymes -- Isotopically labeled adenosine 5'-[γ-^{32}P] triphosphate (~3000 Ci/mmol) was supplied by Amersham, United Kingdom. HPLC-purified oligomer Q, with the IgG switch region sequence 5'-d(TACAGGGGAGCTGGGGTAGA)-3' was purchased from Operon Technologies, Alameda, CA. Sigma, St. Louis, MO. provided all the reagents except DEAE-cellulose (DE52), phosphocellulose (P11) and DE81 filter paper which were purchased from by Whatman, Kent, UK. Bacteriophage T4 polynucleotide kinase was provided by United States Biochemical Cleveland, OH. Reagents for polyacrylamide gel electrophoresis (PAGE) were supplied by IBI, New Haven, CT.

Isolation of G4 DNA -- Oligomer Q was end labeled with [^{32}P] and its G4 form was prepared and purified as we described (17). A portion of the isolated tetrahelix dissociated into mono-stranded DNA during storage. Although a mix of G4 and mono-strand oligomer Q was thus used in protein binding assays, it was only the G4 form to which *QUAD* bound (17).

Purification of QUAD Protein -- *QUAD* protein was purified from an extract of non histone proteins from rabbit hepatocytes. Extract preparation and purification of *QUAD* by Sepharose 6B-CL gel filtration and column chromatography on DE52, P11 and phenyl sepharose were conducted as described (17). PAGE-SDS electrophoresis of the phenyl sepharose fraction of *QUAD* and silver staining of the resolved protein indicated a 80-90% purity of *QUAD* protein. This fraction was used throughout the work.

Mobility Shift Electrophoresis -- The formation of a *QUAD*-G4 oligomer Q complex was monitored by the electrophoretic retardation of the protein-bound G4-DNA on a non-denaturing gel. Conditions for *QUAD*-G4 DNA complex formation and its resolution by mobility shift electrophoresis were as recently described (17). Gels were dried on DE81 filter paper and *QUAD* binding to G4-DNA was quantified by counting Cerenkov radioactivity in bands cut of the dried gel. Amounts of free and protein-bound DNA were deduced from the predetermined specific activity of the DNA. One unit of G4 DNA binding activity was defined as the activity that binds 0.1 ng of G4 oligomer Q under standard binding conditions.

RESULTS

QUAD is a heat-stable protein -- In studying physical-chemical properties of highly purified *QUAD* protein, we noted its remarkable resistance to heat. Purified *QUAD* was incubated at different temperatures for 10 min and then added into a binding mixture together with a 30:70 mix of [^{32}P]-G4 oligomer Q:mono-strand oligomer Q. Formation of a G4 DNA-protein complex was monitored by mobility shift electrophoresis. As seen in Figure 1, the quadruplex DNA binding activity of *QUAD* is remarkably heat stable. *QUAD* incubated for 10 min at 4 to 65 °C yields similar amounts of a major and a minor complex bands with G4 oligomer Q. Although the minor band is reduced when *QUAD* is heated at 75 or 100 °C, the major complex band remains largely undiminished (Fig. 1). Quantification of *QUAD*-associated [^{32}P]-G4 oligomer Q reveals that merely ~10% of the complex disappears after the protein is heated at

100 °C for 10 min (results not shown). *QUAD* loses its capacity to form a complex with the G4 DNA only after it is heated at 100 °C for 30 min (Fig. 1).

QUAD-G4 DNA complex is heat stable -- Utilizing the relative heat stability of *QUAD*, we assessed the heat resistance of its complex with G4 DNA. A preformed *QUAD*-[^{32}P]-G4 oligomer Q complex was heated at 100 °C for different periods of time, protein-bound and free G4 DNA were resolved by mobility shift electrophoresis and their respective amounts were determined. As seen in Figure 2, the complex is remarkably heat stable, its amount decreasing by only 11 and 15% after heating at 100 °C for 1 and 7 min, respectively.

QUAD protects G4 DNA against heat denaturation -- In view of the heat resistance of the *QUAD*-G4 DNA complex, we examined whether the tetrahelical structure of the DNA component is conserved within the boiled complex. Naked G4 oligomer Q or its complex with *QUAD* were heated at 100 °C for 3 min, rapidly cooled to 4 °C and digested at 37 °C for 30 min by trypsin. Electrophoretic analysis shows that following trypsin digestion of the protein residue

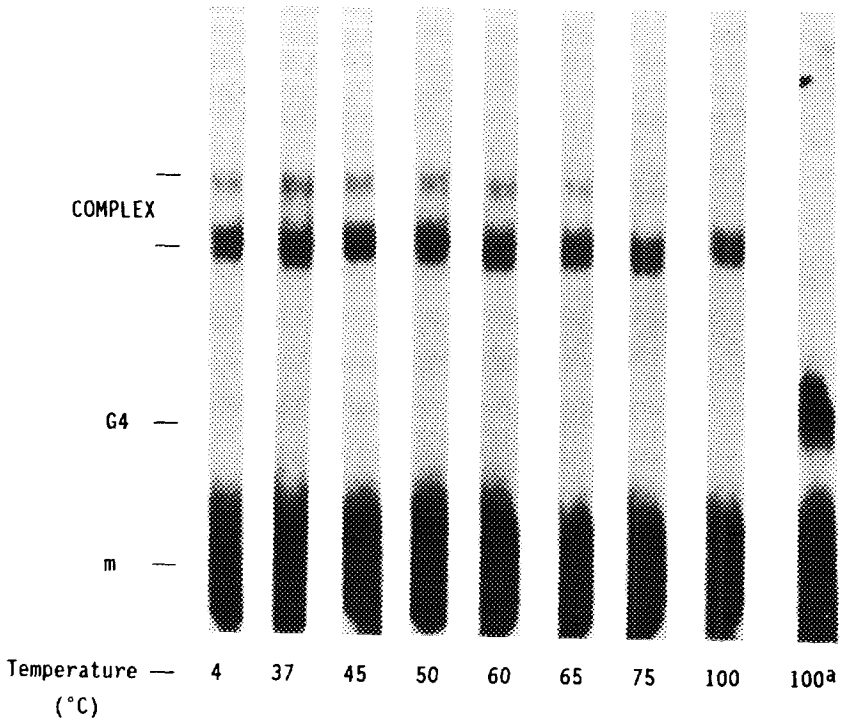

Fig. 1. **Heat resistance of *QUAD* protein.** Purified *QUAD* protein (11.5 units) was incubated for 10 min at the indicated temperatures, rapidly cooled to 4 °C and added to a 30:70 mixture of [^{32}P]-G4:mono-strand oligomer Q in a DNA binding reaction mix. *QUAD*-G4 DNA complex and free DNA were resolved by gel shift electrophoresis (see Methods). *m*, mono strand oligomer Q; *G4*, quadruplex form of oligomer Q; *complex*, *QUAD*-bound G4 oligomer Q. [a] *QUAD* protein preincubated at 100 °C for 30 min.

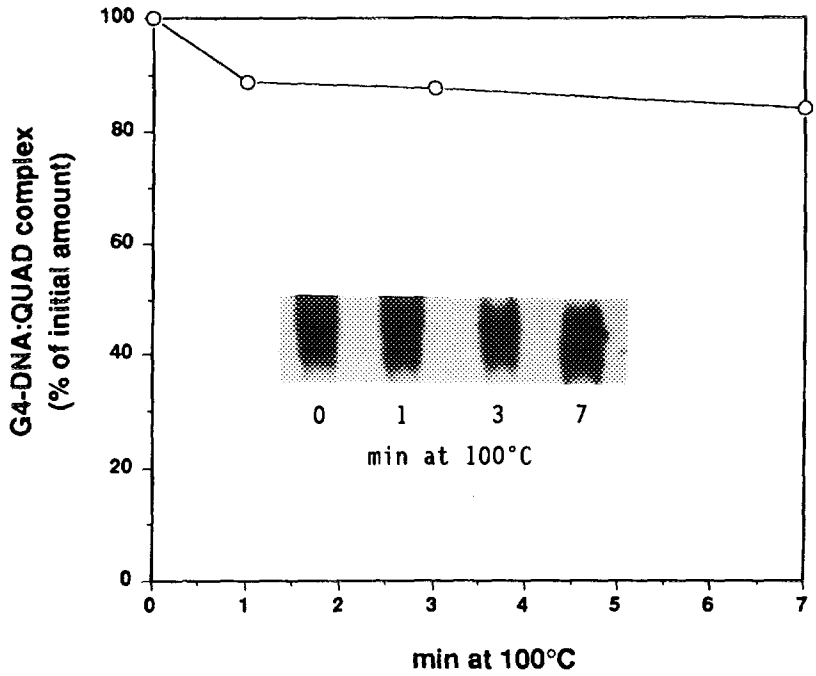

Fig. 2. **Heat stability of the *QUAD*-G4 oligomer Q complex.** Purified *QUAD* protein (11.5 units) was incubated at 4 °C in a DNA binding reaction mixture with 30:70 mixture of [^{32}P]-G4:mono-strand oligomer Q. After the complex was formed, the mixtures were heated at 100 °C for the indicated periods of time. *QUAD*-G4-oligomer Q complex was resolved from free DNA by mobility shift electrophoresis and the relative amount of G4-DNA in complex was determined (see Methods). Binding of 100% represents the amount of bound G4 oligomer Q in a mixture not exposed to 100 °C. Inset: *QUAD*-G4 oligomer Q bands.

of the complex, the recovered DNA component is largely maintained in a tetrahelical form (Fig. 3). By clear contrast, naked G4 DNA that is similarly heated is completely melted into mono-strand oligomer Q (Fig. 3). The conservation of oligomer Q in a tetrahelical form within the heated complex was further substantiated by comparing the kinetics of melting at 100 °C of naked and *QUAD*-associated G4-oligomer Q. As seen in Figure 4, more than 90% of naked G4 oligomer Q is denatured after it is exposed to 100 °C for 1 min and the tetrahelix becomes undetectable after 2 to 6 min at this temperature. By contrast, more than 80% of the *QUAD*-bound DNA remains in quadruplex form when the complex is heated to 100 °C for 1 or 2 min and about 50 to 55% of the tetrahelix remains intact after heating of the complex at 100 °C for 4 or 6 min (Fig. 4). We conclude, therefore, that the relative heat resistance of the complex between *QUAD* and G4 oligomer Q (Fig. 2), reflects stabilization of the DNA tetrahelix by *QUAD*.

DISCUSSION

Several lines of evidence argue that tetrahelical structures which are formed by interaction between guanine-rich segments in DNA may exist in the cell. First, guanine quartets are

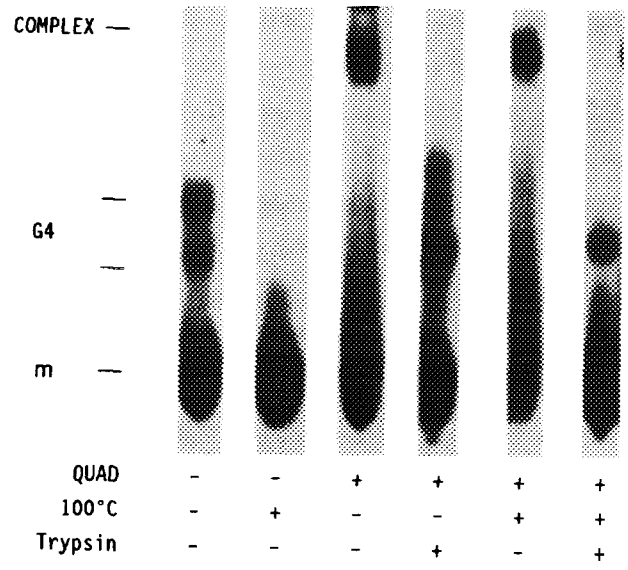

Fig. 3. ***QUAD* stabilizes bound G4-oligomer Q against heat denaturation.** A 40:60 mixture of naked [^{32}P]-G4:mono strand oligomer Q and a complex of the G4 DNA with *QUAD* were kept at 4 °C or heated at 100 °C for 3 min and then cooled rapidly in an ice bath to 4 °C. All the mixtures were subsequently incubated for 30 min at 37 °C with trypsin (375 μg/ml) and the proteolytic digestion was terminated by the addition of soy bean trypsin inhibitor (2.0 mg/ml). *QUAD*-G4 oligomer Q complex, mono-strand oligomer Q and its G4 form were resolved by mobility shift electrophoresis. *m*, mono strand oligomer Q; *G4*, quadruplex form of oligomer Q; *complex*, *QUAD*-bound oligomer Q.

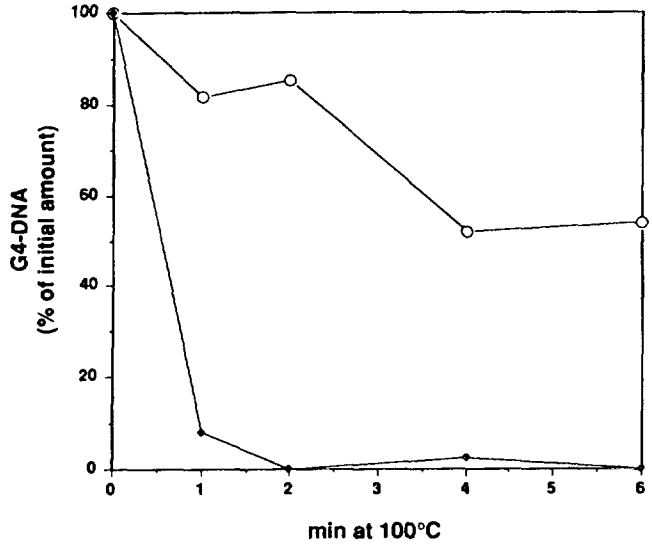

Fig. 4. **Kinetics of denaturation by heat of naked and *QUAD*-bound G4-oligomer Q.** Free or *QUAD*-bound G4-oligomer Q were heated at 100 °C for the indicated periods of time, rapidly cooled to 4 °C in an ice bath and exposed for 30 min at 37 °C to trypsin (375 μg/ml). Proteolytic digestion was terminated by the addition of soy bean trypsin inhibitor (2.0 mg/ml), G4 and mono-stranded oligomer Q were resolved by non-denaturing gel electrophoresis and their relative amounts were determined. O-----O G4-oligomer Q recovered from its complex with *QUAD* protein; ●-----● naked G4-oligomer Q.

generated under physiological conditions (11,13). Second, indirect evidence suggests that G4 DNA may form along a region in c-Ha-*ras* gene that spans the highly mutable codon 12 (23), along the expanded $d(CGG)_n$ stretch in fragile X syndrome DNA (24), and within the dimeric RNA of the HIV-1 genome (25). Last, several proteins bind to or interact specifically with quadruplex DNA (17,19-22). It was thus conjectured that quadruplex DNA is transiently formed *in vivo* to play a role in normal cellular functions. Sen and Gilbert (13), were the first to suggest a possible role of guanine quartets in the generation of parallel tetrahelical synapses between homolog chromosomes during meiosis. It was further argued that a precise alignment of homologous chromatids could be mediated by tetrahelical synapses that are generated between tracts of contiguous guanine residues at the telomeric ends and at intrachromosomal sites (13). The recent finding that a quadruplex DNA dependent nuclease in yeast is essential for meiosis (22), gives credence to this model. However, it is hard to see how the relatively vast homologous chromosomes can be stably held together by a limited number of tetrahelices, each consisting of a single or a few repeats of 3-4 guanine quartets. We proposed, therefore, that once tetrahelical synapses are formed, they are further stabilized by an association with G4 DNA binding proteins such as rabbit liver *QUAD* (17). Results presented in this paper show that the complex between G4 oligomer Q and the heat stable *QUAD* is heat resistant - remaining largely intact after 10 min at 100 °C (Fig. 2). This stability contrasts the fragility of naked G4 oligomer Q which melts completely after 1 to 2 min at 100 °C (Fig. 4). Most interestingly, we show that the relative heat resistance of the complex reflects an increased stability of the bound quadruplex form of oligomer Q, as demonstrated by the direct recovery of conserved G4-DNA from its heated complex with *QUAD* (Fig. 3 and Fig. 4). Hence, short stretches of Hoogsteen-bonded guanine quartets are stabilized when in complex with *QUAD* protein. These data support the notion that *QUAD* may act *in vivo* to stabilize interchromosomal quadruplex synapses.

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