QUAD, a Protein from Hepatocyte Chromatin That Binds Selectively to Guanine-rich Quadruplex DNA*

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The single-stranded oligomer Q, whose nucleotide sequence 5'-d(TACAGGGAGCTGGGGTAGA)-3' corresponds to the IgG switch region, forms in concentrated solutions and in the presence of alkali metal ions, parallel four-stranded complexes termed G4 DNA (Sen, D., and Gilbert, W. (1988) Nature 334, 364-366). We show that G4 DNA was also formed during storage of dried oligomer Q. This quadruplex complex migrated more slowly than mono-strand oligomer Q during nondenaturing gel electrophoresis, the rate of its formation depending on the mass of stored oligomer Q, and N7 positions of guanine residues were involved in its stabilization. Here we report the purification of a protein designated QUAD that binds specifically to the G4 form of oligomer Q, from nonhistone protein extracts of rabbit hepatocytes. QUAD was 80-90% purified by sequential steps of column chromatography on Sepharose 6B, DEAE-cellulose, phosphocellulose, and phenyl-Sepharose. Purified QUAD migrated on SDS-polyacrylamide gel electrophoresis as a 58 ± 2.6-kDa polypeptide and had a native molecular mass of 57 ± 2.5 kDa as determined by Sepharose 6B gel filtration. The dissociation constant of G4 DNA binding to QUAD was in the range of 2.5 to 7.0 × 10^{-9} M/liter. Excess unlabelled mono-stranded oligomer Q did not compete with 5'-32P-labeled G4 DNA on its binding to QUAD. Further, that QUAD recognized the G4 DNA structure rather than a DNA sequence was also demonstrated by the inefficient competition on the binding of 5'-[32P]G4 DNA to QUAD by excess unlabelled single- or double-stranded DNA molecules that contained guanine clusters of different length or various other nucleotide sequences.

A growing body of experimental evidence indicates that unusual DNA structures such as cruciform DNA, alternating B-Z regions, bent DNA, and triplex and quadruplex DNA may be involved in specific cellular processes (Yagil, 1991; Palecek, 1991). Clusters of guanine residues appear in many chromosomal locations such as telomers (Zakian, 1989; Blackburn, 1990; 1991), in gene promoters (Evans et al., 1984; Kilpatrick et al., 1986; Clark et al., 1990), and in the immunglobulin switch region (Shimizu and Honjo, 1984). Single-stranded DNA fragments from such regions are capable of aggregating into four stranded structures that are stabilized by Hoogsteen-bonded guanine quartets (Henderson et al., 1987; Sen and Gilbert, 1988, 1990, 1992; Williamson et al., 1989; Sundquist and Klug, 1989; Panyutin et al., 1989, 1990; Hardin et al., 1991; Lu et al., 1992). Oligomeric fragments of telomers can form in the presence of low concentrations of KCl or NaCl fold-back structures which migrate in a nondenaturing gel more rapidly than the mono-stranded input oligomer (Henderson et al., 1987; Williamson et al., 1989). Two such hairpin fold-back telomeric fragments of Oxycricha and Tetrahymena can combine to generate guanine quartet-stabilized dimeric quadruplex structure (Williamson et al., 1989; Sundquist and Klug, 1989; Hardin et al., 1991). Further, stretches of 27-37 guanine residues in denatured plasmid DNA are able to fold into antiparallel tetrahedrical structures (Panyutin et al., 1989, 1990). Lastly, single-stranded oligomers that contain short runs of guanine form in highly concentrated solutions, and in the presence of sodium ions, a four-stranded structure, termed G4 DNA, in which the strands run in a parallel orientation (Sen and Gilbert, 1988, 1990, 1992; Lu et al., 1992).

Formation of four-stranded DNA under physiological conditions invites suggestions as to the potential in vivo role of this unusual structure. Generation of parallel, Hoogsteen-bonded quadruplex DNA during the interaction of four double helices has been proposed to tie together the four chromatids at meiosis (Sen and Gilbert, 1988). If involved in the formation of tight synapses between homologous chromosomes at their telomeric ends and elsewhere in the chromosome (Sen and Gilbert, 1988; Lu et al., 1992), such tetrahedrical structure at guanine-rich regions in DNA may be instrumental in promoting recombination.

In view of the relative fragility of Hoogsteen-bonded quadruplex DNA, it is conceivable that specific DNA binding proteins may promote its formation or stabilization. In this paper, we describe the purification of a protein, designated QUAD, from chromatin of hepatocytes that specifically binds a quadruplex form of oligomer Q, whose nucleotide sequence 5'-d(TACAGGGAGCTGGGGTAGA)-3' corresponds to the IgG switch region. An observed high affinity binding of QUAD protein to tetrahedrical but not to mono-stranded oligomer Q or to other single- or double-stranded DNA molecules, might indicate its involvement in the stabilization of quadruplex DNA.

**EXPERIMENTAL PROCEDURES**

Nucleotides, Oligonucleotides, and Polynucleotides—Radioactively labeled adenosine 5'-[γ-32P]triphosphate (5000 Ci/mmol) was the product of Amersham, United Kingdom. The following HPLC-purified chemicals were used: dTTP, dThd, dATP, dGTP, dCTP, 3',5'-diphosphate; ddATP, ddTTP, ddCTP, ddGTP, 3',5'-diphosphate; 3',5'-dGTP; ATP, 3',5'-diphosphate, and 3',5'-cyclic AMP; GMP, 3',5'-cyclic GMP; c, d, and L ribose and deoxyribose 3',5'-cyclic monophosphates; and 3',5'-cyclic UMP, 3',5'-cyclic CMP, 3',5'-cyclic GMP, and 3',5'-cyclic AMP. Stock solutions of each compound were made in 100 mM HEPES (pH 7.5) and stored at -70°C. The specific activities of 3',5'-cyclic GMP and 3',5'-cyclic AMP were determined by measurement of cGMP and cAMP released from ribonucleoside 5'-triphosphates (Christen et al., 1980).

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1 The abbreviations used are: HPLC, high performance liquid chromatography; DTT, dithiothreitol; MalNEt, N-ethylmaleimide; pTTP, thymidine 3',5'-diphosphate; STI, soybean trypsin inhibitor; TEMED, N,N,N',N'-tetramethylthylendiamine; PAGE, polyacrylamide gel electrophoresis.
rified synthetic oligomers were purchased from Operon Technologies, Alameda, CA: oligomer Q, 5'-d(TACAGGGAGCTGGGGTAGA)-3'; Tet, 5'-d(T[GC]3)-3'; d(AATT(AG)3)-5', 5'-d(AATT(AG)3)-3', (dG)10. Sigma supplied (dE)-3', poly(dA-TI), poly(dC-GI), poly(dG)-poly(dC), and poly(dA)-poly(dT). Mammalian cell mass marker proteins, trypsin, and micro- 

vocal nucleases were supplied by Sigma. DEAE-cellulose (DE52), phosphocellulose (P-11), and DE51 filter paper were the products of Whatman. Bacteriophage T4 polynucleotide kinase was provided by United States Biochemical Corp. Acry1:Bis (19:1 or 30:1.2) was pur- 

chased from Amresco, Solon, OH. Kodak XAR autographic film, uae, TEMED, bromphenol blue, and xylene cyanol FF were 

supplied by IBI, New Haven, CT.

Isolation of G4 DNA—Quadruplex G4 DNA was formed by inter-

strand association of oligomer Q. Firstly, aliquots (1.0 µg/lane) of 5% polyacrylamide gel were prepared from isolated nuclei of hepatocytes of 

One unit of G4 binding activity was defined as the activity that binds 

protein-DNA binding mixtures were electrophoresed at room temper-

ature under 10 V/cm for 1.5 h through a 6% polyacrylamide gel 

4°C for 20 min with 0.5-3.0 ng of 5'-32P-labeled G4 DNA. The 

result was terminated by the addition of EDTA to a final con- 

centration of 20 mM, and the column was washed with 2-column volume of wash with PS buffer. Forty fractions were collected, 0.2 mg/ml STI and 0.05% Nonidet P40 were added, and each fraction was dialyzed against DE buffer. DNA binding activity of QUAD protein was detected in fractions that were 

eluted in the final PS buffer wash. Purified QUAD protein remained active for at least 2 months when stored at -70°C in DE buffer that contained 0.2 mg/ml STI, 0.05% Nonidet P-40.

Determination of the Molecular Mass of QUAD Protein—The mole-

cular mass of phenyl-Sepharose-purified, denatured QUAD protein was determined by its relative electrophoretic migration through a 10% SDS-PAGE (Laemmli, 1970). Molecular mass marker proteins were: phosphorylase B, 92.5 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45.0 kDa; carbonic anhydrase, 31.0 kDa; STI, 21.5 kDa. Ammoniacal silver was used to stain the protein bands (Switzer et 

al., 1979). The molecular mass of native, phenyl-Sepharose-purified QUAD protein was determined by its gel partition coefficient (Kv) in Sepharose 6B-CL (0.7 X 48.1 cm column, 18.5 ml of packed column volume, 14.1 ml void volume). Molecular mass marker proteins were: 

β-amyelobin, 200 kDa; bovine serum albumin, 66 kDa; ovalbumin, 48 kDa; carbonic anhydrase, 29 KDa; STI, 21.5 kDa.

Determination of the Amount of Protein—The Bio-Rad protein assay kit was used to determine the amount of protein.

RESULTS

Generation and Properties of Quadruplex G4 DNA—Oligo-

mer Q, whose nucleotide sequence 5'-d(TACAGGGAGCTGGGGTAGA)-3' corresponds to the IgG switch region (Sen and Gilbert, 1988), has been shown to form an interstrand association of its two dG oligomers. This protein was eluted by 140- 

215 mM KCl, and the pooled active fractions were dialyzed overnight against PS buffer (25 mM Tris-Cl buffer, pH 7.5, 0.5 mM EDTA, 1 mM DTT, 20% 

glycerol).

Phosphocellulose Chromatography—DEAE-cellulose-resolved QUAD protein was loaded onto a phenyl-Sepharose column equilibrated in PS buffer that contained 4.0 mM KCl at a ratio of 1.0 mg of protein/ml of packed column volume. The column was washed with 2 column volumes of the equilibration buffer, and proteins were eluted by a 10-column volume gradient of 0.0 to 0.5 M NaCl in PS buffer followed by 3-column volume wash with PS buffer. 

Fifty fractions were collected, 0.2 mg/ml STI and 0.05% Nonidet P40 were added, and each fraction was dialyzed against DE buffer. DNA binding activity of QUAD protein was detected in fractions that were 

eluted in the final PS buffer wash. Purified QUAD protein remained active for at least 2 months when stored at -70°C in DE buffer that contained 0.2 mg/ml STI, 0.05% Nonidet P-40.
Experimental Procedures. Formation of gel-purified oligomer Q at were suspended in DE buffer to through a oligomer Q stored under desiccation. Oligomer Q was purified by denaturing gel electrophoresis and stored under desiccation. Variable amounts of oligomer Q were stored desiccated at −20 °C for 48 h as described under "Experimental Procedures." Results were plotted as the log of the amount of G4 DNA formation and stability on DNA concentration suggest that it is formed by interaction between multiple DNA strands (Sen and Gilbert, 1989). The two types of DNA were incubated above. A value of 100% G4 DNA represents 52% of the total mono-strand and G4 oligomer Q mixture in the initial 1.4 μg/ml solution.

G4 DNA that was formed under desiccation was suspended in TE buffer to concentrations ranging between 1.4 and 0.14 μg of DNA/ml and electrophoresed through a nondenaturing gel. As shown in Fig. 5B, the slowly migrating G4 DNA remained stable in solutions of 1.4 to 0.35 μg of DNA/ml, but, upon dilution to 0.14 μg/ml, about 50% of the G4 DNA was dissociated to mono-strand oligomer Q. Thelower mobility of G4 DNA relative to oligomer Q, its heat denaturation, and lack of rapid self-renaturation as well as the dependence of G4 formation and stability on DNA concentration suggest that it is formed by interaction between multiple DNA strands (Sen and Gilbert, 1988, 1990, 1992).

To demonstrate the involvement of N° positions of guanine residues in the formation of G4 DNA, methylation-protection was compared for mono-strand oligomer Q and its G4 form. Aliquots, 70 ng each, of gel-purified desiccated G4 form of oligomer Q were suspended in 20 μl of TE buffer and either denatured at 100 °C for 5 min to generate mono-strand oligomer or left untreated as a 70:30 mixture of G4 and mono-stranded oligomer Q, respectively. In a procedure derived from Williamson et al. (1989), the two types of DNA were incubated...
at room temperature for 10 min with 0.1% dimethyl sulfate and then heated at 90 °C for 15 min with 2 M pyrrolidine. Following desiccation and two washes with water, the intact and hydrolyzed DNA samples were electrophoresed through a 12% polyacrylamide denaturing gel. Measurement of Cernekov radioactivity in bands of undegraded and hydrolyzed DNA indicated that without dimethyl sulfate treatment, 15% of the G4-enriched DNA were broken down under the described conditions. However, following methylation, 16.7% of denatured oligomer Q were hydrolyzed, whereas only 1.5% of the G4-enriched DNA were broken down. The apparent inaccessibility of the N7 position in guanines of G4 DNA indicated their involvement in Hoogsteen bonding (Hoogsteen, 1959) and affirmed the tetrahelical nature of G4 DNA (Sen and Gilbert, 1988, 1990; Williamson et al., 1989; Sundquist and Klug, 1989).

Purification of the G4 DNA Binding Protein QUAD—Electrophoretic mobility shift analysis revealed the presence of a G4 DNA binding activity in crude extracts of non-histone proteins from rabbit hepatocytes. This activity was purified by sequential steps of column chromatography on Sepharose 6B, DEAE-cellulose, phosphocellulose, and phenyl-Sepharose. As shown in Fig. 4A, proteins resolved in Sepharose 6B fractions 42 to 55 associated with both mono-stranded oligomer Q and its G4 form. Upon subsequent purification of these fractions by DE52 chromatography, proteins were eluted at 145 to 245 mM KCl (fractions 12–20, Fig. 4B) that also associated with both mono-strand and G4 DNA. Chromatography of these fractions on P-11 removed most of the mono-strand binding activity and yielded a protein that we termed QUAD and which was eluted from the cation exchanger at 140 to 215 mM KP. As seen in Fig. 4C, QUAD associated with both the major and minor G4 DNA forms but bound only negligibly mono-strand oligomer Q. Final purification of P-11-resolved QUAD protein was performed by hydrophobic phenyl-Sepharose column chromatography. QUAD activity was eluted in the final wash with buffer devoid of salt (Fig. 5A). Whereas SDS-PAGE analysis showed that the QUAD-containing fractions of Sepharose 6B, DE52, and P-11 contained a progressively decreasing number of proteins (Fig. 5B), a single major polypeptide band of 58.7 kDa was discerned in fractions of phenyl-Sepharose-resolved QUAD (Fig. 5C). The apparent identity between the elution profiles of the 58.7-kDa protein (Fig. 5C) and the G4 DNA binding activity (Fig. 5A) strongly suggested that the 58.7-kDa polypeptide was QUAD. Multiple electrophoresis analyses indicated that the 58.7-kDa band consisted of at least 80–90% of the total protein in phenyl-Sepharose-resolved fractions of QUAD. This highly purified fraction of QUAD was used throughout this work.

Chemical-Physical Properties of QUAD—Some properties of QUAD protein are summarized in Table I. As demonstrated by its heat lability and inactivation by SDS or trypsin digestion, QUAD is a protein. By contrast, QUAD activity was not diminished by nucleolytic digestion with micrococcal nuclease and thus it did not contain an essential nucleic acid component. In fact, nuclease digestion somewhat increased G4 DNA binding by QUAD (Table I), possibly by removing some competing DNA contamination. Binding of G4 DNA by QUAD was decreased only slightly in the presence of 4–10 mM MalNEt (Table I), and thus reduced sulfhydryl groups are probably not involved directly in G4 DNA binding.

The highly purified QUAD protein migrated on SDS-PAGE as a 58.7 ± 2.6-kDa polypeptide (average of determinations in six independent QUAD preparations). Sepharose 6B gel filtration of QUAD yielded a native molecular mass of 57.0 ± 2.45 kDa (average of determinations in four independent QUAD preparations). QUAD is, therefore, most probably a monomeric protein.

Specificity of G4 DNA Binding by QUAD—To examine the extent of DNA sequence and structure specificity of the association of QUAD with G4 DNA, molar excesses of a variety of oligonucleotides and polynucleotide were used to compete with 5'-32P-labeled G4 DNA on its binding to QUAD. Results of this experiment are summarized in Table II. It is
Fig. 5. Phenyl-Sepharose column purification of QUAD protein. A phosphocellulose-purified fraction of QUAD was chromatographed through a phenyl-Sepharose column as described under "Experimental Procedures." G4 DNA binding activity was eluted from the column in the PS buffer final wash (fractions 4-6). Shown are fractions eluted only by 0.5 to 0.0 M NaCl. A, mobility shift electrophoresis of fractions eluted from the phenyl-Sepharose column. m, mono-strand oligomer G4; Q, G4, quadruplex form of oligomer Q; complex, protein-bound G4 DNA. B, silver-stained proteins in SDS-PAGE resolved QUAD fractionated by Sepharose 6B, DEAE-cellulose, and phosphocellulose chromatographies. C, silver-stained proteins in SDS-PAGE-resolved QUAD fractionated by the phenyl-Sepharose column chromatography shown in A. Arrowheads indicate positions of molecular mass marker proteins. STI, stabilizing STI protein.

TABLE I

Chemical-physical properties of QUAD protein

| Treatment          | % initial activity |
|--------------------|-------------------|
| None               | 100.0             |
| 100 °C, 30 min     | 2.5               |
| Trypsin digestiona| 4.3               |
| 0.2% SDS          | 4.7               |
| Micrococcal nucleaseb | 145.0            |
| 4 mM MalNEtc      | 86.5              |
| 10 mM MalNEtc     | 87.5              |

a QUAD protein was incubated at 37 °C for 60 min with 375 μg/ml trypsin, and the proteolytic digestion was terminated by the addition of STI to a final concentration of 2.0 mg/ml. Shown is an average result of three independent determinations.

b QUAD protein was incubated at 37 °C for 30 min with 50 μg/ml micrococcal nuclease in the presence of 1.0 mM CaCl₂, and digestion was terminated by the addition of EDTA and pTP to final concentrations of 3.3 mM and 0.25 mM, respectively. Shown is an average result of two experiments.

c QUAD protein was incubated at 4 °C for 15 min with MalNEt, and the reaction was terminated by the addition of 20 mM DTT. Average result of two experiments.

Table II

| Competitor DNA | 5'-[32P]G4-Q DNA bound |
|----------------|-----------------------|
| None          | 100                   |
| G4-Q          | 3                     |
| Mono-strand Q | 80                    |
| G4 (dG)₃₅     | 120                   |
| Mono-strand Tet | 108                |
| Mono-strand Tet | 80                  |
| d[AATTC(A)₃₅G] | 120                   |
| d[AATTC(C)₃₅G] | 75                    |
| (dT)₂₀        | 54                    |
| Poly[dA-T]    | 130                   |
| Poly[dG-C]    | 97                    |
| Poly[dG-poly(dC)] | 80               |
| Poly[dG]-poly[dC-T] | 85             |

a Unlabeled G4 forms of oligomer Q and of (dG)₃₅ were isolated as described under "Experimental Procedures" except that bands of unlabeled G4 DNA were identified for excision from nondenaturing gels by labeled G4 DNA which was run in parallel lanes. Amounts of DNA that were extracted from the gel slices were determined spectrophotometrically.

In the light of the apparent high degree of the specificity of the association of QUAD protein with G4 DNA, we measured the binding affinity of highly purified QUAD to G4 DNA. A constant amount of phenyl-Sepharose-purified QUAD protein was incubated at 4 °C for 20 min with increasing amounts of 5'-[32P]labeled G4 DNA, and DNA-protein complexes were resolved by mobility shift electrophoresis. Radioactivity in free and protein-bound G4 DNA was measured in their respective cut bands to determine their amounts (see "Experimental Procedures"). A typical Scatchard plot of the results of such a measurement is presented in Fig. 6. The range of values of dissociation constants that were derived in different experiments from the negative reciprocal of the slope was between 2.5 × 10⁻⁶ to 7.0 × 10⁻⁹ M/liter (four determinations).

DISCUSSION

Single-stranded DNA that contains guanine-rich motifs has been shown to self-associate in the presence of monovalent salt in a concentration-dependent fashion, to form a four-stranded G4 DNA structure in which the strands assume parallel orientation (Sen and Gilbert, 1988, 1990, 1992; Lu et al., 1992). In agreement with these reports, we found that when stored in solution and in the presence of 0.4–1.0 M NaCl, oligomer Q aggregated in a concentration-dependent manner to generate a form with a lower electrophoretic mobility in a nondenaturing gel than that of the mono-stranded oligomer (Fig. 1). In this work, we showed evidence that a similar slowly migrating and heat-labile G4-like form of oligomer Q...
oligomer Q to QUAD protein (Table II). Hence, rather than being a sequence-specific DNA binding protein, QUAD behaved as a DNA structure-specific binding protein.

By associating specifically with quadruplex G4 DNA, QUAD differs from previously described DNA binding proteins that bind guanine runs in DNA. Some such proteins are complexed with the guanine-rich telomeric DNA. A heterodimeric protein from *Oxytricha* binds specifically to the telomeric d(G,T)₆ single strand tail and to its adjacent duplex region (Gottschling and Zakian, 1986; Price and Cech, 1989; Raghuraman et al., 1989). This telomere binding protein has been shown, however, to be unable to bind a folded, presumably guanine quartet-stabilized form of the telomeric sequence (Raghuraman and Cech, 1990). Other guanine cluster binding proteins associate with single- or double-stranded DNA. In yeast, the abundant transcriptional regulator RAP1 binds to the duplex portion of telomeric DNA but not to its single-stranded protrusion (Conrad et al., 1990; Lustig et al., 1990). The intermediate filament subunit vimentin binds at a low ionic strength single-stranded synthetic oligonucleotides that correspond to a single repeat unit of telomers from *Oxytricha* (T₄G₄, Saccharomyces (TGTGTG₄), or *Tetrahymena* (T₄G₄)) (Shoeman et al., 1988). BGP1, a zinc-dependent double strand DNA binding protein from erythroid cells associates specifically with a string of 7 or more guanine residues (Lewis et al., 1988; Clark et al., 1990). None of these proteins, however, was reported to bind tetrahelical guanine-rich DNA.

After this manuscript was submitted for publication, Walsh and Gualberto (1992) reported that recombinant MyoD, a transcription factor that initiates myogenesis, binds guanine tetrad s formed within a single strand creatinine kinase enhancer probe or within a telomeric DNA probe. In addition to the different physical properties of QUAD and MyoD and their dissimilar tissue localization, they display different DNA binding properties. Although MyoD binds guanine tetrad s at a high affinity, it also binds single-stranded and double-stranded probes (Walsh and Gualberto, 1992). By contrast, QUAD binds to the tetrahelical form of oligomer Q but does not bind significantly to various single- or double-stranded DNA probes that do or do not contain guanine clusters (Table II). Yet, the fact that these two proteins associate at a high affinity with quadruplex DNA argues for a physiological occurrence and a biological role for this unusual DNA conformer.

It has been proposed that formation of tetrahelical synapses between chromatids aligns homologous chromosomes in preparation for eventual exchange of genetic material (Sen and Gilbert, 1988). It might be that whereas the initial alignment of chromatids is mediated by tetrat formation at guanine clusters in DNA, proteins are required to stabilize the weakly bonded DNA synapses. It is tempting to speculate that QUAD protein, by virtue of its specific binding to tetrahelical DNA, stabilizes quadruplex DNA synapses to allow recombination to occur.

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