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Human Ku Antigen Tightly Binds and Stabilizes a Tetrahelical Form of the Fragile X Syndrome d(CGG)$_n$ Expanded Sequence*

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Hairpin and tetrahelical structures of a d(CGG)$_n$ sequence in the *FMR1* gene have been implicated in its expansion in fragile X syndrome. The identification of tetraplex d(CGG)$_n$ destabilizing proteins (Fry, M., and Loeb, L. A.(1999) *J. Biol. Chem.* 274, 12797–12803; Weisman-Shomer, P., Naot, Y., and Fry, M. (2000) *J. Biol. Chem.* 275, 2231–2238) suggested that proteins might modulate d(CGG)$_n$ folding and aggregation. We assayed human TK-6 lymphoblastoid cell extracts for d(CGG)$_8$ oligomer binding proteins. The principal binding protein was identified as Ku antigen by its partial amino acid sequence and antigenicity. The purified 88/75-kDa heterodimeric Ku bound with similar affinities (K_d ~1.8–10.2 × 10^{-9} mol/liter) to double-stranded d(CGG)$_8$·d(CCG)$_8$, hairpin d(CGG)$_8$, single-stranded d(CII)$_8$, or tetraplex structures of telomeric or IgG switch region sequences. However, Ku associated more tightly with bimolecular G′2 tetraplex d(CGG)$_8$ (K_d ~0.35 × 10^{-9} mol/liter). Binding to Ku protected G′2 d(CGG)$_8$ against nuclease digestion and impeded its unwinding by the tetraplex destabilizing protein qTBP42. Stabilization of d(CGG)$_n$ tetraplex domains in *FMR1* by Ku or other proteins might promote d(CGG) expansion and *FMR1* silencing.

Fragile X syndrome is the single most common inherited cause of mental impairment. Recent studies suggest that fragile X affects 1 in 2000 males and 1 in 4000 females regardless of race or ethnicity (1). The syndrome is associated with a substantial expansion of a d(CGG) trinucleotide repeat in the 5′-untranslated region of the first exon of the *FMR1* gene (2). This gene is located at a site coincident with a folate-sensitive Xq27.3 fragile site in cells of affected males (2, 3). Whereas normal subjects carry 2–50 repeats of the d(CGG) trinucleotide and phenotypically unaffected carriers have up to 200 repeats, this tract is expanded to >200–2000 d(CGG) copies in affected individuals (2, 4–8). Subsequent to d(CGG) expansion that takes place in maternal oocytes or early in development (9) the repeat sequence itself (10–13) and an adjacent CpG island (12) become hypermethylated. As a result of d(CGG) expansion and/or the ensuing hypermethylation, transcription of the *FMR1* gene is silenced (12, 14, 15). In addition, replication of the mutated *FMR1* alleles and of a chromosomal region extending hundreds of kilobases 5′ and 3′ to the amplified trinucleotide tract becomes delayed (16, 17).

Oligonucleotides having a d(CGG)$_n$ sequence readily generate *in vitro* under physiological conditions tetrahelical structures (18–20) and hairpin formations (21–24). It was conjectured that tetraplex or hairpin structures of the expanded d(CGG)$_n$ tract arrest the progression of DNA polymerase during DNA replication and promote slippage of the enzyme and expansion of the repeated sequence (18, 25–28). Once expanded, stable hairpin or tetraplex structures are likely to block the transcriptional machinery and thus prevent *FMR1* gene expression (21, 23).

It was proposed that the formation and stability of d(CGG)$_n$ tetraplex or hairpin structures may be modulated by proteins (19, 23). Following this suggestion, we demonstrated that human Werner syndrome DNA helicase (29) and two hnRNP-related murine telomeric DNA binding proteins, qTBP42 and uqTBP25 (30), unwind a bimolecular G′2 d(CGG)$_n$ tetraplex structure. In this work we searched for a protein that might act to stabilize secondary structures of d(CGG)$_n$. We fractionated proteins from cultured human lymphoblastoid cells based on their binding to d(CGG)$_8$. We report the purification of a major d(CGG)$_n$ binding protein of 88- and 75-kDa subunits. This heterodimeric d(CGG)$_n$ binding protein was identified as Ku antigen by its fully homologous partial amino acid sequence and recognition by monospecific anti-Ku antigen antibodies. The purified Ku antigen preferentially and tightly bound G′2 d(CGG)$_n$ bimolecular tetraplexes. Association with Ku antigen preferentially protected G′2 d(CGG)$_8$ tetraplex against digestion by micrococcal nuclease and impeded its unwinding by the tetraplex d(CGG)$_n$ destabilizing protein qTBP42. We speculate that Ku antigen or other proteins that tightly bind secondary structures of d(CGG)$_n$ stretches may play a role in stabilizing folded structures of this sequence in genomic DNA.

EXPERIMENTAL PROCEDURES

Materials and Enzymes—Isotopically 5′-labeled [γ-^{32}P]ATP (~3000 Ci/mmol), Bacteriophage T4 polynucleotide kinase, and molecular mass Rainbow® marker proteins were the products of Amersham Pharmacia Biotech. Synthetic DNA oligomers listed in Table I were supplied by Operon Technologies. Micrococcal nuclease, nonimmune goat IgG, bovine serum albumin, soy bean trypsin inhibitor (STI),[1] gel filtration molecular weight protein markers, proteinase K, dithiothreitol (DTT), *N*-ethylmaleimide, leupeptin, phenylmethylsulfonyl fluoride, SDS, Nonidet P-40, cyanogen bromide-activated Sepharose 4B, and protein

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[1] The abbreviations used are: STI, soy bean trypsin inhibitor; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

TABLE I
DNA oligomers used in this study

Oligomer designation	Length	Nucleotide sequence
d(CGG)[a]	21-mer	5′-d(CGGCGGCGGCGGCGGCGGCGG)-3′
$d(CGG)_8$[a]	24-mer	5′-d(CGGCGGCGGCGGCGGCGGCGGCGG)-3′
3′-tail $d(CGG)_8$[a]	32-mer	5′-d(CGGCGGCGGCGGCGGCGGCGGCGGGTGGACTG)-3′
Hook $d(CGG)_8$[a]	39-mer	5′-d(GTCGTGACGCGGCGGCGGCGGCGGCGGCGGCGTGGACTC)-3′
Hook $d(CCG)_8$[a]	39-mer	5′-d(GAGTCCACGCGGCGGCGGCGGCGGCGGCGGCGTCACGAC)-3′
TeR2[b]	12-mer	5′-d(TTAGGGTTAGGG)-3′
TeR4[b]	24-mer	5′-d(TTAGGGTTAGGGTTAGGGTTAGGG)-3′
TeT4[c]	24-mer	5′-d(TTGGGGTTGGGGTTGGGGTTGGGG)-3′
Q[d]	20-mer	5′-d(TACAGGGGAGCTGGGGTAGA)-3′
$\sim d(G)_{17}$	22-mer	5′-d[AATTC$(G)_{17}$]-3′

[a] Oligomers containing the fragile X expanded trinucleotides d(CGG) or d(CCG).
[b] Oligomer containing the vertebrate telomeric repeat d(TTAGGG).
[c] Oligomer containing the *Tetrahymena* telomeric repeat d(TTGGGG).
[d] Oligomer containing an IgG class switching nucleotide sequence.

G-agarose were provided by Sigma. DEAE-cellulose (DE-52), phosphocellulose (P-11), and DE-81 and 3MM filter paper were purchased from Whatman. Amresco supplied acryl/bisacrylamide (19:1 or 30:1.2). CalBiochem provided Aprotinin and benzamidine. *N*,*N*,*N*′,*N*′-tetramethylenediamine, bromphenol blue, and xylene cyanol FF were the products of IBI. Sep-Pak cartridges were purchased from the Waters Division of Millipore. Superdex© 200 high performance liquid chromatography (HPLC) gel filtration column was the product of Amersham Pharmacia Biotech. Santa Cruz Biotechnology provided affinity-purified goat polyclonal anti-Ku 70 and anti-Ku 86 antibodies. A nearly homogeneous tetraplex $d(CGG)_n$ destabilizing hnRNP-related protein qTBP42 (30, 33) was prepared, and its units of activity were defined as in Ref. 33.

Cells—A human lymphoblastoid TK-6 cell line was the gift of Dr. R. Monnat, University of Washington. The cells were grown at 37 °C under a humidified 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cells seeded into roller bottles at a density of 7.5×10^4 cells/ml were harvested at a density of 5.0×10^6 cells/ml by centrifugation at 4 °C at $1000 \times g$ for 5 min. The cell pellet was rinsed twice with 10 volumes of ice-cold phosphate buffered saline and was immediately frozen and stored at −80 °C until used. A human SK-N-MC neuroblastoma cell line was the gift of Dr. George M. Martin (University of Washington). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells seeded in 75-cm^2 flasks were grown at 37 °C under 5% CO_2 and were harvested by exposure to 0.25% trypsin after reaching 85% confluence. The cells were collected, rinsed, and stored as described above.

Preparation of Single-stranded, Double-stranded, and Tetraplex DNA Oligomers—DNA oligomers were purified by electrophoresis through an 8.0 M urea, 15% polyacrylamide denaturing gel (acrylamide: bisacrylamide, 19:1) in 0.5× TBE buffer (1.25 mM EDTA in 45 mM Tris borate buffer, pH 8.3). The DNA was isolated from the gel as described previously (31), except that salt and acrylamide residues were removed from the isolated DNA either by precipitation and wash of the DNA by ethanol or by Sep-Pak column chromatography. Purified DNA oligomers were 5′ end labeled with ^{32}P in a T4 polynucleotide kinase catalyzed reaction and maintained in their single-stranded conformation by being stored as 0.25 μM solution in water and by boiling immediately prior to use. Double-stranded hook$(CGG)_8$·hook$(CCG)_8$ DNA (oligomer sequences are listed in Table I) was prepared by heating at 90 °C for 2 min an equimolar mixture of the complementary 5′ end labeled oligomers (36 μM each in TE buffer), followed by slow cooling to room temperature. Bimolecular quadruplex DNA structures of ^{32}P-5′-labeled $d(CGG)_8$ were formed by incubating 30–60 μM oligomers at 4 °C for ~20 h in 10 μl of TE buffer containing 300 mM NaCl. Bimolecular tetraplex DNA structure of ^{32}P-5′-labeled TeR2 were similarly generated except that the oligomers were incubated at 37 °C for ~20 h in TE buffer containing 1 M KCl. Tetramolecular quadruplex DNA structure of oligomer Q was prepared by incubating ^{32}P-5′-labeled oligomer at 50 °C for ~20 h in TE buffer, 300 mM NaCl. Formed tetraplex DNA structures and residual single-stranded oligomers were precipitated and washed by ethanol and resuspended and stored at 4 °C in TE buffer containing 100 mM of the respective salt. For some experiments the tetraplex DNA structures were enriched by nondenaturing gel electrophoresis (29, 30). That the formed DNA secondary structures were Hoogsteen hydrogen-bonded tetrahelices was demonstrated by dimethylsulfate protection assay of guanine N7 groups as described previously (19, 32). The stoichiometry of quadruplex DNA forms was determined as described previously (19, 30, 32).

Electrophoretic Mobility Shift Assay, SDS-PAGE, and UV Cross-linking of Protein-DNA Complexes—Binding of proteins isolated from a human TK-6 cell extract to DNA oligomers was monitored by electrophoretic mobility shift assay (31). Briefly, 1.0–2.0 ng of ^{32}P-5′-labeled $d(CGG)_8$ was incubated at 4 °C for 20 min with crude or purified protein fractions in 10 μl of final volume of buffer D (0.5 mM DTT, 1.0 mM EDTA, 20% glycerol in 25 mM Tris-HCl buffer, pH 7.5). Protein-DNA complexes were resolved from unbound DNA by electrophoresis of the reaction mixture at 4 °C and at a constant current of 15 mA through a nondenaturing 6% polyacrylamide gel in 0.5× TBE running buffer. Formed protein-DNA complexes were visualized in gels that were dried on Whatman 3MM filter paper and exposed to x-ray film. To quantify the electrophoretically resolved free and protein-bound DNA, the gels were dried on DE-81 filter paper and exposed to a PhosphorImager plate (Fuji). Amounts of protein-bound and unbound DNA were calculated by phosphorimaging quantification of their corresponding radioactive bands and the predetermined specific activity of the labeled DNA. One unit of DNA binding activity was defined as the amount of protein that formed a complex with 0.01 ng of ^{32}P-5′-labeled $d(CGG)_8$. SDS-PAGE and silver or Coomassie Blue staining of resolved proteins were conducted as described previously (31).

Covalent cross-linking of protein-DNA complexes by UV light was performed as described (31, 32). Briefly, 10-μl aliquots of DNA binding reaction mixtures placed in microtiter plate wells at a distance of 6 cm from a UV light source (UVP, San Gabriel) were irradiated at 4 °C for 5 min at 254 nm (580 microwatts/cm^2 at 6 inches). An equal volume of denaturing electrophoresis-loading buffer was added to the irradiated samples, which were than boiled for 5 min and resolved by SDS-PAGE.

Purification of a $d(CGG)_n$ Binding Protein from TK-6 Human Cells—Frozen lymphoblastoid TK-6 cells (packed volume, 20 ml) or neuroblastoma SK-N-MC cells (packed volume, 10 ml) were thawed at 4 °C and suspended in an equal volume of ice-cold buffer S (0.8 M NaCl, 1.0 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml STI, 10 μg/ml aprotinin, 0.1 mM benzamidine, 0.5 mM DTT, 1.0 mM EDTA, 20% glycerol, 25 mM Tris-HCl buffer, pH 7.5). All the subsequent steps of protein purification were conducted at 4 °C. The cells were disrupted in a Dounce homogenizer equipped with a B pestle, and the cell extract was centrifuged at $20,000 \times g$ for 45 min. The supernatant was collected, and the pellet was resuspended in an equal volume of the above buffer containing 0.4 M NaCl (buffer S1), homogenized, and centrifuged as described above. The combined two supernatant fractions were chromatographed through a DE-52 column equilibrated in buffer D containing 0.4 M NaCl to remove residual DNA (31). Following overnight dialysis against ~200 volumes of buffer D, proteins were precipitated by addition of ammonium sulfate to a final concentration of 55%, and the protein precipitate was suspended in and dialyzed against buffer D. Electrophoretic mobility shift analysis detected most of the activity of a major $d(CGG)_8$ binding protein in the 55% $(NH_4)_2SO_4$ precipitate. This fraction was chromatographed on a DE-52 column as described (33) except that absorbed proteins were eluted from the column by a linear gradient of 50–400 mM NaCl in buffer D. To stabilize the eluted $d(CGG)_8$ binding activity and to prevent its adsorption to glass or plastic, fractions were collected at each consecutive step of column chromatography into STI and Nonidet P-40 at final concentrations of 0.2 mg/ml and 0.05%, respectively. Aliquots of the collected fractions were dialyzed against ~200 volumes of buffer D, and $d(CGG)_8$ binding activity was detected in fractions that were eluted from the column by 120–250 mM NaCl. Pooled active fractions were dialyzed overnight

against ~200 volumes of buffer P (0.5 mM DTT, 1.0 mM EDTA, 20% glycerol, 50 mM KPO_4 buffer, pH 7.3) and loaded onto a P-11 column equilibrated with the same buffer (33). Adsorbed proteins were eluted by a linear gradient of buffer P containing 50–500 mM KPO_4, and aliquots of each collected fraction were dialyzed against buffer D. The major portion of the $d(CGG)_8$ binding activity was detected in fractions that were eluted from P-11 by 130–380 mM KPO_4. Pooled active fractions were dialyzed overnight against ~200 volumes of buffer D and loaded onto an affinity chromatography column of $d(CGG)_{16}$ covalently linked to Sepharose 4B. To prepare the affinity matrix, CNBr-activated Sepharose 4B was suspended in water and washed over a Millipore funnel by 30 volumes each of 1.0 mM HCl and H_2O followed by 10 volumes of 10 mM KPO_4 buffer, pH 8.0. The matrix, suspended in the last wash buffer was mixed with a solution that contained in the same buffer, 250 μg/ml of unlabeled $d(CGG)_{16}$ and 0.4 ng/ml of ^{32}P-5′-labeled $d(CGG)_{16}$, which served to monitor the efficacy of DNA binding to the matrix. The mixture was slowly rotated at room temperature overnight, and the matrix was subsequently washed over a Millipore funnel by 20 volumes of H_2O and 10 volumes of 1.0 M ethanolamine-HCl buffer, pH 8.0. After suspending the matrix in the last wash buffer it was rotated at room temperature for additional 4 h and washed by 10 volumes each of 10 mM KPO_4 buffer, pH 8.0; 1.0 M KPO_4 buffer, pH 8.0; 1.0 M KCl; and 300 mM NaCl, 1.0 mM EDTA, 0.02% NaN_3 in 10 mM Tris-HCl buffer, pH 7.5. The washed matrix was packed into a column and stored at 4 °C under the final wash buffer until used. About 20% of the ^{32}P-5′-labeled $d(CGG)_{16}$ became covalently bound to the activated Sepharose to 20 μg of $d(CGG)_{16}$/ml of packed Sepharose 4B matrix. After slowly loading the protein solution onto a column pre-equilibrated with buffer D, it was washed with one column volume of buffer D, and adsorbed proteins were eluted stepwise with a single column volume each of solutions that contained 0.05, 0.1, 0.2, 0.3, 0.5, and 1.0 M NaCl in buffer D. Collected fractions were dialyzed against buffer D, and the $d(CGG)_8$ binding activity was detected by electrophoretic mobility shift analysis in fractions eluted by 200–500 mM NaCl. The active fractions were stored at −80 °C in the presence of 0.2 mg/ml STI and 0.05% Nonidet P-40. Under these conditions the DNA binding activity remained undiminished for at least 6 months.

Protein Analysis—Amounts of protein were determined using the Bio-Rad protein assay kit. Partial amino acid sequence of a $d(CGG)_{16}$-Sepharose purified fraction of the $d(CGG)_n$ binding protein was determined following its resolution by SDS-PAGE and Coomassie Blue staining. Bands of 75 and 88 kDa that corresponded to the $d(CGG)_8$ binding activity were excised, and protein was eluted from the gel slices. The isolated proteins were digested by Lys C protease, and resulting peptides were separated by reverse-phase HPLC. Amino acid sequences of selected peptides were determined by standard automated procedures, utilizing Peptide Sequencer 476A (PerkinElmer Life Sciences).

Immunochemical Identification of the $d(CGG)_n$ Binding Protein—Two immunoassays were used to verify the identification of the $d(CGG)_n$ binding protein as Ku antigen: (*a*) Gel supershift. P-11 purified fraction of the binding protein was incubated with ^{32}P-5′-labeled $d(CGG)_7$ under standard DNA binding conditions followed by incubation at 4 °C for 30 min with 0.2–0.6 μg of nonimmune goat IgG or goat antibodies directed against a peptide corresponding to amino acids 713–730 of the 86-kDa subunit of human Ku antigen or to amino acids 577–595 of the 70-kDa subunit of this protein. Shifted and supershifted ^{32}P-5′-$d(CGG)_7$-protein complexes were resolved by electrophoresis at 4 °C through a nondenaturing 6% polyacrylamide gel in 0.5× TBE buffer. (*b*) Immune precipitation. Aliquots of the P-11 fraction of the $d(CGG)_n$ binding protein were incubated at 4 °C for 45 min with 0.6 μg of either nonimmune goat IgG or goat anti-Ku 86 antibodies. Swollen protein G-agarose beads were added at 1.5 mg/reaction mixture and adsorption of immune complexes onto protein G was conducted at 4 °C by rotation for 4 h. Protein G-adsorbed immune complexes were removed by centrifugation at 6,000 × *g* for 5 min, and aliquots of the supernatant fractions were analyzed by mobility shift electrophoresis for their capacity to bind ^{32}P-5′-labeled $d(CGG)_7$.

Assay of Tetraplex $d(CGG)_n$ Unwinding by qTBP42—G′2 $d(CGG)_8$ destabilizing activity of qTBP42 with or without added Ku antigen or STI was assayed as described (30).

RESULTS

Purification of a $d(CGG)_n$ Binding Protein—In a search for proteins that affect the conformation of the fragile X expanded sequence $(CGG)_n$, we fractionated proteins from human TK-6 lymphoblastoid cells based on their binding to oligomeric $(CGG)_n$. A major TK-6 cell $d(CGG)_8$ binding activity was precipitated by 55% ammonium sulfate and was further purified by chromatography on columns of DE-52, P-11, and $d(CGG)_{16}$-Sepharose (see "Experimental Procedures"). Fig. 1*A* shows a typical elution pattern of ^{32}P-5′-$d(CGG)_8$ binding proteins from a $d(CGG)_{16}$-Sepharose column. These results demonstrated that $d(CGG)_8$ formed a major electrophoretically retarded complex with a protein that was eluted from the column by 200–500 mM NaCl, with maximum binding activity detected in the second 300 mM NaCl fraction. A lower sized complex was also discerned in fractions eluted by 500 mM NaCl (Fig. 1*A*). However, this lower complex was eliminated when an excess of unlabeled ~$d(G)_{17}$~ competing oligomer was added to the binding mixture, whereas the intensity of the major complex remained undiminished (results not shown). SDS-PAGE of the $d(CGG)_{16}$-Sepharose resolved proteins revealed in the 200–500 mM NaCl fractions two major subunits of 72 and 87 kDa. The

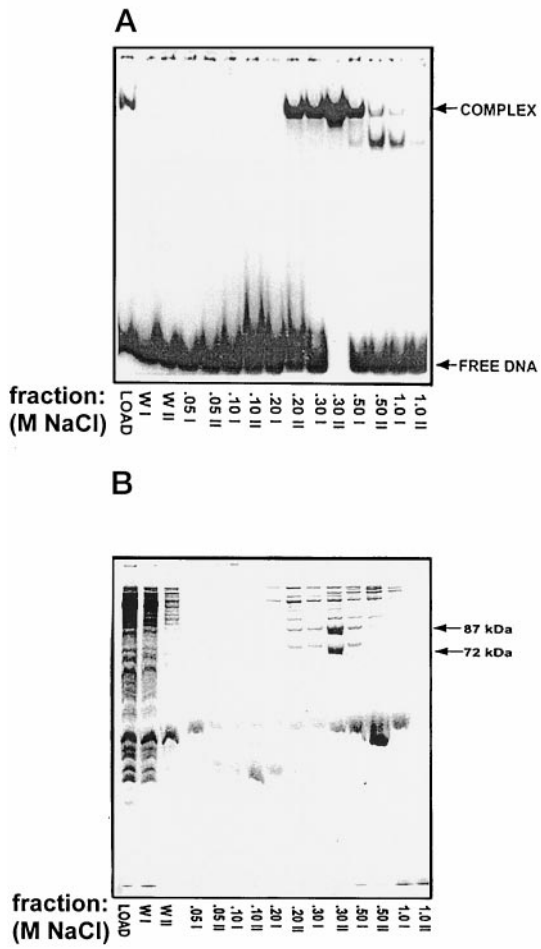

FIG. 1. **Resolution of $d(CGG)_8$ binding activities by $d(CGG)_{16}$-Sepharose affinity chromatography and SDS-PAGE of protein fractions.** Phosphocellulose-purified fraction of the $d(CGG)_8$ binding protein was loaded onto a $d(CGG)_{16}$-Sepharose affinity column, and proteins were eluted by a stepwise gradient of 0.05–1.0 M NaCl in buffer D. Each eluting salt solution (a single column volume) was collected in two fractions that were stabilized by 0.2 mg/ml STI and 0.05% Nonidet P-40. *A*, mobility shift electrophoresis of the affinity-purified protein. Fractions were assayed for binding of ^{32}P-5′-labeled $d(CGG)_8$ as described under "Experimental Procedures." Concentrations of eluting salt are indicated for each fraction in the abscissa. *B*, Coomassie Blue staining of SDS-PAGE resolved affinity purified protein fractions. Electrophoresis and protein staining were conducted as described under "Experimental Procedures." To better separate high molecular mass proteins, lower mass proteins including the added STI stabilizer were run out of the gel. *Arrows* indicate the positions of the 72- and 87-kDa protein bands whose distribution in the eluted fractions was in concordance to the binding activity (*A*).

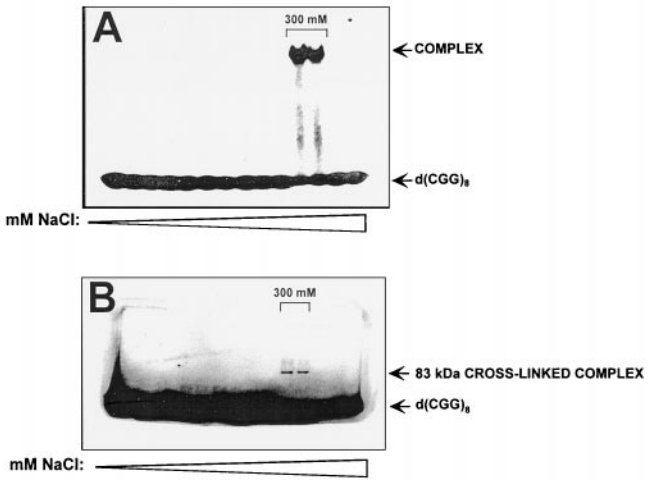

FIG. 2. **Covalent cross-linking of the binding protein to d(CGG)$_8$.** Resolution of the d(CGG)$_8$ binding activity by d(CGG)$_{16}$-Sepharose affinity column chromatography was conducted as described in the legend to Fig. 1. Aliquots of each of the d(CGG)$_{16}$-Sepharose resolved fractions were incubated with ^{32}P-5′-labeled d(CGG)$_8$ under binding conditions as described under "Experimental Procedures" except that a 100-fold molar excess of unlabeled ~d(G)$_{17}$~ competing oligomer was present in the reaction mixtures. The protein-d(CGG)$_8$ complexes were either directly resolved by nondenaturing mobility shift electrophoresis or were covalently cross-linked by UV light (see "Experimental Procedures"). Following electrophoresis through an SDS-12% polyacrylamide gel, the dried gels were exposed to autoradiographic film. Molecular mass of the cross-linked complex was estimated from its migration relative to that of molecular size marker proteins. *A*, nondenaturing mobility shift electrophoresis. *B*, SDS-PAGE of a UV-cross-linked complex.

relative amounts of these polypeptides that constituted more than 50% to the total protein content of the second 300 mM NaCl eluate were in concordance with their binding activity (Fig. 1*B*). Repeated SDS-PAGE analyses yielded average sizes of 75 and 88 kDa for these two polypeptide chains, respectively (see below).

To further establish the identity of the d(CGG)$_n$ binding protein, the affinity purified protein was cross-linked to ^{32}P-5′-d(CGG)$_8$ by UV light and the covalently bonded complex was resolved by SDS-PAGE. Fractions collected from the d(CGG)$_{16}$-Sepharose affinity matrix were assayed for ^{32}P-5′-d(CGG)$_8$ binding activity, and protein-DNA complexes were irradiated by UV light. As seen in Fig. 2*A*, fractions that were eluted from the affinity matrix by 300 mM NaCl contained d(CGG)$_8$ binding activity and also displayed a corresponding covalently linked complex of 83 kDa (Fig. 2*B*). Notably, whereas SDS-PAGE revealed two protein bands that conformed with the d(CGG)$_8$ binding activity (Fig. 1*B*), only a single band of UV cross-linked d(CGG)$_8$-protein complex was discerned (Fig. 2*B*). The presence of a single complex band was likely due to the confinement of the DNA binding capacity to the 75-kDa protein subunit of the dimeric protein which together with the associated d(CGG)$_8$ oligomer, yielded the observed 83-kDa complex (see "Discussion").

By eliminating the d(CGG)$_{16}$-Sepharose chromatography step, a major d(CGG)$_n$ binding protein was purified to a lower degree from cultured SK-N-MC neuroblastoma cells. Properties of the binding activities isolated from TK-6 or SK-N-MC cells were indistinguishable, and results shown hereto forth were obtained with the TK-6 cell protein.

Properties of the d(CGG)$_n$ Binding Activity—Initial characterization of the d(CGG)$_{16}$-Sepharose affinity purified d(CGG)$_n$ binding activity from TK-6 cells indicated that it is a protein. Digestion of the binding activity with 133 μg/ml proteinase K as described (32) or its incubation at 4 °C for 5 min with 0.13% SDS reduced by 80% or 100%, respectively, the formation of an electrophoretically retarded complex with ^{32}P-5′-labeled d(CGG)$_8$. Similarly, incubation of the affinity purified activity at 50 or 54 °C for 10 min resulted in loss of 91 or 97%, respectively, of the d(CGG)$_8$ binding activity. By contrast, digestion of the protein with 4 units/μl micrococcal nuclease (33), stimulated binding of ^{32}P-5′-labeled d(CGG)$_8$, probably as a result of hydrolysis of residual protein-bound cellular DNA. Incubation of the protein at 4 °C for 15 min with 8.5 mM *N*-ethylmaleimide and termination of the reaction by the addition of 22 mM DTT, resulted in a 80–100% reduction in the binding ^{32}P-5′-labeled d(CGG)$_8$. Hence, reduced sulfydryl groups thus appeared to be essential for the DNA binding activity.

TABLE II

Amino acid sequences of peptides derived from the 75- and 88-kDa subunits of the d(CGG)$_n$ binding protein and sequences of the 70- and 85-kDa subunits of human Ku autoantigen

The 75- and 88-kDa subunits of the d(CGG)$_n$ binding protein, designated p75 and p88, respectively, were resolved by SDS-PAGE, stained with Coomassie Blue, and isolated as described under "Experimental Procedures." Amino acid sequences of HPLC-resolved Lys C peptides were determined and a search through GenBank™ identified the 70-kDa (48) and 85-kDa (36) subunits of Ku autoantigen as the only homologs of the corresponding d(CGG)$_n$ binding protein subunits. Positions of the identified amino acid residues within each Ku autoantigen subunit are indicated.

Protein	Peptide	Sequence
d(CGG)$_n$ binding protein p75	I	TRTFNTSTGGLLLPSDT
Ku autoantigen p70		^{300}TRTFNTSTGGLLLPSDT^{316}
d(CGG)$_n$ binding protein p75	II	RILELDQFKGQQGQ
Ku autoantigen p70		^{115}RILELDQFKGQQGQ^{128}
d(CGG)$_n$ binding protein p75	III	SQIYGSRQIILEKEET
Ku autoantigen p70		^{319}SQIYGSRQIILEKEET^{334}
d(CGG)$_n$ binding protein p88	I	KDQVTAQEIFQDN
Ku autoantigen p85		^{545}KDQVTAQEIFQDN^{557}
d(CGG)$_n$ binding protein p88	II	TDTLEDLFPTTK
Ku autoantigen p85		^{470}TDTLEDLFPTTK^{481}
d(CGG)$_n$ binding protein p88	III	TLFPLIEAKK
Ku autoantigen p85		^{535}TLFPLIEAKK^{544}

Two independent determinations of the native molecular mass of the protein by Superdex© 200 gel filtration yielded values of 170 and 162 kDa. SDS-PAGE resolution of the affinity-purified protein and its staining with silver or Coomassie Blue indicated molecular sizes of 75 ± 3.0 and 88 ± 2.0 kDa for the two prominent protein bands seen in Fig. 1*B* (average of three independent determinations). The measured native mass of the binding protein and the denatured sizes of its two related polypeptides suggested that it is a heterodimer of ~75- and ~88-kDa subunits.

The d(CGG)$_n$ Binding Protein Is Ku Autoantigen—To identify the d(CGG)$_n$ binding protein, we determined partial amino acid sequences of three Lys C peptides derived from each of its two isolated subunits. A search through GenBank™ revealed that the two subunits of human Ku autoantigen were the only proteins to contain sequences homologous to the similarly sized subunits of the d(CGG)$_n$ binding protein. Ku autoantigen, as well as closely homologous or identical nuclear DNA binding proteins (35, 38–43), are heterodimers of a 68–75-kDa polypeptide and a larger subunit of 83–87 kDa (Refs. 36–38, 41, and 43; for a recent review see Ref. 44). As shown in Table II, sequences of peptides derived from the 75- and 88-kDa subunits of the d(CGG)$_n$ binding protein with cumulative lengths of 47 and 35 amino acid residues, respectively, were 100% homologous to sequences of the two corresponding Ku autoantigen subunits.

The designation of the d(CGG)$_n$ binding protein as a Ku antigen was further verified by demonstrating its specific interaction with goat antibodies directed against the subunits of

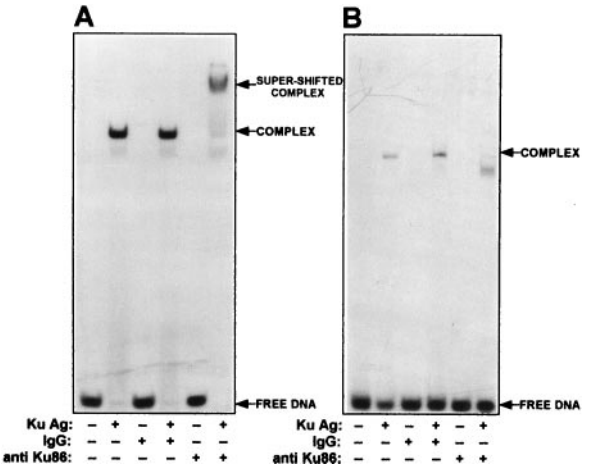

FIG. 3. **The d(CGG)$_n$ binding protein is recognized by anti-Ku antigen antibodies.** *A*, electrophoretic mobility supershift by anti-Ku 86 antibody. P-11 purified d(CGG)$_n$ binding protein was incubated with ^{32}P-5′-labeled d(CGG)$_7$ under standard DNA binding conditions, 0.2 μg of nonimmune goat IgG or goat antibodies directed against the 86-kDa subunit of human Ku antigen were added, and the mixtures were further incubated at 4 °C for 30 min. Control mixtures contained the respective immunoglobulins without d(CGG)$_n$ binding protein. Protein-DNA complexes were resolved by electrophoresis at 4 °C through a nondenaturing gel of 6% polyacrylamide in 0.5× TBE buffer. *Arrows* indicate the positions of free ^{32}P-5′-d(CGG)$_7$, a complex of the binding protein with ^{32}P-5′-d(CGG)$_7$ and of a complex supershifted by anti-Ku 86 antibody. *B*, immunoprecipitation of the binding protein by anti-Ku 86 antibody. Aliquots of P-11 purified d(CGG)$_n$ binding protein were incubated at 4 °C for 45 min with 0.6 μg of either nonimmune goat IgG or goat anti-Ku 86 antibodies. Protein G-agarose beads were added at 1.5 mg/reaction mixture, and the mixtures were rotated at 4 °C for 4 h. Protein-G adsorbed immune complexes were removed by 5 min of centrifugation in the cold at 6,000 × *g*, and aliquots of the supernatant fractions were assayed for ^{32}P-5′-d(CGG)$_n$ binding by mobility shift gel electrophoresis. *Arrows* mark positions of unbound ^{32}P-5′-d(CGG)$_n$ and of a ^{32}P-5′-d(CGG)$_7$-protein complex.

human Ku antigen. Aliquots of the P-11 purified fraction of the d(CGG)$_n$ binding protein were preincubated with ^{32}P-5′-d(CGG)$_7$ under standard DNA binding conditions and then incubated at 4 °C for 30 min with no added protein or with either nonimmune goat IgG or a goat antibody directed against the 86-kDa subunit of Ku antigen. Formed protein-DNA complexes or complexes of antibody-protein-DNA were resolved by nondenaturing gel electrophoresis. As seen in Fig. 3*A*, neither nonimmune IgG nor anti-Ku 86 antibodies by themselves formed a detectable complex with ^{32}P-5′-d(CGG)$_7$. Additionally, nonimmune IgG did not affect the formation or electrophoretic migration of the complex of the binding protein with ^{32}P-5′-d(CGG)$_7$. However, anti-Ku 86 antibody retarded the electrophoretic mobility of the DNA-binding protein complex (Fig. 3*A*). A similar supershifting of the protein-d(CGG)$_7$ complex was induced by an antibody directed against the 70-kDa subunit of human Ku antigen (result not shown). To test whether anti-Ku antibodies immunoprecipitated the d(CGG)$_n$ binding protein, it was incubated with either nonimmune IgG or with anti-Ku 86 antibody. Immune complexes were adsorbed onto protein G-agarose beads, and nonprecipitated DNA binding activity was measured by electrophoretic mobility shift assay. As seen in Fig. 3*B*, whereas binding activity was detected in samples that were not exposed to immunoglobulin or were incubated with nonimmune IgG, it was eliminated following incubation with anti-Ku 86 antibody. A more rapidly migrating faint band that was detected after immune precipitation (Fig. 3*B*) represented either a complex of the DNA with unprecipitated 75-kDa Ku subunit or with a weaker DNA binding activity that was ordinarily competed out by the more strongly binding Ku antigen. By virtue of their similar native

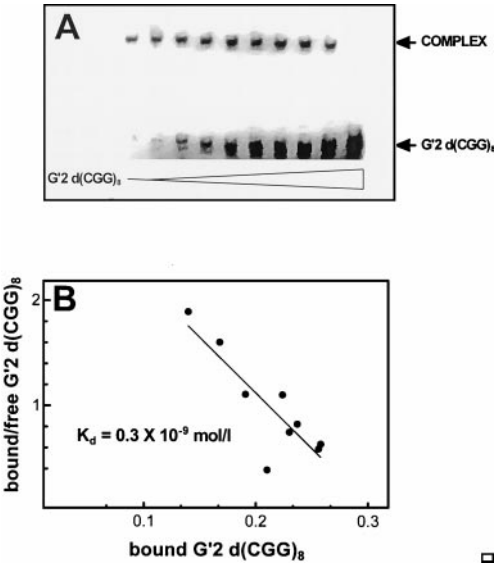

FIG. 4. **Determination of the dissociation constant of Ku antigen-G′2 d(CGG)$_8$ complex.** Affinity purified Ku antigen (32 activity units) was incubated at 4 °C for 20 min with increasing amounts of 5′-^{32}P-labeled G′2 d(CGG)$_8$ under standard DNA binding conditions. Ku-G′2 d(CGG)$_8$ complexes were resolved from unbound G′2 d(CGG)$_8$ by mobility shift electrophoresis through a nondenaturing 8% polyacrylamide gel in 0.5× TBE buffer (see "Experimental Procedures"). *A*, mobility shift electrophoresis pattern of mixtures of Ku antigen with increasing amounts of G′2 d(CGG)$_8$. *B*, Scatchard plot of results shown in *A* and quantified by phosphorimaging. The K_d value was calculated as the negative reciprocal of the slope of the Scatchard plot. □

and subunit molecular masses, fully homologous partial amino acid sequences and specific interaction with anti-Ku antigen antibodies, the d(CGG)$_n$ binding protein was identified as Ku antigen (see "Discussion").

DNA Sequence and Structure Binding Preferences of Ku Antigen—Mammalian Ku antigen binds *in vitro* ends of double-stranded or hairpin DNA in a largely DNA structure- and sequence-independent fashion (reviewed in Ref. 44). To quantitatively assess the nucleotide sequence and DNA structure binding preferences of the d(CGG)$_n$ binding Ku, we determined dissociation constants (K_d) of its complexes with selected DNA structures and sequences.

A typical Scatchard plot of the binding of increasing amounts of 5′-^{32}P-labeled G′2 d(CGG)$_8$ to affinity purified TK-6 cell Ku and the deduced K_d value are shown in Fig. 4. Average K_d values were similarly determined for complexes of Ku with various single-stranded, double-stranded, and tetraplex DNA sequences and are listed in Table III. Whereas d(CGG)$_n$ exists in solution in a hairpin form (21–24), its analog (CII)$_8$ maintained a single-stranded conformation under a variety of conditions as a result of its inability to form a hydrogen bond at the inosine C2 group.[2] As seen in Table III, Ku formed a complex with (CII)$_8$ that had a similar K_d value to dissociation constants of complexes with other guanine or cytosine-rich single strands. However, the complex of Ku with hairpin d(CGG)$_8$ had a 2–3-fold higher K_d value. In line with its reported preferential binding to ends of double-stranded DNA (44), Ku bound d(CGG)$_8$·d(CCG)$_8$ at a K_d that was 1.8–2.9-fold lower than the K_d value of Ku complexes with single-stranded oligomers (Table III). Tetramolecular tetraplex structure of the IgG switch region sequence d(TACAG$_4$AGCTG$_4$TAGA) and bimolecular and unimolecular tetraplex structures of the vertebrate telomeric sequence d(TTAGGG)$_n$ were bound at affinities that ranged between those of single-stranded and hairpin DNA.

[2] M. Fry, unpublished results.

TABLE III
Dissociation constants of complexes of Ku antigen with preferred DNA sequences and structures

Dissociation constants (K_d) for the listed protein-DNA complexes were inferred from Scatchard plots of the binding of increasing amounts of DNA by affinity-purified Ku antigen as shown in Fig. 4.

DNA ligand	K_d (n)[a]
	10^{-9} *mol/liter*
Single-stranded DNA	
$d(CII)_8$[b]	3.4 ± 1.0 (3)
$d(GCC)_8$	3.2 ± 1.8 (3)
$d(TTAGGG)_4$	5.3 ± 2.3 (7)
$d(TTGGGG)_4$	4.4 ± 1.1 (3)
Hairpin DNA	
$d(CGG)_8$[c]	9.6 ± 1.8 (4)
Double-stranded DNA	
hook $d(CGG)_8$ · hook $d(CCG)_8$	1.8 ± 0.7 (5)
Tetraplex DNA[d]	
G′2 $d(CGG)_8$	0.35 ± 0.15 (4)
G′2 $d(TTAGGG)_2$	10.2 ± 3.5 (2)
G′4 $d(TTGGGG)_4$	6.15 ± 1.7 (3)
G4 d(TACAGGGGAGCTGGGGTAGA)	3.0 ± 1.8 (3)

[a] *n*, number of independent binding curves generated to determine a K_d value for a complex with a specific DNA ligand.
[b] $d(CII)_8$ maintained a single-stranded conformation as verified by its migration in a nondenaturing polyacrylamide gel (see "Results").
[c] $d(CGG)_8$ maintained hairpin structure under the binding conditions (23).
[d] Unimolecular, bimolecular, and four-molecular tetraplex structures of the listed guanine-rich oligomers were prepared as detailed under "Experimental Procedures."

Most notable was the significantly higher affinity of the Ku protein for a G′2 bimolecular tetraplex structure of $d(CGG)_8$. The measured average K_d value of 0.35×10^{-9} mol/l for the Ku-G′2 $d(CGG)_8$ complex was 27.4-fold lower than the K_d of a complex with hairpin $d(CGG)_8$ (Table III). Further, Ku-G′2 $d(CGG)_8$ complexes had a K_d that was 29- and 8.6-fold lower than the K_d values of complexes with G′2 $d(TTAGGG)_2$ and G4 $d(TACAG_4AGCTG_4TAGA)$. Also, the dissociation constant of the Ku-G′2 $d(CGG)_8$ complex was 9.1–15.1 lower than the K_d values of complexes with the four examined single-stranded sequences and >5-fold lower than the K_d of the complex with double-stranded $d(CGG)_8$·$d(CCG)_8$ (Table III). Thus, the TK-6 cell Ku displayed preferentially tight binding to tetraplex G′2 $d(CGG)_8$.

Ku Antigen Protects G′2 d(CGG)$_8$ Tetraplex against Nuclease Digestion—We next examined whether the preferential tight binding of tetraplex G′2 $d(CGG)_8$ by TK-6 cell Ku affected the resistance of this tetrahelix to nuclease digestion. End-labeled G′2 $d(CGG)_8$ was incubated under binding condition with either affinity purified Ku antigen containing 200 μg/ml STI stabilizing protein or with a similar amount of STI alone. A set of control DNA samples was similarly incubated with no protein added. The mixtures were than digested by micrococcal nuclease for different periods of time. Fig. 5 shows results of electrophoretic separation of intact G′2 $d(CGG)_8$ from its degradation products. The presented data indicated that 1 min of digestion with micrococcal nuclease with or without added STI led to degradation of about 35% of the tetraplex DNA structure. By contrast, only 15% of this DNA was digested in mixtures that contained Ku antigen. Likewise, throughout the time course of the experiment, Ku-bound G′2 $d(CGG)_8$ remained more resistant to degradation relative to DNA incubated without or with STI (Fig. 5). Hence, Ku specifically increased the nuclease resistance of the tightly bound G′2 $d(CGG)_8$.

Ku Antigen Impedes Unwinding of G′2 d(CGG)$_8$ Tetraplex by the Destabilizing Protein qTBP42—Bimolecular tetraplex structures of $d(CGG)_n$ oligomers were shown to be unwound by human Werner syndrome DNA helicase (29) and by the murine hnRNP-related proteins qTBP42 and uqTBP25 (30). We inquired whether formation of a complex with Ku affected the destabilization of G′2 $d(CGG)_8$ by qTBP42. Following incubation of G′2 ^{32}P-5-$d(CGG)_8$ under binding conditions with either STI-containing Ku or with a similar amount of STI alone, the binding reaction mixtures were incubated with qTBP42 under tetraplex unwinding conditions. Results presented in Fig. 6 indicated that up to ~20% of the G′2 $d(CGG)_8$ was destabilized in the presence of STI under the tested conditions. However, no significant unwinding was detected in mixtures that contained Ku. Thus, in line with its preferential tight binding to G′2 $d(CGG)_8$ (Table III) and protection against nuclease attack (Fig. 5), Ku also hindered qTBP42-mediated destabilization of this tetraplex DNA.

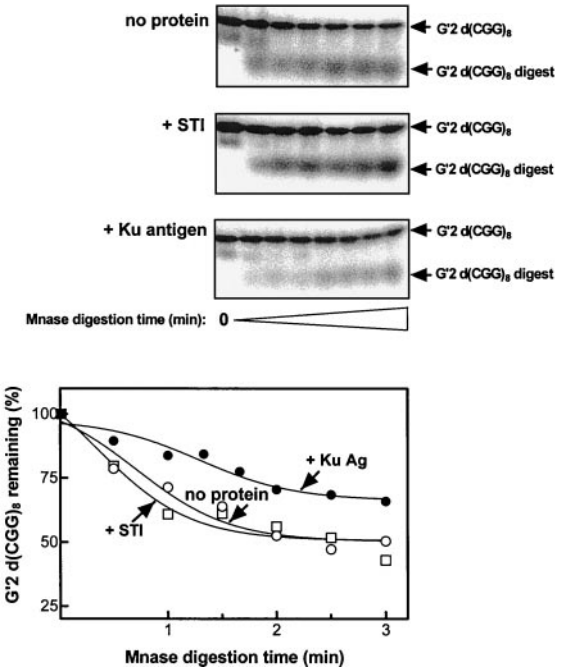

FIG. 5. **Ku antigen protects G′2 d(CGG)$_8$ against nuclease digestion.** DNA binding mixtures at a volume of 9 μl each, contained 2.0 ng of G′2 ^{32}P-5′ $d(CGG)_8$ and affinity purified Ku antigen (300 activity units containing 0.6 μg of STI) or 0.6 μg of STI alone in buffer D. A third set of control mixtures contained each 2.0 ng of ^{32}P-5′ G′2 $d(CGG)_8$ in buffer D with no protein added. Following incubation at 4 °C for 20 min, 1 μl of a solution containing micrococcal nuclease and $CaCl_2$ at final concentrations of 0.2 μg/μl and 1 mM, respectively, was added, and the mixtures were transferred to 20 °C. Digestion by the nuclease was terminated at the indicated time points by adding to each mixture 3 μl of a solution of 40% glycerol, 50 mM EDTA, 2 mM SDS, 3% bromphenol blue, and 3% xylene cyanol. Intact G′2 $d(CGG)_8$ was resolved from its degradation products by electrophoresis through a nondenaturing 6% polyacrylamide gel in 0.5× TBE buffer. *Upper panels,* autoradiograms of the progressive nucleolytic digestion of ^{32}P-5′ G′2 $d(CGG)_8$ without added protein or in the presence of STI containing Ku antigen or STI alone. *Lower panel*, kinetics of ^{32}P-5′ G′2 $d(CGG)_8$ digestion inferred from phosphorimaging quantification of results shown in the *upper panels*.

DISCUSSION

Ku protein, originally identified as a nuclear autoantigen (35), is mainly recognized for its essential roles in the repair of DNA double-stranded breaks and in site-specific recombination of the V(D)J gene segments (45). Identical or very closely related proteins were characterized as sequence-independent or sequence-selective DNA binding proteins (35, 38, 40, 43, 46, 47). Additionally, human DNA helicase II was also equated with Ku antigen (37, 41). Evidence suggested that the ~70-kDa DNA binding subunit of Ku antigen is encoded by multiple, closely homologous but not identical genes and that minimal variations in the amino acid sequence of this subunit in mem-

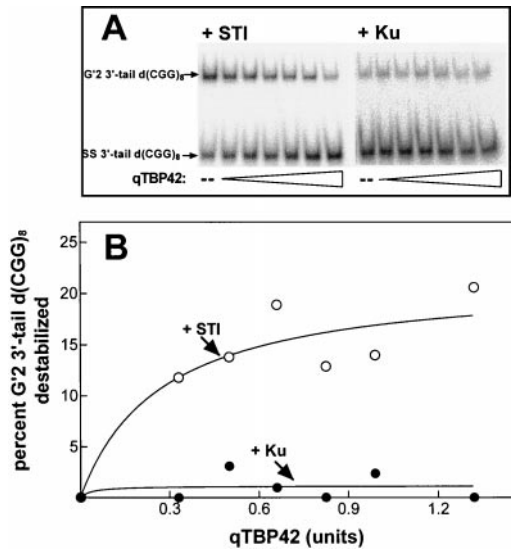

FIG. 6. **Ku antigen impedes destabilization of G′2 d(CGG)$_8$ by qTBP24.** DNA binding mixtures at a volume of 6 μl each, contained 2.0 ng of G′2 ^{32}P-5′-d(CGG)$_8$ and affinity purified Ku antigen (300 activity units containing 0.6 μg ofSTI) or 0.6 μg of STI alone in buffer D. Following incubation at 4 °C for 20 min, 0.3–1.3 units of qTBP42 in buffer D were added to the reaction mixtures to a final volume of 10 μl, and incubation continued at 37 °C for 30 min. Destabilization of the G′2 ^{32}P-5′ d(CGG)$_8$ tetraplex by qTBP42 was terminated by the addition to each mixture of 3 μl of 40% glycerol, 50 mM EDTA, 2 mM SDS, 3% bromphenol blue, and 3% xylene cyanol and displacement of single strands from the G′2 d(CGG)$_8$ tetraplex was monitored by electrophoresis through a nondenaturing 12% polyacrylamide gel in 0.5× TBE buffer. *A*, autoradiograms of the destabilization of G′2 d(CGG)$_8$ by increasing amounts of qTBP42 in the presence of STI-containing Ku antigen or STI alone. *B*, phosphorimaging quantification of the extent of G′2 d(CGG)$_8$ destabilization by increasing amounts of qTBP42 in the presence of STI-containing Ku antigen or STI alone.

bers of the Ku autoantigen family, dictate different DNA binding specificity of the proteins (48). The classification of the TK-6 cell d(CGG)$_n$ binding protein as Ku antigen was indicated most strongly by the synonymous amino acid sequences of Lys C peptides from each of the two subunits of the d(CGG)$_n$ binding protein and sequences of corresponding Ku autoantigen subunits (Table II). The identity of the d(CGG)$_n$ binding protein was confirmed by its specific binding and immunoprecipitation by antibodies directed against the 70- and 86-kDa subunits of human Ku antigen (see Fig. 3 and "Results"). Three additional lines of evidence supported the classification of the d(CGG)$_n$ binding protein as Ku antigen: (*a*) Similarly to the heterodimeric Ku antigen that constitutes of 68–75- and 83–87-kDa subunits (37, 38, 41, 43) the native ~170-kDa d(CGG)$_n$ binding protein displayed subunits of 75 and 88 kDa (Fig. 1 and "Results"). (*b*) UV cross-linking analysis attributed the DNA binding activity of Ku antigen-related proteins to their 70–75-kDa subunit (38, 44). The 83-kDa molecular mass of the single band of UV cross-linked d(CGG)$_8$-protein complex (Fig. 2*B*) suggested that it was composed of the 75-kDa polypeptide of the binding protein and the ~ 7.5-kDa d(CGG)$_8$. (*c*) *N*-ethylmaleimide, which obliterated the d(CGG)$_n$ binding activity of the TK-6 cell protein (see "Results"), was also reported to abolish DNA binding by of Ku autoantigen (49). These cumulative data warranted the identification of the TK-6 d(CGG)$_n$ binding protein as Ku antigen.

Preferentially Tight Binding of G′2 d(CGG)$_n$ by Ku Antigen—The TK-6 cell Ku protein was purified based on its binding to d(CGG)$_n$. However, results summarized in Table III indicated that it bound DNA in a largely sequence- and structure-independent fashion. The dissociation constants of complexes of Ku with inosine-, guanine-, or cytosine-rich single-stranded oligomers were very similar, ranging between 3.2 ± 1.8 and 5.3 ± 2.3 × 10^{-9} mol/liter (Table III). These values were in good accord with a dissociation constant of 3.5 ± 1.3 nM that was determined for a complex of Ku with single-stranded fragment of the long terminal repeat of mouse mammary tumor virus (50). Double-stranded hook d(CGG)$_8$·hook d(CCG)$_8$ was bound to the TK-6 cell Ku protein more tightly, as reflected by a measured K_d of 1.8 ± 0.7 × 10^{-9} mol/liter. This dissociation constant was in the same range as K_d values of 0.84 ± 0.24 (55) or 2.4 × 10^{-9} mol/liter (51) that were reported for complexes of Ku with other short DNA double strands. Dissociation constants of Ku complexes with tetrahelical structures of vertebrate and *Tetrahymena* telomeric DNA and of an IgG switch region sequence ranged between 3.0 ± 1.8 and 10.2 ± 3.5 × 10^{-9} mol/liter (Table III). These values are in some conflict with recently documented results, obtained by binding competition assay, of tighter association of Ku with tetraplex and single-stranded forms of the *Oxytricha* telomeric repeat than with double strands of this sequence (52). This discrepancy might be due to the different modes of measurement employed or to the specific sequence or structure of the *Oxytricha* telomeric tetraplex structure.

Most notable was the preferentially tight binding of Ku to a bimolecular tetraplex structure of d(CGG)$_8$. The measured K_d of 0.35 ± 0.15 × 10^{-9} mol/liter for a Ku-G′2 d(CGG)$_8$ complex was 27- and 9.7-fold lower than dissociation constants for complexes with hairpin d(CGG)$_8$ and single-stranded d(CII)$_8$, respectively (Table III). Similarly, Ku bound G′2 d(CGG)$_8$ significantly more tightly than any of the DNA sequences or structures that were tested, suggesting a preferred affinity of this protein for bimolecular tetraplex d(CGG)$_n$. That this selectively tight binding reflected preferred recognition by Ku of both the sequence and structure of G′2 d(CGG)$_8$ was demonstrated by the tighter binding of G′2 d(CGG)$_8$ than hairpin d(CGG)$_8$ or tetraplex structures of other guanine-rich sequences.

Protection of G′2 d(CGG)$_n$ by Ku Antigen—A number of proteins have been shown to associate *in vitro* with different structures of d(CGG) repeats. These include HMG box proteins that bind branched structures of (GCC)$_{15}$·d(CGG)$_{10}$ (54) and a 20-kDa human nuclear protein that associates with double-stranded but not single-stranded d(CGG)$_n$ oligomers (55, 56) and affects the activity of the *FMR1* gene promoter (57). An additional protein from mouse brain binds several single-stranded trinucleotide repeats including d(CGG)$_n$ (58). Although these proteins were not studied for their potential effect on the structure of the bound DNA, human Werner syndrome DNA helicase (29) and the murine hnRNP-related proteins qTBP42 and uqTBP25 (30) were found to efficiently unwind bimolecular tetraplex forms of d(CGG)$_n$. Results presented in this work indicated that by tightly associating with bimolecular tetraplex d(CGG)$_n$, human Ku antigen was capable of stabilizing this tetrahelical structure of the fragile X expanded repeat sequence.

As our results indicated, the preferential tight binding by Ku of G′2 d(CGG)$_n$ affected the stability of the protein-associated bimolecular tetraplex. Data shown in Fig. 5 showed that binding to Ku increased the relative resistance of G′2 d(CGG)$_8$ to digestion by micrococcal nuclease. Further, association with Ku rendered G′2 3′-tail d(CGG)$_8$ resistant to unwinding by qTBP42 (Fig. 6). Qualitatively similar results were obtained when Ku protected G′2 3′-tail d(CGG)$_8$ against unwinding by human Werner syndrome helicase (results not shown). However, in line with a recent report (53) we found that Ku stimulated the helicase-associated 3′ → 5′ exonuclease, leading to

degradation of part of the tetraplex 3′-tail $d(CGG)_8$ substrate in the course of the unwinding reaction (data not presented).

Significance of $d(CGG)_n$ Binding and Protection by Proteins—Expansion of the d(CGG) trinucleotide repeat in the *FMR1* gene (2, 4–8) and ensuing hypermethylation of the amplified sequence and an adjacent CpG island (10–13) silence *FMR1* transcription (12, 14, 15) and delay its replication (16, 17) in fragile X cells. The formation of hairpin (21–24, 34) and tetrahelical structures (18–20) of $d(CGG)_n$ results in blocking of DNA polymerases *in vitro* at $d(CGG)_n$ template tracts (26, 27) and in the obstruction of replication *in vivo* (59). Evidence suggested that hairpin or tetraplex structures of $d(CGG)_n$ may cause polymerase slippage and expansion of the d(CGG) trinucleotide repeat (46).

Previously we showed that proteins, Werner syndrome helicase (29) and qTBP42 and uqTBP25 (30), mediated unwinding of bimolecular tetraplex forms of $d(CGG)_n$ into their single-stranded constituents. The demonstrated capability of the TK-6 cell Ku protein to impede nucleolytic digestion of G′2 $d(CGG)_n$ and to block its destabilization by qTBP42 raises the possibility that proteins might also prevent the removal or melting of $d(CGG)_n$ secondary structures. *In vivo* stabilization of secondary structures of the *FMR1* gene $d(CGG)_n$ stretch by Ku or similar DNA binding proteins, might thus contribute to polymerase slippage and to trinucleotide repeat expansion. Further, proteins that protect and stabilize hairpin or tetraplex formations of $d(CGG)_n$ might also exacerbate the blocking of *FMR1* transcription in fragile X cells. Hence, in assessing the formation and stability of secondary structures of $d(CGG)_n$, the potential contribution of both hairpin and tetraplex $d(CGG)_n$ stabilizing and destabilizing nuclear proteins should be taken into account.

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