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The fragile X syndrome d(CGG)$_n$ nucleotide repeats form a stable tetrahelical structure

(tetrahelical DNA/methylcytosine/quadruplex DNA)

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ABSTRACT **The fragile X mental retardation syndrome is associated with the expansion of trinucleotide 5′-d(CGG)-3′ repeats within the *FMR1* gene and with hypermethylation of the cytosine residues of these repeats. The expansion and hypermethylation may account for the suppression of the transcription of the *FMR1* gene and for the delay of its replication during the cell cycle. Here we show that d(CGG)$_n$ oligomers can form a stable Hoogsteen-bonded structure that exhibits properties consistent with those of tetraplex DNA. Oligomers, d(^{m}CGG)$_n$, (n = 4, 5, or 7), at pH 8.0 and in the presence of an alkali metal ion form stable species exhibiting a reduced electrophoretic mobility in nondenaturing polyacrylamide gels. These species are denatured by heating at 90°C for 10 min. With a short d(^{m}CGG)$_5$ oligomer, the slowly migrating species is formed only when the cytosine residue is 5-methylated, whereas with the longer d(CGG)$_7$ it is generated whether or not cytosine is 5-methylated. By contrast, complementary cytosine-rich oligomers do not form analogous complexes. The second-order association kinetics of the formation of the slowly migrating species of d(^{m}CGG)$_5$ suggests that it is an interstrand complex. Formation of intermediate-size complexes between d(^{m}CGG)$_5$ and d(^{m}CGG)$_7$ indicates that the stoichiometry of the slowly migrating structures is tetramolecular. Protection of the complex from methylation by dimethyl sulfate indicates the involvement of the N-7 positions of the guanine residues in Hoogsteen hydrogen bonding, a characteristic of quadruplex structures. If formed *in vivo* along the expanded and hypermethylated d(^{m}CGG)$_n$ stretch, this tetraplex structure could suppress transcription and replication of the *FMR1* gene in the fragile X syndrome cells.**

The fragile X syndrome is the most common cause of inherited mental retardation, with an incidence of 1 in 1500 males and 1 in 2500 females (1, 2). This syndrome is associated with a folate-sensitive fragile site at Xq27.3 which contains an expanded stretch of a d(CGG)$_n$ trinucleotide repeat located at the 5′ region of the chromosome X-linked housekeeping *FMR1* gene (3–6). Whereas in normal individuals the number of d(CGG) repeats ranges between 6 and 54, in phenotypically normal "premutation" carriers the number of repeats is increased to >50–200 and in individuals affected with fragile X syndrome the number is expanded to >200–2000 (reviewed in refs. 7 and 8). The *FMR1* alleles in patients with the fragile X syndrome are transcriptionally inactive (9), and their CpG island (10) and adjacent d(CGG) repeats (10–13) are at least partially methylated. Further, the replication of the mutant *FMR1* gene and of chromosomal segments ≥150 kb 5′ and ≥34 kb 3′ from the expanded repeat is delayed (14). The mechanisms that link d(CGG) expansion and hypermethylation with the suppression of the transcription of the *FMR1* gene and the delay in its replication are not known.

Guanine-rich single-stranded sequences found in telomeres and in other chromosomal locations can form, in the presence of alkali metal ions, parallel or antiparallel tetrahelical structures (reviewed in refs. 15 and 16). The core of these tetrahelices are cyclic arrays of four hydrogen-bonded guanine bases in which each base acts as both donor and acceptor of two hydrogen bonds with other guanines and the pairing between the bases is of the Hoogsteen type (15, 16). The formation of tetraplex structures under physiological conditions (15, 16), as well as the selective interaction or binding of several specific proteins with quadruplex DNA (17–21), suggests that tetrahelical DNA may be formed *in vivo* at some guanine-rich loci along the genome. Here we demonstrate that model d(CGG)$_n$ oligonucleotides form under physiological conditions a complex structure whose properties conform with those of tetrahelical DNA and that 5-methylation of the cytosine residues of a short d(^{m}CGG)$_n$ tract stabilizes this structure. If generated *in vivo*, a d(CGG)$_n$ tetrahelix could obstruct transcription and replication.

MATERIALS AND METHODS

Formation and Electrophoretic Analysis of d(CGG)$_n$ Oligomers. DNA oligomers were prepared and purified by HPLC by Operon Technologies, Alameda, CA. The 5-methyldeoxycytidine cyanoethyl phosphoramidite used for the synthesis of the 5-methylcytosine (^{m}C)-containing oligomers was the product of Glen Research, Sterling, VA. Oligomers were labeled with ^{32}P at their 5′ termini in a polynucleotide kinase-catalyzed reaction; solutions containing 4–15 μM labeled oligomer were denatured at 90°C for 10 min; and, to generate intermolecular complexes, the DNA was incubated for 90 h at 4°C in a final volume of 5 μl of 10 mM Tris·HCl, pH 8.0/1 mM EDTA (TE buffer) and 200 mM KCl. To resolve complex from single-stranded oligomer, 1 or 2 μl of the DNA solution was added to 10 μl of 25 mM Tris·HCl, pH 8.0/1 mM EDTA/0.5 mM dithiothreitol/20% (vol/vol) glycerol (T buffer) and immediately electrophoresed through a nondenaturing 12% polyacrylamide gel (30:1.2 ratio of acrylamide to bisacrylamide) using 0.5× TBE buffer (45 mM Tris borate buffer, pH 8.3/1 mM EDTA). The gels were run at room temperature at 12 V/cm until a bromophenol marker dye migrated 7 cm. The dried gel was autoradiographed by exposure to Kodak XAR-5 x-ray film, and the amounts of single-strand and complex DNA were determined by cutting out the bands and measuring Cerenkov radiation.

Methylation-Protection Analysis of Monomeric and Multimeric d(CGG)$_n$ Species. Single-stranded 5′-^{32}P-labeled d(^{m}CGG)$_5$ was generated by heat denaturation of oligomer solution in TE buffer with no salt. A mixture of single-

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Abbreviation: DMS, dimethyl sulfate.

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stranded and slowly migrating DNA was formed by the incubation at 4°C for 144 h of 5'-^{32}P-labeled d(^{m}CGG)$_5$ at 70 ng·μl^{-1} in the presence of 200 mM KCl in TE buffer in a final volume of 5 μl. Single-stranded d(^{m}CGG)$_5$ and a mixture of single-stranded DNA and the slowly migrating species were each exposed to 1% dimethyl sulfate (DMS) at room temperature for 0, 3, or 5 min and the reaction was stopped by the addition of 2 vol of T buffer. The DNA preparations were immediately resolved by electrophoresis through a nondenaturing 6% polyacrylamide gel. After autoradiography, bands of single-strand and complex d(^{m}CGG)$_5$ were excised from the gel and the DNA was extracted by an overnight incubation at 4°C of each of the gel slices in 100 μl of TE buffer. The yield of extracted DNA was 65–95% and it was precipitated at −20°C by 70% (vol/vol) ethanol with 2.0 M ammonium acetate. Each dried DNA sample was resuspended in 20 μl of TE buffer, and the DNA was hydrolyzed by adding to each sample 20 μl of 2.0 M pyrrolidine and heating at 90°C for 15 min. The DNA was dried by Speed Vac centrifugation and washed twice with 20 μl of water, and equivalent amounts of radioactivity for each sample of any type of DMS treatment were resolved by electrophoresis through a denaturing 8 M urea/15% polyacrylamide gel.

RESULTS

Oligomers of the d(CGG)$_n$ Type Form Slowly Migrating Electrophoretic Species. The generation of tetraplex DNA *in vitro* was found to normally require clusters of at least three contiguous guanine residues (15), although an oligomer that contains clusters of only two contiguous guanines was reported to also form a tetraplex structure (22). In consideration of the high content of guanines in the fragile X syndrome d(CGG)$_n$ stretch, we undertook to investigate its capacity to form quadruplex structure. First we examined whether d(CGG)$_n$ tracts generate a complex that displays aberrant electrophoretic mobility in nondenaturing polyacrylamide gels. Different guanine- or cytosine-rich oligomers were incubated at 4°C for 90 h in the presence of 200 mM KCl and then analyzed by electrophoresis through a nondenaturing 12% polyacrylamide gel as described in *Materials and Methods*. As seen in Fig. 1, a substantial proportion of d(^{m}CGG)$_5$ exhibits a slower electrophoretic mobility than does the single-strand oligomer. This slowly migrating species is relatively stable as judged by repetitive electrophoresis, but it can be denatured by heating for 10 min at 90°C (Fig. 1) or by

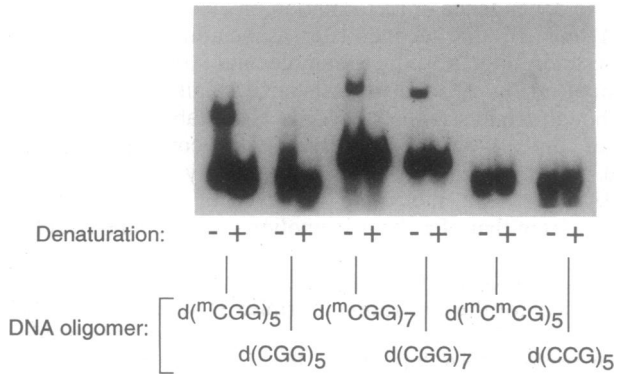

FIG. 1. Electrophoretic resolution of monomeric and slowly migrating forms of d(CGG)$_n$ oligomers and their complementary cytosine-rich strands. The HPLC-purified DNA oligomers were 5'-end-labeled with ^{32}P, and each oligomer at 15 μM was denatured for 10 min at 90°C, rapidly cooled, and then incubated at 4°C for 90 h in a final volume of 5 μl of TE buffer, pH 8.0/200 mM KCl. Aliquots of each oligomer were heated at 90°C for 10 min, and equal amounts of denatured and nondenatured DNA were electrophoresed through a nondenaturing 12% polyacrylamide gel.

electrophoresis through an 8 M urea-containing denaturing polyacrylamide gel (data not shown). Similar results were obtained with the shorter methylated oligonucleotide d(^{m}CGG)$_4$ (data not shown). Under the same conditions, the nonmethylated analogous oligomers d(CGG)$_5$ (Fig. 1) and d(CGG)$_4$ (not shown) did not generate a significant amount of an electrophoretically retarded form. By contrast, a slowly migrating, heat-labile form was obtained with both the methylated and unmethylated variants of the longer d(CGG)$_7$ oligomer (Fig. 1). No detectable slowly migrating form was observed with the complementary cytosine-rich methylated d(^{m}C^{m}CG)$_5$ or unmethylated d(CCG)$_5$ oligomers (Fig. 1). An identical pattern of formation of slowly migrating complexes of d(^{m}CGG)$_4$, d(^{m}CGG)$_5$, d(^{m}CGG)$_7$, or d(CGG)$_7$ was obtained in the presence of 200 mM NaCl or 200 mM LiCl but not with their complementary cytosine-rich oligomers (data not shown).

Ion Dependence and Kinetics of the Formation of the Slowly Migrating Species of d(^{m}CGG)$_5$. Quadruplex DNA formation and stabilization have been found to depend on the presence of monovalent alkali metal ions that are thought to be localized between two guanine quartets and to coordinate the eight guanine O-6 atoms (15, 16, 23–26). As shown in Fig. 2 *A* and *B*, the formation of the slowly migrating d(^{m}CGG)$_5$ complex was dependent on monovalent alkali metal ions. In accord with a previous report on the formation of quadruplex DNA with low millimolar Mg^{2+} (27), we also found that a maximum amount of the slowly migrating complex (12.6% of the total) was generated at 4 mM Mg^{2+}. Complex formation decreased progressively by more than 50% of the maximum as the concentration of Mg^{2+} was increased to 5–15 mM (data not shown). The relationship between the initial rate of formation of the slowly migrating DNA species and the concentration of d(^{m}CGG)$_5$ serves to indicate whether the complex is an interstrand aggregate (26). The graph presented in Fig. 2*C* depicts the amount of the slowly migrating species that is formed as a function of the initial d(^{m}CGG)$_5$ concentration. The slope of the log–log curve is 1.75 in the presence of Na^+ or K^+ (Fig. 1*C*) and 1.55 with Li^+ (not shown). This slope approaches the value of 2.0 that is expected if the formation of an in-register parallel duplex is a rate-limiting step for the formation of the complex as previously reported for tetraplex form of the IgG switch region (26). The lower than 2.0 slope that we observe suggests that an intermediate step in the formation of the complex exists or that our measurements were not conducted at the initial linear phase of complex formation. These results, as well as the slow migration of the formed species, exclude the possibility that it is a monomolecular folded structure and, rather, suggest that it is an interstrand complex.

The Slowly Migrating d(^{m}CGG)$_n$ Species Has a Tetramolecular Stoichiometry. To directly inspect the stoichiometry of the slowly migrating d(^{m}CGG)$_n$ structures, we examined the generation of complexes between oligomers of different length. Mixtures of 5'-^{32}P-labeled d(^{m}CGG)$_5$ and d(^{m}CGG)$_7$ at different molar ratios were incubated at 4°C for 18 h in the presence of 200 mM KCl. Formed complexes were separated from the monomers and resolved by electrophoresis through a nondenaturing polyacrylamide gel. As shown in Fig. 3, complexes of unmixed d(^{m}CGG)$_5$ and of d(^{m}CGG)$_7$ displayed the fastest and slowest mobilities, respectively. Additional complexes of intermediate mobility appeared in mixtures of the two oligomers, and the relative amounts of these species changed in consonance with the changing ratio between the 15- and 21-mer oligonucleotides. The changing intensity of intermediate bands II and III (Fig. 3) indicates that they represent tetramolecular species with the structures [d(^{m}CGG)$_5$]$_3$·[d(^{m}CGG)$_7$]$_1$ and [d(^{m}CGG)$_5$]$_2$·[d(^{m}CGG)$_7$]$_2$, respectively. Thus, complexes formed by unmixed oligomers are, most probably, [d(^{m}CGG)$_5$]$_4$ and [d(^{m}CGG)$_7$]$_4$. Note that the 2:2

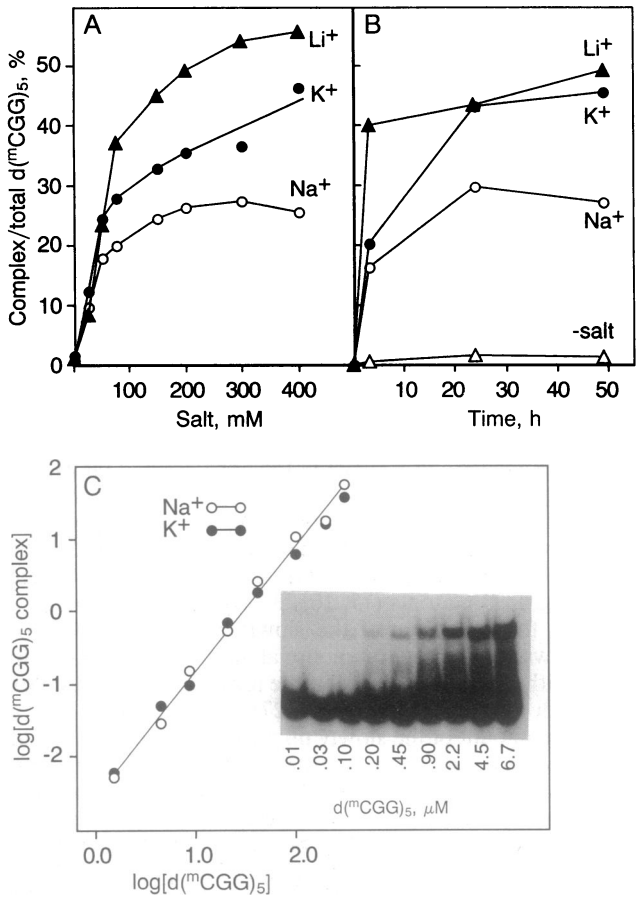

FIG. 2. (*A*) Effect of monovalent alkali metal ions on the formation of the slowly migrating structure of d(mCGG)$_5$. The 5′-^{32}P-labeled oligomer was denatured and subsequently incubated at 4°C for 49 h in TE buffer that contained salts as indicated. Conditions of incubation and of the subsequent electrophoresis were as in Fig. 1. The amounts of the monomeric and of the slowly migrating form of d(mCGG)$_5$ were determined by Cerenkov counting of radioactivity in their respective cut bands (10). (*B*) Kinetics of formation of the slowly migrating form of d(mCGG)$_5$ in the absence or presence of 200 mM alkali metal ions. Incubation at 4°C of 5′-^{32}P-labeled d(mCGG)$_5$ was performed as in *A* for the indicated periods of time. (*C*) Dependence of formation of the slowly migrating species on the initial concentration of d(mCGG)$_5$. A series of samples of d(mCGG)$_5$ at a total concentration of 30–0.05 ng·μl^{-1} (≈6.7–0.01 μM), each containing an equal amount of 5′-^{32}P-labeled oligomer, were incubated at 4°C for 3 h in the presence of either 200 mM NaCl or 200 mM KCl and resolved by electrophoresis through a nondenaturing 12% polyacrylamide gel. Quantification of the amounts of the formed complex and of remaining monomer was conducted as in *B*. (*Inset*) Electrophoretic resolution of d(mCGG)$_5$ incubated in the presence of 200 mM KCl.

intermediate complex (III) is formed at a high efficiency, whereas the 3:1 tetraplex (II) is generated at a lower rate. Moreover, a complex with the structure [d(mCGG)$_5$]$_1$·[d(mCGG)$_7$]$_3$ is not discerned. The different yield of the various hybrids between d(mCGG)$_5$ and d(mCGG)$_7$ is probably due to their different stabilities. Similar results were also obtained when complexes were formed in the presence of 200 mM NaCl (data not shown).

Methylation-Protection Analysis of the d(mCGG)$_5$ Complex. The guanine quartet core of quadruplex DNA is formed through guanine–guanine Hoogsteen hydrogen bonding that involves the N-7 positions. This N-7 bonding renders the guanine residues resistant to alkylation (15, 16, 23, 26). To examine the accessibility of the guanine N-7 positions to methylation, we compared the slowly migrating species of d(mCGG)$_5$ to single-strand d(mCGG)$_5$. Methylation protection

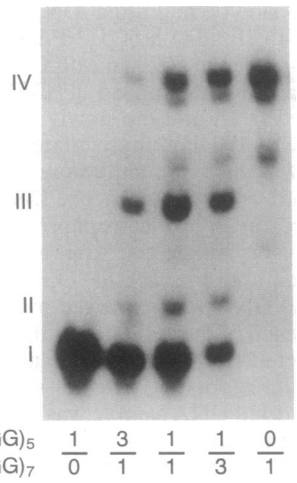

FIG. 3. Stoichiometry of the formation of slowly migrating forms in mixtures of d(mCGG)$_5$ and d(mCGG)$_7$. HPLC-purified and 5′-^{32}P-labeled d(mCGG)$_5$, d(mCGG)$_7$, or mixtures thereof at a final total concentration of 4.5 μM were incubated at 4°C for 18 h in the presence of 200 mM KCl in a final volume of 5 μl. Molar stoichiometry is given below each lane. Aliquots of 1 μl of each incubation mixture were electrophoresed through a nondenaturing 12% polyacrylamide gel (0.4-mm thickness; 40-cm length), until the monomeric forms of the two oligomers migrated off the gel. I, [d(mCGG)$_5$]$_4$; II, [d(mCGG)$_5$]$_3$·[d(mCGG)$_7$]$_1$; III, [d(mCGG)$_5$]$_2$·[d(mCGG)$_7$]$_2$; IV, [d(mCGG)$_7$]$_4$. An unmarked band that migrates ahead of the [d(mCGG)$_7$]$_4$ complex appears in the absence of d(mCGG)$_5$ (rightmost lane) and thus represents an impurity in the d(mCGG)$_7$ oligomer.

was conducted according to Sen and Gilbert (26), with minor modifications as detailed in *Materials and Methods*. As seen in Fig. 4, in contrast to the significant DMS-dependent hydrolysis of the single-strand d(mCGG)$_5$ molecules, the slowly migrating complex remained largely protected against methylation and subsequent pyrrolidine hydrolysis. This resistance to alkylation indicates that the N-7 positions of guanines in the complex are involved in Hoogsteen bond formation and thus are protected against attack by the methylating agent.

DISCUSSION

A multi-strand tetrahelical structure of the d(mCGG)$_5$ complex is indicated by its slowed electrophoretic mobility (Fig. 1), the dependence of its formation on alkali metal ions (Fig. 2 *A* and *B*), the second-order association kinetics of its generation (Fig. 2*C*), the tetramolecular stoichiometry of its structure (Fig. 3), and its resistance to alkylation (Fig. 4). Although triple-stranded DNA is also stabilized by Hoogsteen bonds, the conditions for d(CGG)$_n$ complex formation and its properties make it highly unlikely that it is triplex DNA. First, the nucleotide sequence of the d(CGG)$_n$ run differs from that of polypurine·polypyrimidine stretches that are the common progenitors of triple-helical structure. Second, the pH 8.0 at which the d(CGG)$_n$ aggregates form is not conducive for the generation of triplex DNA. Last, although a guanine-rich telomeric sequence was recently reported to form triplex DNA but unlike the d(CGG)$_n$ complex, that structure had the same electrophoretic mobility as its respective monomer and it was assumed to be the product of the folding-back of a single DNA molecule (28). The multimolecular nature of the d(mCGG)$_5$ complex and the conditions for its formation indicate, therefore, that it is a tetrahelix.

The d(CGG)$_n$ complexes are distinguished as intermolecular tetrahelical DNA aggregates with characteristic properties. Methylation of the cytosine residues is necessary for the stabilization of the quadruplex form of short d(mCGG)$_n$ tracts

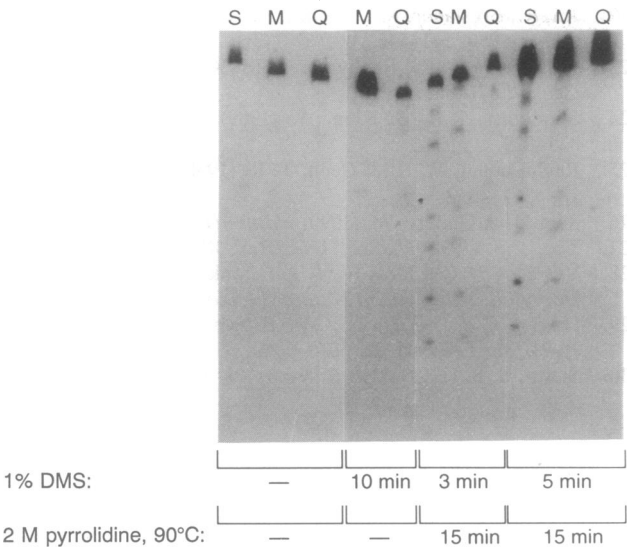

FIG. 4. Methylation protection of the monomeric and of the slowly migrating forms of d(^{m}CGG)$_5$. A mixture of monomeric and slowly migrating forms of 5′-^{32}P-labeled d(^{m}CGG)$_5$ and single-strand d(^{m}CGG)$_5$ were prepared and exposed to 1% DMS for the indicated periods of time as detailed in ref. 14. The DMS-treated DNA samples were resolved by electrophoresis through a nondenaturing 6% polyacrylamide gel; respective bands of monomer and complex forms were excised from the gel; and the DNA was extracted into TE buffer and precipitated by ethanol (14). The purified DNA was resuspended in 20 μl of TE buffer and hydrolyzed at 90°C for 15 min by 20 μl of 2.0 M pyrrolidine. The samples were dried, and each was washed twice with 20 μl of H_2O and electrophoresed through a denaturing 15% polyacrylamide/urea gel. Lanes S, d(^{m}CGG)$_5$ denatured in the absence of salt (this DNA migrated on a nondenaturing gel as a purely monomeric form); lanes M, monomeric form of d(^{m}CGG)$_5$ resolved from its mixture with the slowly migrating form; lanes Q, slowly migrating form of d(^{m}CGG)$_5$ resolved from its mixture with the monomer.

(Fig. 1). Methylation of C-5 of cytosine was recently shown to stabilize guanine quartet structure by producing more favorable stacking interactions (29). This observation serves to explain the requirement for 5-methylcytosine for the stabilization of short d(^{m}CGG)$_n$ tetraplex. Formation of the complex species of d(^{m}CGG)$_5$, d(^{m}CGG)$_7$, and d(CGG)$_7$ was maximally promoted by Li^+ and less by K^+ or Na^+ (Fig. 2 *A* and *B* and results not shown). This is in contrast to the finding that oligomers which contain short runs of guanine within nonguanine sequences form tetraplexes in the presence of Na^+ or K^+ but not with Li^+ (23–26). This ion preference was explained by the size-selective binding of cations within the central cavity of guanosine quartets (15, 16). The preferential generation of quadruplex d(^{m}CGG)$_5$, d(^{m}CGG)$_7$, and d(CGG)$_7$ in the presence of the small Li^+ ion may indicate that the guanosine tetrads are stacked more tightly in this tetraplex than in tetrahelices formed by short guanine tracts that are dispersed among nonguanine sequences. Whereas the generation of quadruplex DNA by short runs of contiguous guanines is usually slow, linear with time, and dependent on relatively high concentrations of DNA (8–800 μM, ref. 26), tetraplex d(^{m}CGG)$_5$ was formed rapidly (≥50% of the maximum within 3 h; Fig. 2*B*) and at relatively low concentrations of DNA (typically, 4–15 μM). The rapid formation of quadruplex d(^{m}CGG)$_5$ at relatively low DNA concentrations may be due to the high content of guanine residues in the repeating tract which could also be responsible for the high melting temperature of the quadruplex form.

The rapid and efficient formation *in vitro* of a tetraplex form of d(^{m}CGG)$_5$ runs and its enhanced stabilization by 5-methylation of the cytosine residues raises the possibility that a structure of this type can be formed in fragile X syndrome cells. Notably, it was hypothesized that 5-methylation of cytosine may be facilitated at regions of unusual structures of DNA, including tetrahelical stretches (30), and thus methylation and quadruplex formation could be mutually promoted. Long stretches of expanded and hypermethylated single strands of d(CGG)$_n$ may be exposed during DNA replication. Back-folding of the d(CGG)$_n$ tract and the formation of Hoogsteen bonds between the looped-back strands could lead to the generation of stable antiparallel hairpin or quadruplex domains. If formed, regions of tetraplex DNA could be involved in the suppression of the transcription of the *FMR1* gene (9). Also, regions of tetrahelical DNA could be involved in the blocking of the progress of replication forks as observed for the fragile X locus (14). These inhibitory effects may be undetectable in cells of normal and "premutation" individuals, since the d(CGG)$_n$ stretch is shorter and largely hypomethylated. It is also possible that specific binding proteins may be available to sufficiently protect the limited number of d(CGG) single-strand repeats in normal and carrier cells against quadruplex-generating DNA–DNA interaction. By contrast, the length of the expanded trinucleotide stretch in affected cells may exceed the capacity of specific binding proteins, and exposed DNA stretches may remain free to interact to form hairpin or tetrahelical complexes.

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