Guanine Tetraplex Formation by Short DNA Fragments Containing Runs of Guanine and Cytosine

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ABSTRACT Using CD spectroscopy, guanine tetraplex formation was studied with short DNA fragments in which cytosine residues were systematically added to runs of guanine either at the 5′ or 3′ ends. Potassium cations induced the G-tetraplex more easily with fragments having the guanine run at the 5′ end, which is just an opposite tendency to what was reported for (G+T) oligonucleotides. However, the present (G+C) fragments simultaneously adopted other conformers that complicated the analysis. We demonstrate that repeated freezing/thawing, performed at low ionic strength, is a suitable method to exclusively stabilize the tetraplex in the (G+C) DNA fragments. In contrast to KCl, the repeated freeze/thaw cycles better stabilized the tetraplex with fragments having the guanine run on the 3′ end. The tendency of guanine blocks to generate the tetraplex destabilized the $d(G_5).d(C_5)$ duplex whose strands dissociated, giving rise to a stable tetraplex of (dG_5) and single-stranded (dC_5). In contrast to $d(G_3C_3)$ and $d(G_5C_5)$, repeated freezing/thawing induced the tetraplex even with the self-complementary $d(C_3G_3)$ or $d(C_5G_5)$; hence the latter oligonucleotides preferred the tetraplex to the apparently very stable duplex. The tetraplexes only included guanine blocks while the 5′ end cytosines interfered neither with the tetraplex formation nor the tetraplex structure.

INTRODUCTION

DNA can adopt many conformations that significantly differ from the Watson-Crick double helix. One of them is stabilized by stacked guanine tetrads and physiological concentrations of potassium or other cations (review, Guschlbauer et al., 1990). Its molecular structure has been determined by x-ray diffraction (Kang et al., 1992; Laughlan et al., 1994) and NMR spectroscopy (Smith and Feigon, 1992; Aboul-ela et al., 1992; Wang and Patel, 1993; Gupta et al., 1993). Although the first reports about the aggregation tendencies of deoxyguanosine DNA fragments (Ralph et al., 1962) and the guanine tetraplex structure (Gellert et al., 1962) were published several decades ago, the tetraplex has become of wider interest only recently when it was demonstrated to be formed by telomeric DNA (Henderson et al., 1987; Sen and Gilbert, 1988; Williamson et al., 1989; Sundquist and Klug, 1989). However, its actual formation and role in vivo have not yet been established. In the past several years, the G-tetraplex has also been studied in connection with topoisomerase inhibition (Chung and Muller, 1991), DNA structure alteration in a polymorphic region linked to the human insulin gene (Hammond-Kosack et al., 1992), generation of a K^+-dependent DNA synthesis arrest site (Woodford et al., 1994), formation in a region of an rRNA gene associated with hydrogen peroxide-mediated mutations (Akman et al., 1991), and a high propensity of appearance in the 5′ end sequences of the retinoblastoma susceptibility gene (Murchie and Lilley, 1992). In addition, the G-tetraplex framework has been found to stand behind potent antithrombotic activities of some DNA fragments (Macaya et al., 1993, 1995; Padmanabhan et al., 1993; Wang et al., 1993), an adenosine and ATP binding by a DNA aptamer (Huizenga and Szostak, 1995), inhibition of HIV cell fusion (Wyatt et al., 1994), and the antiproliferative and other effects caused by a non-antisense mechanism (Burgess et al., 1995). The aggregation tendencies of the G-rich DNA fragments can also be advantageously employed to develop new nanostructures and biomaterials such as G-wires (Marsh et al., 1995) or synapsable DNAs (Venczel and Sen, 1996). Hence there are good reasons to study the tetraplex in rigorous details, which is not easy owing to its very slow kinetics of formation and aggregation that is difficult to control (Sen and Gilbert, 1992).

We have recently started a systematic investigation of the conformations adopted by (G+C)-rich DNA fragments in solution (Vorlíčková et al., 1996). These studies are complicated by a tendency of some of the (G+C)-rich fragments to form conformers or their mixtures, depending on the sample history, fragment concentration, etc. One of the conformers participating in these complex conformational equilibria is the G-tetraplex whose properties are studied here in detail. We are especially interested in how cytosines and their possible pairing with guanines interfere or compete with the G-tetraplex. This is an interesting question because >1% of the human genome consists of tracts at least 28 bp in length with a (G+C) content >85% (MacLeod et al., 1996).

We use CD spectroscopy here because it sensitively reflects the G-tetraplex formation (Gray and Bollum, 1974; Balagurumoorthy et al., 1992; Chen, 1992; Guo et al., 1993). This method provides valuable information about the

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conformational equilibria of DNA if a series of properly selected DNA fragments is studied in parallel. We have chosen $d(G)_3$ and $d(G)_5$ as the common guanine tetraplex-forming strands to study destabilization of the tetraplex in the variants of $d(G)_3$ to which cytosines were added at either its 3′ end or 5′ end, giving $d(G_3C)$, $d(G_3C_2)$, $d(G_3C_3)$, $d(CG_3)$, $d(C_2G_3)$, and $d(C_3G_3)$. As the self-complementary sequences are the most interesting here, we have extended this group to $d(G_5C_5)$ and $d(C_5G_5)$. The present paper shows that repeated freezing/thawing is an effective way of induction of the G-tetraplex, avoiding other interfering conformers or associations of more than four strands. The tetraplex of d(GGGGG) is shown to be much more stable than its duplex with the complementary strand of d(CCCCC) and, remarkably, the self-complementary sequences $d(C_3G_3)$ and $d(C_5G_5)$ even prefer the tetraplex to the apparently very stable duplex.

MATERIALS AND METHODS

Determination of DNA fragment concentrations

The DNA fragments used in this study are listed in Table 1. They were synthesized and purified as described earlier (Kypr et al., 1996). Their stock solutions (~0.02 M in bases) were prepared in 1 mM NaCl and the concentrations were determined in the following way. An appropriate amount of the stock solution was diluted 400-fold by water, the diluted sample was heated for 60 min at 96°C, and its UV absorption was measured at 96°C. The sample concentration was determined from this absorbance value (at 260 nm), taking use of the calculated molar extinction coefficient (Borer, 1975; Table 1). The UV absorption spectra were measured using a Philips PU 8730 spectrophotometer (Philips Analytical, Cambridge, UK).

CD spectra measurements

CD spectra were measured on the Mark IV and VI Jobin-Yvon dichrographs (I.S.A. Jobin Yvon, Longjumeau, France) in quartz cells from Hellma (GmbH & Co., D-7840 Müllheim/Baden, Germany) placed in a thermostated holder. The cell pathlengths varied from 0.001 cm (sandwich cells) to 5 cm. The sample absorption was always within 0.5–0.8 at 260 nm.

CD spectra dependences on the DNA fragment concentration

The measurements were done in 1 mM NaCl. The samples were gradually diluted, kept after each dilution 3 h at room temperature, then 18 h at 4°C, and 3 h at room temperature again. After this treatment, the CD spectra were measured at room temperature.

TABLE 1 DNA fragments studied in this work and their extinction coefficients per one fragment base

DNA Fragment	ϵ_{260} ($M^{-1}cm^{-1}$)
d(GGGGG)	10 380
d(GGG)	10 570
d(GGGC)	9 450
d(CGGG)	9 550
d(GGGCC)	9 000
d(CCGGG)	9 080
d(CCCCC)	7 240
$d(G_3C_3)$	8 700
$d(C_3G_3)$	8 770
$d(G_5C_5)$	8 720
$d(C_5G_5)$	8 680

Elimination of higher aggregates

DNA fragment solutions (0.1 mM in bases) in 2 mM potassium phosphate buffer, pH 7.0, were heated for 60 min at 90°C and then cooled in an ice bath. Because of the thermal stability of their molecular aggregates, d(GGGGG), $d(C_5G_5)$, and $d(G_5C_5)$ were heated in 0.01 mM potassium phosphate and the buffer concentration was only then increased to 2 mM. Hereafter, this procedure will be called "temperature treatment."

Dependences on the KCl concentration

After the temperature treatment, 3 M KCl stock solution and then solid KCl were gradually added to 0.1 mM DNA fragment samples dissolved in 2 mM K-phosphate, pH 7. The sample CD spectra were measured at 20°C.

Freezing/thawing

After the temperature treatment, the DNA fragment solutions (0.1 mM in bases) dissolved in 2 mM potassium buffer, pH 7.0, were kept for 15 min at −35°C and then thawed for 5 min in a water bath, temperature 20°C. CD spectra of the samples were then measured at 20°C.

Annealing $d(G_5)$ with $d(C_5)$

The samples containing different molar ratios of $d(G_5)$ and $d(C_5)$ were prepared in 0.1 M NaCl and 1 mM Tris-HCl buffer, pH 8.0. The total DNA base concentration was kept constant, 5×10^{-5} M, in the mixed samples. By adding 1 M NaOH, the samples were alkalized to pH 12.5, then neutralized by 1 M HCl, kept at 80°C for 60 min, and very slowly (5 h) cooled to room temperature. Their CD spectra were then measured at room temperature.

RESULTS

Treatment of the DNA fragment samples

After a hundredfold dilution of the stock solutions giving the DNA fragment concentrations of ~0.2 mM (in bases), the CD spectra of all the present DNA fragments changed with time. With the exception of d(GGGCC), the fragments yielded CD spectra with a strong positive band in the vicinity of 260 nm, which slowly diminished with time (Fig. 1). Such a CD spectrum shape was provided by intermolecular parallel-stranded guanine tetraplexes (Chen, 1991; Guo et al., 1993; Lu et al., 1993). However, even short G-rich DNA fragments were shown to aggregate and form both liquid crystalline phases (Mariani et al., 1989; Garbesi et al., 1993; Gottarelli and Spada, 1994) and G-wires (Marsh and Henderson, 1994; Marsh et al., 1995) that contributed to the CD spectrum as well. Thus the CD spectra have to be interpreted with caution.

A careful analysis of the CD spectra reveals two processes during the time-dependent dissociation of d(GGG) (Fig. 1). The latter process (Fig. 1, *right*) has a very slow kinetics. The time-dependent CD spectra have isoelliptic points reflecting a two-state nature of the process that includes only the single strand and tetraplex. On the other hand, the preceding spectra do not pass through these isoel-

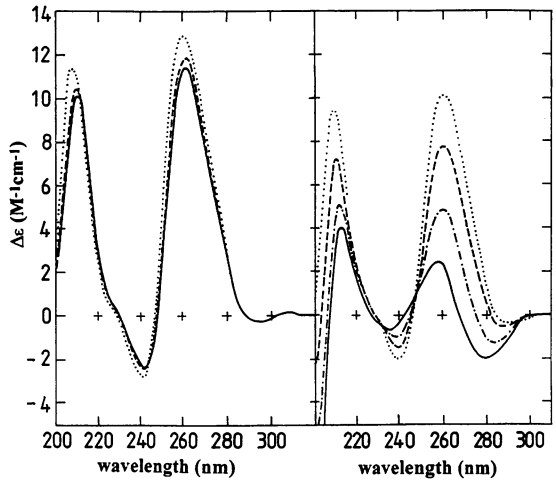

FIGURE 1 Time-dependent changes in the CD spectra of d(GGG). The CD spectra were measured (*left*): (···) 1 min, (- - -) 14 min, (—) 25 min; and (*right*): (···) 3.25 h, (- - -) 13 h, and (- · - · -) 79.5 h after a hundredfold dilution of the stock solution into 1×10^{-5} M NaCl. The final DNA concentration was 1.9×10^{-4} M. Temperature 3°C. *Right* (—): 1.5×10^{-4} M d(GGG), room temperature, the equilibrium spectrum; the sample was treated as in Fig. 2.

liptic points (Fig. 1, *left*) to suggest participation of chiral species different from the two conformers. Most probably, the chiral species are aggregates of more than four d(GGG) strands.

The behavior is still more complex with d(GGGGG), whose molecules associate much more extensively. This follows from the equilibrium CD spectra dependence on the DNA fragment concentration (Fig. 2, *left*). The large ellipticity at 260 nm depends, in a nonmonotonic way, on the d(GGGGG) concentration, indicating the presence of numerous species in the solution, whose chirality is different from that of the tetraplex. The d(GGGGG) strand association is so strong that the high CD amplitudes persist in the whole range of DNA concentrations measurable by CD (up to 2×10^{-6} M DNA base concentration; measured in a 5-cm pathlength cell). The high CD amplitudes only disappeared upon thermal denaturation of the diluted sample (see below, Fig. 3).

It follows from Fig. 2 that the DNA concentration dependence of the CD spectra is a general property of all the present DNA fragments. DNA concentration regions can be found with d(GGG), d(GGGC), and d(CCGGG) (Fig. 2, *b*, *c*, and *f*), where the equilibrium CD spectra exhibit two-state changes upon dilution. Nevertheless, before starting rigorous experiments, the samples had to be devoid of the aggregates and transferred into standard reproducible initial states. The relevant procedure, as described in Materials and Methods, included the DNA fragment incubation for one hour at high temperature in a low ionic strength aqueous buffer. The DNA concentration should also be low. After this treatment, the oligonucleotides provided CD spectra (Fig. 3) at room temperature that were very similar to those calculated for their single strands (Cantor et al., 1970). All

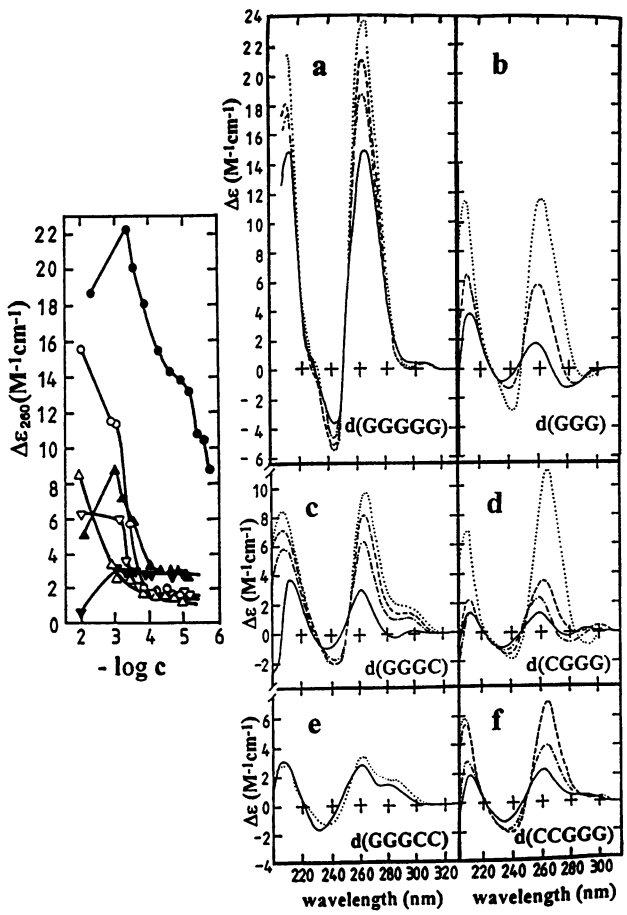

FIGURE 2 Concentration-dependent equilibrium CD spectra of the indicated DNA fragments. All of the spectra were measured in 1 mM NaCl at room temperature. The samples were treated as described in Materials and Methods. *Left*: Ellipticity changes at 260 nm as a function of $-\log c$ (c is the molar concentration of the indicated DNA fragment bases): ● d(GGGGG), ○ d(GGG), ▲ d(GGGC), △ d(CGGG), ▼ d(GGGCC), ▽ d(CCGGG). *Right*: DNA fragment concentrations: (*a*) d(GGGGG): (···) 4.4×10^{-4} M, (- - -) 3.0×10^{-4} M, (- · - · -) 1.3×10^{-4} M, and (—) 1.1×10^{-5} M; (*b*) d(GGG): (···) 8.6×10^{-4} M, (- - -) 3.9×10^{-4} M, and (—) 1.1×10^{-5}M; (*c*) d(GGGC): (···) 9.3×10^{-4} M, (- - -) 6.1×10^{-4} M, (- · - · -) 2.7×10^{-4} M, and (—) 2.3×10^{-5}M; (*d*) d(CGGG): (···) 1.0×10^{-2} M, (- - -) 1.3×10^{-3} M, (- · - · -) 3.9×10^{-4} M, and (—) 1.2×10^{-5}M; (*e*) d(GGGCC): (···) 9.5×10^{-4} M, and (—) 6.8×10^{-6}M; (*f*) d(CCGGG): (- - -) 7.0×10^{-4} M, (- · - · -) 4.6×10^{-4} M, and (—) 2.0×10^{-4}M.

of the following experiments were carried out with samples pretreated in this way.

Tetraplex stabilization by KCl

We studied the oligonucleotides in the presence of KCl, which is the most effective monovalent salt in inducing the G-tetraplex (Guschlbauer et al., 1990). Both d(GGGGG) and d(GGG) underwent (Fig. 4) slow, two-state transitions upon the addition of KCl. The isoelliptic points at 221 and 248 (Fig. 4 *a*), or 233 and 247 nm (Fig. 4 *b*), suggested that the single strand and the tetraplex were the only conformers of d(GGG) and d(GGGGG) participating in the isomeriza-

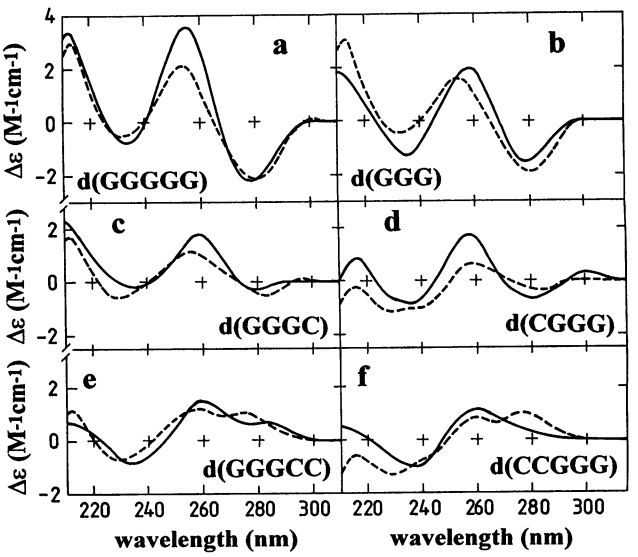

FIGURE 3 Comparison of the (—) measured (room temperature, 2 mM potassium phosphate buffer, pH 7.0, 1×10^{-4} M base concentration, after the "temperature treatment" (see Materials and Methods), and (- - -) calculated (Cantor et al., 1970) CD spectra of the indicated DNA fragments.

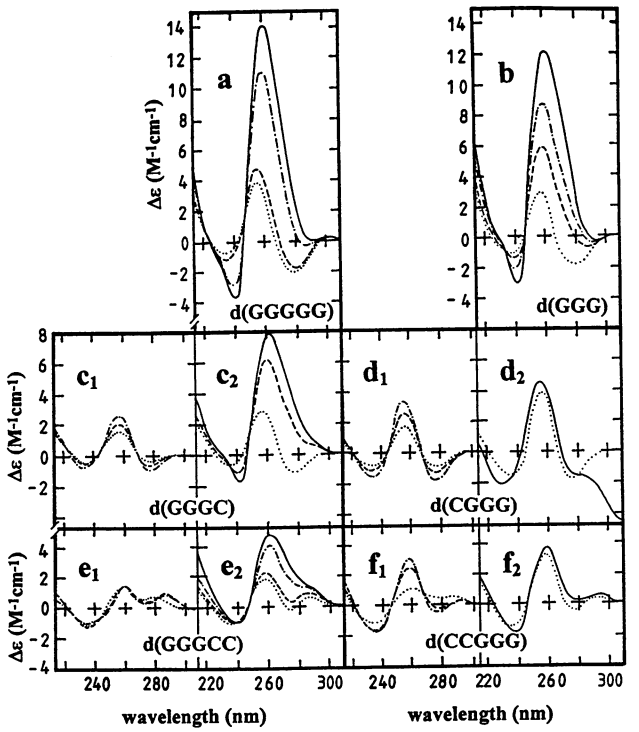

FIGURE 4 KCl-dependent and time-dependent changes in the CD spectra of (*a*) d(GGGGG) in 17 mM KCl after (···) 0, (- - -) 1, (- · - · -) 18, and (—) 130 h; (*b*) d(GGG) in 0.66 M KCl after (···) 0, (- - -) 55 min, (- · - · -) 5 h, and (—) 75.5 h; (c_1) d(GGGC) in (···) 0 M, (- - -) 0.36 M, and (- · - · -) 1.56 M KCl; (c_2) d(GGGC) in 3 M KCl after (···) 0, (- - -) 22 h, and (—) 91 h; (d_1) d(CGGG) in (···) 0 M, (- - -) 0.33 M, and (- · - · -) 1.50 M KCl; (d_2) d(CGGG) in 3 M KCl after (···) 0 and (—) 22 h; (e_1) d(GGGCC) in (···) 0 M, (- - -) 0.76 M, and (- · - · -) 1.20 M KCl; (e_2) d(GGGCC) in 3 M KCl after (···) 0, (- - -) 14 h, (- · - · -) 139 h, and (—) 313 h; (f_1) d(CCGGG) in (···) 0 M, (- - -) 0.35 M, and (- · - · -) 0.79 M KCl; (f_2) d(CCGGG) in 3 M KCl after (···) 0 and (—) 139 h.

tions. The limiting ellipticities (at 260 nm) were ~14 M^{-1} cm^{-1} and 12 M^{-1} cm^{-1} with the tetraplex of d(GGGGG) and d(GGG), respectively. These values corresponded to the respective plateaus in the concentration dependences of d(GGGGG) and d(GGG) (Fig. 2) as well as to the CD spectrum of the tetraplex of d(GGG) (Fig. 1) obtained during time-dependent dissociation of its aggregates present in the stock solution.

Interpretation of the KCl-induced CD changes was more difficult with DNA fragments containing cytosine (Fig. 4, *c–f*). The changes consisted of two steps. At relatively low KCl concentrations, the changes were fast (Fig. 4, c_1–f_1) but overlapped by slow changes at higher KCl concentrations. This especially concerned the DNA fragments containing the guanine run at the 5′ end (Fig. 4 c_2, e_2), whereas the CD spectrum of d(CCGGG) changed only slightly (Fig. 4 f_2) and d(CGGG) aggregated with time (Fig. 4 d_2). The time-dependent changes were more extensive with DNA fragments containing 5′ end guanines for various KCl concentrations. They were only less pronounced and slower at salt concentrations lower than 3 M KCl. The presence of more than two chiral states made interpretation of the KCl-induced changes difficult. That is why we looked for another way to induce the guanine tetraplex.

Tetraplex stabilization by repeated freezing/thawing

If the present samples were deposited in a freezer, they provided CD spectra after thawing that indicated an increased content of the guanine tetraplex. This phenomenon had earlier been noticed by Gray and Bollum (1974) with $d(G)_5$ and interpreted as a self-complexation of its strands. At very low ionic strength, repeated freezing/thawing proved to be an efficient way to induce exclusively tetraplexes with the present oligonucleotides (see below). That is why we studied this phenomenon in more detail.

We found that freezing, rather than low temperature, promoted the tetraplex. This follows from an experiment when two identical samples of d(GGG) were exposed to −9°C and only one was frozen by an abrupt shaking. After transferring the samples to room temperature, only that undergoing freezing exhibited the characteristic CD signs of an increased guanine tetraplex content.

Repeated freezing/thawing induced incomparably more tetraplexes than keeping the sample frozen for a long period of time. The sample of d(GGG) undergoing 22 freezing (15 min each)/thawing cycles provided the ellipticity of 12.8 M^{-1} cm^{-1} at 260 nm, while the same sample frozen for 6 h had the ellipticity of 4.8 M^{-1} cm^{-1}, which corresponded to only two freezing/thawing cycles. Hence it was the process of freezing and not the frozen state that induced the tetraplex formation.

Freezing dehydrates the oligonucleotides because most of the water molecules participate in the ice lattice. Alcohols dehydrate DNA like freezing but we found that even 80% trifluoroethanol did not induce the tetraplex with d(GGGGG). Even freezing the sample of d(GGGGG) containing 60% TFE had no effect. Hence trifluoroethanol hindered rather than promoted the tetraplex. Thus dehydration hardly was the factor standing behind the tetraplex stabilization by freezing.

Repeated freezing/thawing induced a two-state transition of $d(G_5)$ (Fig. 5 *a*) and $d(G_3)$ (Fig. 5 *b*) into the tetraplex. The CD spectra observed during this process, including the limiting spectra, were the same as those measured in the course of the time-dependent tetraplex stabilization by KCl (Fig. 4, *a* and *b*). The samples of d(GGGGG) and d(GGG) reached the limiting spectra after ~7 and 15 freeze/thaw cycles, respectively. Repeated freezing/thawing induced the tetraplex in the present cytosine-containing oligonucleotides as well. In contrast to the tetraplex induction by KCl, the freeze/thaw cycles operated in a two-state manner because the CD spectra (Fig. 5) intersected in isoelliptic points. Dependences of the ellipticity at 260 nm on the number of the freeze/thaw cycles indicated (not shown) that the tetraplex was generated more easily with d(CGGG) than with d(GGGC), although the limiting ellipticity values were similar (Fig. 5, *c* and *d*). The tetraplex forming propensity was much larger with d(CCGGG) as compared to d(GGGCC) that unwillingly associated into the tetraplex (Fig. 5, *e* and *f*). Thus the DNA fragments starting with dC on the 5′ end formed the tetraplex more easily than the DNA fragments having dC on the 3′ end.

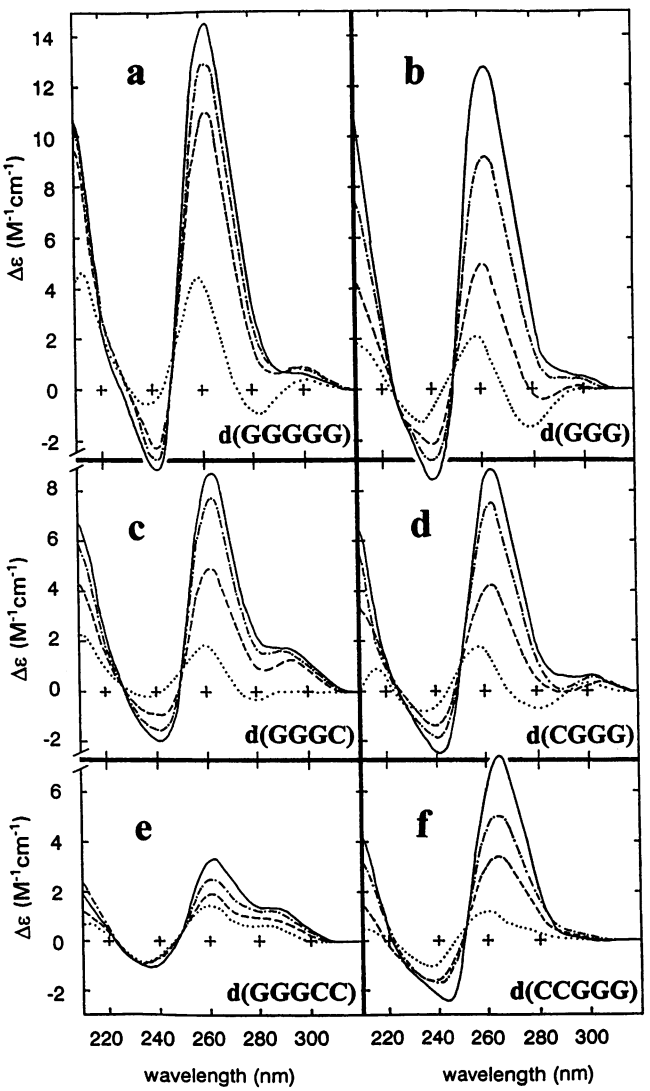

FIGURE 5 CD spectra of the indicated DNA fragments as dependent on the number of the freeze/thaw cycles. DNA fragments were dissolved in 2 mM potassium phosphate buffer, pH 7.0, temperature 20°C. Numbers of the freeze/thaw cycles were the following (from dotted to bold spectra): (*a*) d(GGGGG): 0, 1, 2, 15; (*b*) d(GGG): 0, 1, 4, 22; (*c*) d(GGGC): 0, 2, 7, 19; (*d*) d(CGGG): 0, 1, 3, 19; (*e*) d(GGGCC): 0, 1, 4, 13; (*f*) d(CCGGG): 0, 2, 4, 13.

The unstable d(GGGGG).d(CCCCC) duplex

One might expect that the complementary d(GGGGG) and d(CCCCC) pentamers would associate to form a stable Watson-Crick duplex. Trying to prepare the duplex, we used the procedure of Marck and Thiele (1978) as described in Materials and Methods. Fig. 6 (*left*) shows CD spectra of mixtures of single-stranded $d(G_5)$ with various amounts of single-stranded $d(C_5)$ measured immediately after mixing. The spectra can be reproduced by a linear combination of three reference CD spectra corresponding to single-stranded $d(C_5)$, single-stranded $d(G_5)$, and the $d(G_5).d(C_5)$ duplex. The duplex naturally dominates at the 1:1 $d(G_5):d(C_5)$ ratio. Its spectrum has the same shape (Fig. 6, *insert a*) as that determined for poly(dG).poly(dC) (Marck and Thiele, 1978). The amplitudes are, however, about three times smaller.

The CD spectra of the $d(G_5)$ + $d(C_5)$ mixtures underwent extensive changes with time (Fig. 6, *insert b*). The equilibrium spectra, taken after 14 days (Fig. 6, *right*), were very different from those taken immediately after mixing (Fig. 6, *left*). They could be reproduced by linear combinations of the spectra corresponding to the duplex of $d(G_5).d(C_5)$, tetraplex of $d(G_5)$, and single-stranded $d(C_5)$ (Fig. 6, *insert c*). However, the duplex only contributed significantly in the excess of $d(C_5)$. At the $d(G_5)$-to-[$d(G_5)$ + $d(C_5)$] molar ratios higher than 0.2 (and $d(G_5)$ nucleotide residue concentrations higher than 1×10^{-5} M), the duplex population no longer increased because the added $d(G_5)$ strands preferred to associate into the tetraplex. At ratios higher than 0.4, the population of the duplex of $d(G_5).d(C_5)$ even started decreasing because the added $d(G_5)$ dissociated the duplex to allow for its $d(G_5)$ strands' association into the tetraplex (Fig. 6, *insert c*).

Tetraplex formation by $d(C_nG_n)$

The shapes and magnitudes of the CD spectra strongly depended on the DNA fragment concentration even with the

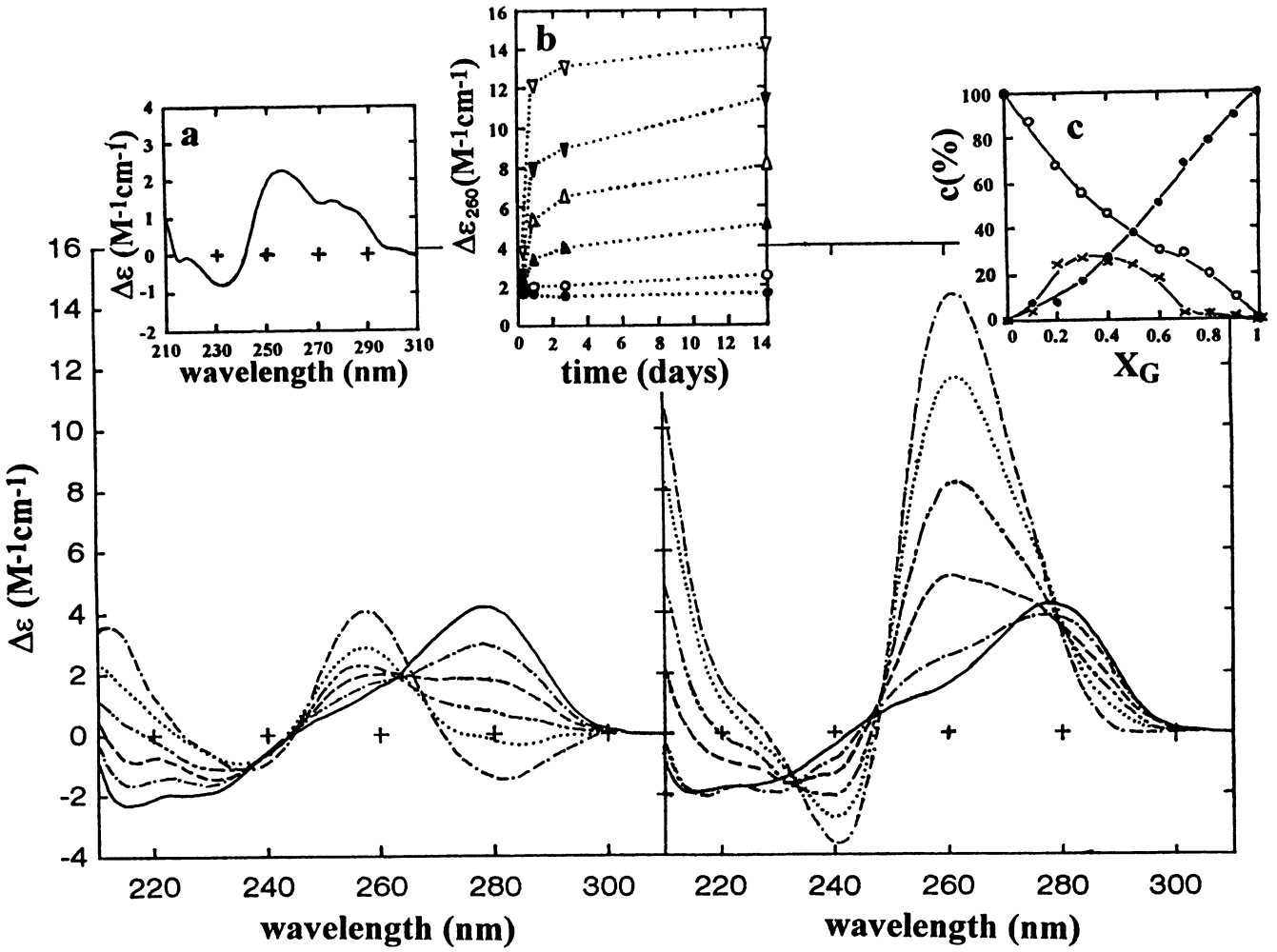

FIGURE 6 Interactions of d(GGGGG) with d(CCCCC). The mixtures of d(GGGGG) and d(CCCCC) were prepared as described in Materials and Methods. In mixed samples, the molar fraction X_G (the ratio of the d(GGGGG) concentration to the [d(GGGGG) + d(CCCCC)] concentration) was the following: (—) 0, (- · · · -) 0.2, (- - -) 0.4, (- ·· - ·· -) 0.6, (· · ·) 0.8, and (- · - · -) 1. The CD spectra were measured (*left*): immediately after mixing $d(G_5)$ with $d(C_5)$; (*right*) 14 days after mixing $d(G_5)$ with $d(C_5)$. Insert (*a*) calculated CD spectrum of the $d(G_5).d(C_5)$ duplex (see text). Insert (*b*) time dependence of the ellipticity at 260 nm of the $d(G_5)$ mixtures with $d(C_5)$. Molar fractions of d(GGGGG): ● 0, ○ 0.2, ▲ 0.4, △ 0.6, ▼ 0.8, and ▽ 1. Insert (*c*) percentage of ○ single-stranded d(CCCCC), × duplex of $d(G_5).d(C_5)$, and ● tetraplex of d(GGGGG) in mixed $d(G_5)+d(C_5)$ samples measured 14 days after their mixing.

self-complementary DNA fragments $d(G_3C_3)$, $d(C_3G_3)$, $d(G_5C_5)$, and $d(C_5G_5)$ (not shown) that apparently should associate to form stable WC duplexes. The concentration dependences forced us to apply the procedure eliminating the associations (see Materials and Methods) to get reproducible CD spectra with these oligonucleotides as well. All of the following experiments were carried out with these pretreated samples.

Repeated freezing/thawing almost did not change the CD spectra of $d(G_3C_3)$ and $d(G_5C_5)$ (Fig. 7). On the other hand, the amplitudes of the CD spectra of $d(C_3G_3)$ and $d(C_5G_5)$ increased with the number of the freeze/thaw cycles. The spectra had a two-state origin. These experiments suggested that the strands of $d(C_3G_3)$ and $d(C_5G_5)$ were tetraplexed. The tetraplex formation was comparable with $d(G_3)$, $d(CG_3)$, $d(C_2G_3)$, and $d(C_3G_3)$. The diagnostic CD band at 260 nm reached a plateau after ~12–15 freeze/thaw cycles with all four oligonucleotides. Thus, the presence of 5′ end dC did not significantly influence the guanine tetraplex formation. Eight cycles sufficed with $d(C_5G_5)$ and $d(G_5)$ (not shown).

Fig. 8 compares the CD spectra of the tetraplexes of d(GGG) and d(GGGC) (Fig. 8 *a*), and of the tetraplexes of d(GGG) and d(CGGG) (Fig. 8 *b*) after 15 freeze/thaw cycles. Here the DNA fragment ellipticities are given in their strand molarities to demonstrate the CD effects of the 3′ end and the 5′ end cytosine residues. The [d(GGGC)-d(GGG)] and [d(CGGG)-d(GGG)] difference CD spectra (Fig. 8, *a* and *b*) differ and, not surprisingly, both are different from the CD spectrum of the dC monomer (Cantor et al., 1970). However, the difference CD spectra of [d(CGGG)-d(GGG)] and [d(CCGGG)-d(CGGG)] (all three

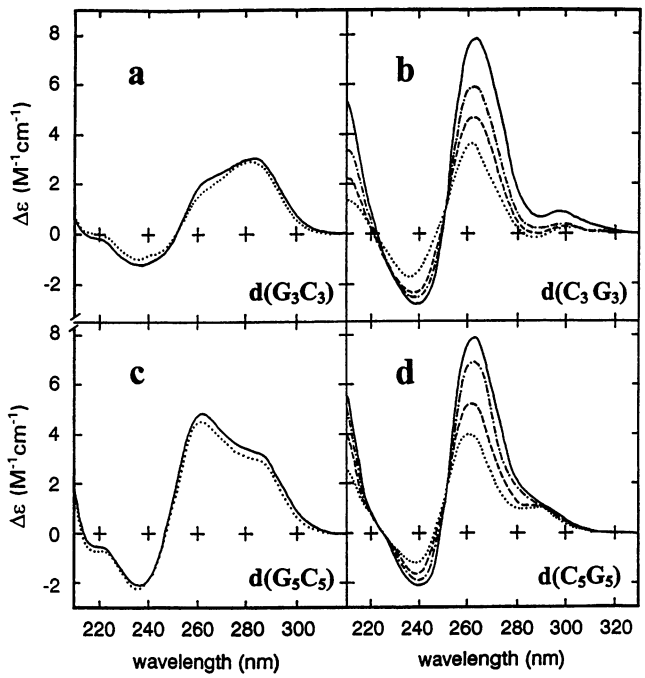

FIGURE 7 CD spectra of the indicated DNA fragments in 2 mM potassium buffer, pH 7.0, after repeated freezing/thawing (temperature 20°C). The numbers of freeze/thaw cycles (from the dotted to the bold spectrum) were the following: (*a*) $d(G_3C_3)$: 0, 15; (*b*) $d(C_3G_3)$: 0, 2, 4, 14; (*c*) $d(G_5C_5)$: 0, 15; (*d*) $d(C_5G_5)$: 0, 1, 3, 14.

samples were exposed to 15 freeze/thaw cycles) are fairly similar (not shown) so that the CD spectrum of the tetraplex of $d(G_3)$ plus twice the difference spectrum of the 5′ end dC gives the CD spectrum of the tetraplex of d(CCGGG) (Fig. 8 *d*). In other words, all of the dC residues in d(CGGG) and d(CCGGG) equally contribute to the CD spectrum of the tetraplex formed by the three consecutive guanines at the 3′ end of the DNA fragment. Even the CD spectra of $d(C_3G_3)$ and $d(C_5G_5)$ (Fig. 8, *f* and *h*) can reasonably be reproduced by a sum of the CD spectrum of the G-tetraplex plus three or five times the CD spectrum of the 5′ end cytosine. Hence all of the cytosines of $d(C_nG_n)$ have the same, presumably unstacked, geometry and do not influence the guanine tetraplex conformation. On the other hand, there are significant dissimilarities between the calculated and experimental CD spectra of $d(G_3C_2)$, $d(G_3C_3)$, and $d(G_5C_5)$ (Fig. 8, *c*, *e*, and *g*).

DISCUSSION

The guanine tetraplex has been extensively studied in the last decade. However, the analyzed DNA fragments mostly contained guanine, thymine, and adenine. In contrast, studies of the guanine tetraplex formed by (G+C)-rich DNA fragments have been much less abundant (Hardin et al., 1992, 1993; Goodlett et al., 1993; Fry and Loeb, 1994; Deng and Braunlin, 1995; Usdin and Woodford, 1995; Kettani et al., 1995; Chen, 1995), although (G+C)-rich sequences exist inside genomic DNAs, have adopted unusual conformations, and may play important biological functions (e.g., Nickol and Felsenfeld, 1983; Akman et al., 1991; Chung and Muller, 1991; Murchie and Lilley, 1992; Wang et al., 1992; Hammond-Kosack et al., 1992; Traub et al., 1992; Fry and Loeb, 1994; Woodford et al., 1994; Kettani et al., 1995; Usdin and Woodford, 1995; MacLeod et al., 1996).

Recently we have started systematic studies of (G+C)-rich DNA fragments (Vorlíčková et al., 1996) to understand their conformational polymorphism in which the G-tetraplex plays a significant role. First of all, we had to find a reproducible procedure of the G-tetraplex stabilization as an exclusive conformer as well as a procedure for its total dissociation. Both procedures are described here. Secondly, we had to analyze the suitability of methods to study conformational polymorphism of the (G+C)-rich DNA fragments. Polyacrylamide gel electrophoresis has been used in many published papers describing tetraplex formation by (G+T)-rich sequences, but it is unsuitable for (G+C) DNA fragments as short as we used in this study. Fortunately, CD spectroscopy proved to be self-sufficient here because of the parallel use of several oligonucleotides whose sequences were systematically varied. Nevertheless, we had to be careful to search for isoelliptic points to discriminate between separate processes and, at the same time, patient enough to wait even weeks until some of the present samples attained the thermodynamic equilibrium.

This paper demonstrates that repeated freezing/thawing is a suitable approach for an exclusive tetraplex induction with the (G+C)-rich DNA fragments. KCl is the most frequently used inducer of the tetraplex in the literature, but it stabilizes other structures as well with the present (G+C) oligonucleotides. KCl presumably stabilizes WC pairing between G and C, which then interferes with tetraplex formation and contributes to the complex association behavior of the (G+C) oligonucleotides. In contrast, tetraplex stabilization by freezing is a two-state process because single strands do not interact at low ionic strength and directly associate to form tetraplexes upon freezing (Fig. 5) that concentrates DNA in cavities generated in the ice (Mizoguchi, 1993).

Repeated freezing/thawing induces tetraplexes even with the self-complementary $d(C_3G_3)$ and $d(C_5G_5)$. In contrast, no tetraplexes were formed by $d(G_3C_3)$ and $d(G_5C_5)$, while Deng and Braunlin (1995) observed a tetraplex with $d(G_4C_4)$ at physiological concentrations of KCl and at 2 mM strand concentration. At this high DNA concentration, $d(G_3C_3)$ and $d(G_5C_5)$ also generated high-molecular associations in our experiments. On the other hand, it is not excluded that $d(G_4C_4)$ adopts the tetraplex more easily than $d(G_3C_3)$ and $d(G_5C_5)$ because the tetraplex stability is not a monotonic function of the $d(C_nG_n)$ length (Vorlíčková et al., unpublished results). This aspect is being investigated in our laboratory.

The DNA fragment $d(C_3G_3)$ has unusual NMR properties (Wolk et al., 1989). Perhaps the participation of a fraction of $d(C_3G_3)$ strands in the G-tetraplex at the high DNA con-

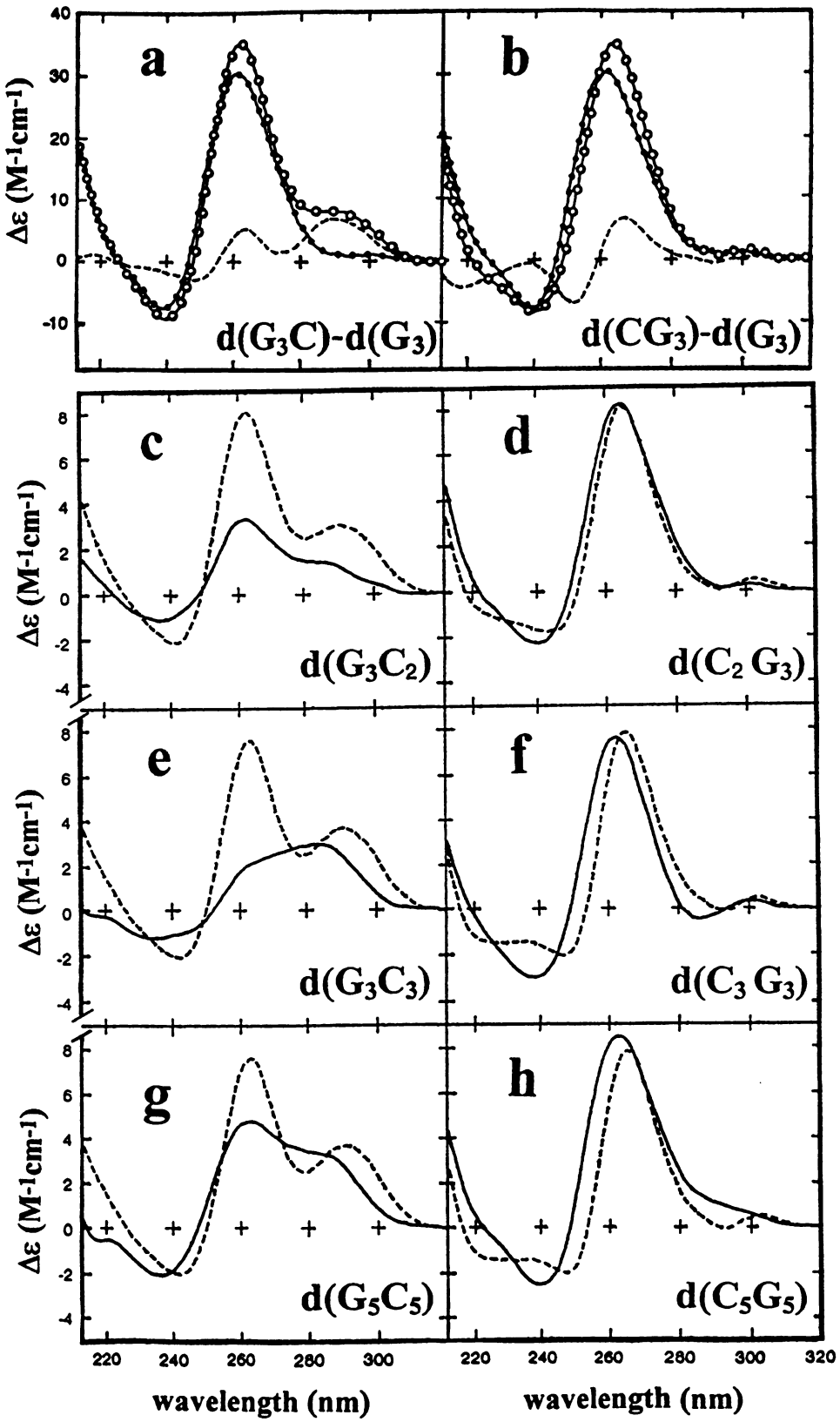

FIGURE 8 Measured (at 20°C) and calculated CD spectra, and difference CD spectra of the indicated DNA fragments dissolved in 2 mM potassium phosphate buffer, pH 7.0, after fifteen freeze/thaw cycles: (*a*) (-●-●-) d(GGG), (-O-O-) d(GGGC), and (- - -) their difference spectrum; (*b*) (-●-●-) d(GGG), (-O-O-) d(CGGG), and (- - -) their difference spectrum. Ellipticities in (*a*) and (*b*) are expressed in DNA fragment strand molarities. (*c–h*) (—) measured CD spectra of DNA fragments and (- - -) their CD spectra calculated as explained in the text (given in their base molarities).

centrations used in the NMR experiments accounts for this behavior. We had an opposite problem, i.e., to eliminate a possibility that a fraction of $d(C_3G_3)$ or $d(C_5G_5)$ participates in a duplex after the limiting number of the freeze/thaw cycles. We also asked whether the tetraplex of $d(C_3G_3)$ was the same as that formed by $d(G_3)$ and what was the 5′ end

cytosine conformation in the tetrads of the $d(C_3G_3)$ molecules. Fig. 8 provides an answer. The CD spectra of $d(C_3G_3)$ and $d(C_5G_5)$ (measured after the limiting number of the freeze/thaw cycles) were reproduced by a sum of the CD spectrum of the tetraplex of $d(G_3)$ or $d(G_5)$ plus the CD spectrum of the 5′ end cytosine residues multiplied by the number of the cytosine residues at the 5′ end parts of the oligonucleotides. This indicates that the tetraplex is the only conformer formed by these self-complementary DNA fragments after the limiting number of the freeze/thaw cycles. The tetraplex only involves the guanines while the 5′ end cytosine residues influence neither the G-tetraplex formation nor its structure.

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