Structural Switch from Hairpin to Duplex/Antiparallel G-Quadruplex at Single-Nucleotide Polymorphism (SNP) Site of Human Apolipoprotein E (APOE) Gene Coding Region

Swati Chaudhary,† Mahima Kaushik,‡ Saami Ahmed,† Ritushree Kukretil,§ and Shrikant Kukretil,†§

†Nucleic Acids Research Lab, Department of Chemistry and ‡Cluster Innovation Center, University of Delhi, Delhi 110007, India
§CSIR-Institute of genomics and Integrative Biology, Delhi 110007, India

ABSTRACT: A gradual dementia, which leads to the loss of memory and intellectual abilities, is the main characteristics of Alzheimer’s disease. Amyloid-β (Aβ) plaques are the main components that accumulate and form clumps in the brains of people suffering from Alzheimer’s disease. Apolipoprotein E (APOE), an amyloid-binding protein is considered as one of the main genetic risk factor of the late-onset Alzheimer’s disease. Different isoforms of APOE gene named APOE2, APOE3, and APOE4 are known to exist, which differ from each other at certain positions involving single-nucleotide polymorphisms (SNPs). Out of these isoforms, APOE4 increases the risk of developing late-onset Alzheimer’s disease, whereas APOE3 is the most common among the general population. APOE4 differs from the common APOE3 by only one nucleotide at position +2985 (T to C), which results in immense alteration in the structure and function of the APOE gene. A combination of gel electrophoresis (polyacrylamide gel electrophoresis, PAGE), circular dichroism (CD), CD melting, thermal difference spectra and UV-thermal denaturation (Tm) techniques was used to investigate the structural polymorphism associated with T → C single-nucleotide polymorphism (SNP) at the GC-rich sequence (d-TGGAGGACGTG. This toxic Aβ protein is known to play a major role in the clearance of Aβ. APOE gene exists in three different isoforms ε2, ε3, and ε4 (APOE2, APOE3, and APOE4) that are very well studied till now. It has been reported that ε4 allele of the APOE gene increases the risk of developing late-onset Alzheimer’s disease, whereas ε3 is the most common among the general population.5,7 Frequency of ε2, ε3, and ε4 APOE alleles was found to be approximately 8.4, 77.9, and 13.7%, respectively, worldwide. But in Alzheimer’s patients, the frequency of ε4 increases dramatically up to 40%.1

Several reports suggest that APOE4 disturbs the clearance mechanism of Aβ, resulting in higher number of Aβ plaques in the brain.6,9 Another report evidently shows that it is not the binding that causes the build-up of Aβ plaques, but APOE blocks the pathway involved in the cellular degradation and clearance of Aβ from the brain by binding to the receptor, lipoprotein receptor-related protein 1 (LRP1). Aβ also competes for the same receptor to enter the clearance pathway.10

INTRODUCTION

Alzheimer’s disease (AD) is a neurodegenerative disease that has been explored as a most common form of dementia, resulting in the depletion of memory due to loss of brain cells and intellectual abilities among adults aged 65 or above.1 In United States, AD is considered the sixth main cause of all deaths, and the death rate increased up to 66% between 2000 and 2008.9 Various reports suggest that instability in the brain takes place between the production and clearance of amyloid-β (Aβ) peptides, which leads to the accumulation and aggregation of toxic Aβ. This toxic Aβ destroys synapses and results in neurodegeneration and dementia.2,5

Most of the cases of Alzheimer’s disease occurs after 65 years of age and hence it is known as late-onset Alzheimer’s disease (LOAD). According to early reports, apolipoprotein E (APOE) gene is the major risk factor that has been associated with LOAD.3,5 It also helps in maintaining normal cholesterol levels by combining with lipids to form lipoproteins that remove excess cholesterol from the blood. Increased cholesterol levels have been found to be associated with plaques in the brain of the AD patient. High accumulation of Aβ has been observed in rabbits taking high-cholesterol diets. On the other hand, low cholesterol level inhibits the generation of Aβ in mice and guinea pigs.6 APOE protein is known to play a major role in the clearance of Aβ. APOE gene exists in three different isoforms ε2, ε3, and ε4 (APOE2, APOE3, and APOE4) that are very well studied till now. It has been reported that ε4 allele of the APOE gene increases the risk of developing late-onset Alzheimer’s disease, whereas ε3 is the most common among the general population.5,7 Frequency of ε2, ε3, and ε4 APOE alleles was found to be approximately 8.4, 77.9, and 13.7%, respectively, worldwide. But in Alzheimer’s patients, the frequency of ε4 increases dramatically up to 40%.1

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All of the three APOE isoforms differ by only two nucleotide bases at positions +2985 (T/C) and +3123 (C/T). APOE3 contains thymine residue at position +2985 and cytosine residue at position +3123. Moreover, the polymorphic version APOE4 differs from the common ε3 type by only one nucleotide base change at position +2985. It contains cytosine residue at position +2985 defined by SNP rs429358, whereas APOE2 is defined by SNP rs7412, which contains thymine residue at position +3123. These changes can immensely alter the structure and function of APOE gene and modify its ability to bind lipids, receptors, and Aβ. It has become extremely essential to gather more knowledge about the modifications, which occur in the mechanism involved in the regulation of APOE gene, due to single-nucleotide polymorphism (SNP) site.

RESULTS AND DISCUSSION

In this paper, bioinformatic, biochemical, and biophysical studies of 22-mer sequences (APOE22T, d-TGGAGGACGTGCGCGGCCGCCT and its SNP version APOE22C, d-TGGAGGACGTGCGCGGCCGCCT) have been carried out for their structural determination. Bioinformatic analysis revealed that APOE22T sequence is present in vivo, and an in-depth knowledge of their structural and physical properties is of crucial importance. Keeping in mind the above facts, we have aimed to investigate the effect of SNP on the secondary structures adapted by APOE3 and APOE4 polymorphic sequences. Using biophysical and biochemical methods, we studied a 22-mer sequence [d(TGGAGGACGTGCGCGGCCGCCT); APOE22T, d-TGGAGGACGTGCGCGGCCGCCT] found in the coding region (exon 4) of APOE gene representing ε3 allele and its SNP counterpart [d-TGGAGGACGTGCGCGGCCGCCT]; APOE22C] representing ε4 allele.
in the Exon 4 region of APOE gene. The diagrammatic representation of three isoforms of APOE gene is shown in Figure 1. In this report, we investigated the structures formed by the sequences APOE22T and APOE22C under physiological pH and salt conditions. The hairpin and antiparallel bimolecular G-quadruplex structural models were proposed for APOE22T at different oligomer concentrations, whereas APOE22C was found to form hairpin as well as duplex structures at all of the oligomer concentrations.

Nondenaturating Gel Electrophoresis. The molecularity of the DNA structures can be established by carrying out native polyacrylamide gel electrophoresis. A 15% gel electrophoretogram having the DNA samples of APOE22T as well as APOE22C along with their duplexes was run in 20 mM sodium cacodylate buffer (pH 7.4) containing 100 mM NaCl and 0.1 mM ethylenediaminetetraacetic acid (EDTA, Figure 2a). 12-, 24-, and 70-mer perfect duplex, moving parallel to the slower band of APOE22C (lane 1), and [M35 + M35c] (lane 6) migrate as 12-, 24-, and 70-mer perfect duplex, respectively. The electrophoretogram showed a single band for APOE22T at 10, 20, and 30 μM (lanes 2, 3, and 4, respectively) oligomer concentrations, which migrated slightly faster than the 12-mer duplex of PAL12, suggesting the presence of an intramolecular species or a hairpin structure. Quite interestingly, as the strand concentration reached 40 μM (lane 5), two bands were observed. The mobility of both the bands of APOE22T was slightly retarded than that of the bands of PAL12 and PAL24, respectively. This confirmed the formation of a hairpin and a bimolecular structure by APOE22T at a higher oligomer concentration, i.e., 40 μM.

Similarly, Figure 2c depicts the gel electrophoretogram of APOE22C in increasing (from 10, 20, 30, and 40 μM) oligomer concentration. It is clear from the figure that there is no change in the structural status of APOE22C at various oligomer concentrations, as it showed two bands exactly similar to that of the previous gel (Figure 2a, lane 3). These two bands suggested the formation of a hairpin (fast-moving band) and a duplex (slow-moving band) structure.

For confirming the exact status of the secondary structures adapted by studied DNA sequences, circular dichroism and UV-thermal melting studies were also performed, which have been discussed in the following sections.

Circular Dichroism Studies. The CD spectra of the studied DNA oligonucleotides were recorded to study the conformation adopted by the secondary structures of these sequences. Figure 3a shows the CD spectra of APOE22T and APOE22C at 3 μM strand concentration in 20 mM sodium cacodylate buffer (pH 7.4) comprising 100 mM NaCl and 0.1 mM EDTA. Both APOE22T and APOE22C displayed a strong positive peak at 287 nm and a strong negative peak at 249 nm, which are the characteristic CD signatures of B-form of DNA.

Oligomer Concentration Dependence in CD Studies. Because the results obtained from oligomer dependence in gel electrophoresis experiments suggested the formation of a new structure at a high oligomer concentration in case of APOE22T, further CD experiments were performed to get more clarity on the same. The CD spectra of APOE22T and APOE22C in 20 mM sodium cacodylate buffer (pH 7.4) containing 100 mM NaCl.

Figure 2b shows the effect of increasing (from 10, 20, 30, and 40 μM) oligomer concentration of APOE22T on its secondary structure. PAL12 (fast-moving band, lane 1), PAL24 (slow-moving band, lane 1), and [M35 + M35c] (lane 6) migrate as 12-, 24-, and 70-mer perfect duplex, respectively. The electrophoretogram showed a single band for APOE22T at 10, 20, and 30 μM (lanes 2, 3, and 4, respectively) oligomer concentrations, which migrated slightly faster than the 12-mer duplex of PAL12, suggesting the presence of an intramolecular species or a hairpin structure. Quite interestingly, as the strand concentration reached 40 μM (lane 5), two bands were observed. The mobility of both the bands of APOE22T was slightly retarded than that of the bands of PAL12 and PAL24, respectively. This confirmed the formation of a hairpin and a bimolecular structure by APOE22T at a higher oligomer concentration, i.e., 40 μM.

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For confirming the exact status of the secondary structures adapted by studied DNA sequences, circular dichroism and UV-thermal melting studies were also performed, which have been discussed in the following sections.
NaCl and 0.1 mM EDTA were recorded at the increasing oligomer concentrations of 4, 10, 20, and 30 μM and are displayed in Figure 3b,c, respectively. With the increase in the oligomer concentration, the CD spectra of APOE22T in Figure 3b shows an appearance of new positive peak around 295 nm at higher oligomer concentrations, which is a characteristic of the antiparallel guanine quadruplex. Gel experiments from previous section indicated that at a higher oligomer concentration of APOE22T, two species exist. The new species was suggested to have a bimolecular structure, which might be guanine quadruplex with antiparallel conformation, as indicated by the CD studies also.

Figure 3c shows the CD spectra of APOE22C at different oligomer concentrations, which indicates an increase in the ellipticity of positive peak at 286 nm with the increase in the oligomer concentration from 4 to 20 μM, signifying an increment in the population of the hairpin as well as duplex structure formed by the APOE22C sequence. At 30 μM oligomer concentration, shift in the wavelength from 286 to 289 nm can be seen along with the increase in the ellipticity. This indicates that at a higher oligomer concentration, the duplex structure is more in population as compared to hairpin structure and is predominantly a B-form of duplex.

CD Melting Studies of APOE22T. Denaturation of DNA can also be investigated by studying the temperature-induced changes in CD, which is known as CD melting. The CD melting profiles of APOE22T at lower (4 μM) and higher (30 μM) oligomer concentration were investigated at wavelengths 286 and 295 nm, respectively, which displayed maximum ellipticity in Figure 3b. The samples were prepared in 20 mM sodium cacodylate buffer (pH 7.4), 100 mM NaCl, and 0.1 mM EDTA solution.

Figure 4 displays the monophasic melting profiles for APOE22T at 4 and 30 μM oligomer concentrations with constant Tm value 72 °C. No significant difference was observed between the melting profiles of APOE22T at lower and higher oligomer concentrations. This observation suggests the melting of a unimolecular hairpin structure, which shows the oligomer concentration independence in melting (similar results were obtained in case of UV-thermal melting too; Figure 6b). The melting of G-quadruplex structure could not be observed in this CD melting, which may be because of the very low concentration of G-quadruplex species in the solution.

UV-Thermal Melting Studies. By utilizing UV spectroscopy, the thermal melting studies were carried out, which describe the melting stability of the sequences under study. An absorbance versus temperature profile was recorded at 260 nm to determine the melting temperature (Tm) at half of the DNA structure unwind. Figure 5a,b shows the denaturation curves attributed to APOE22T and APOE22C, respectively, in 20 mM sodium cacodylate buffer (pH 7.4) having 100 mM NaCl and 0.1 mM EDTA. The melting curve of APOE22T at 3 μM strand concentration is monophasic, indicating the unwinding of single structural species and its Tm value of 73 °C, which was determined by first derivative curve. The monophase melting profile of APOE22T correlates well with the gel electrophoresis study (showing only one band for the sequence), thus suggesting the formation of a hairpin structure only. Further, the melting curve of APOE22C also displayed a monophasic curve with Tm value of 72 °C.

The gel experiment (Figure 2) demonstrated the presence of two structural species of variable molecularity, but the biphasic thermal transition was not observed in UV melting studies. On correlating gel and thermal studies, it can be inferred that the Tm of 72 °C might be due to the denaturation of an intramolecular species, i.e., hairpin structure. It can also be assumed that the duplex structure (slow-moving band of lane 3, Figure 2) formed by APOE22C might be low in concentration as compared to the hairpin structure (major species) at low oligomer concentration of sequence in thermal melting experiments. As a consequence, a prominent Tm curve is not detected for the denaturation of minor species at a low strand concentration.

Oligomer Concentration Dependence of APOE22C. It is clear from the thermal melting experiments discussed above (Figure 5a,b) that the melting of bimolecular structure could not be detected at low strand concentration, so an oligomer concentration dependence in UV-thermal melting was carried out to investigate the formation of the bimolecular structure by APOE22C. Similar buffer conditions were employed to carry out the oligomer concentration dependence studies. The melting profiles displayed in Figure 6a are of APOE22C at various strand concentrations. The thermal melting curves were monophasic at low strand concentration, but as the oligomer concentration increased, the monophasic curve turned into biphasic transition, revealing the melting of two different species. The Tm of lower temperature transition was increased with increase in oligomer concentration (from 4 to 30 μM), whereas the Tm of higher transition remained constant. The lower transition was found to be highly dependent on oligomer concentration, whereas the upper transition was independent of oligomer concentration. This also confirmed that the lower transition is due to an intermolecular species, i.e., duplex, whereas the upper transition is due to the thermal melting of an intramolecular species, i.e., hairpin. It can be clearly seen that the melting curve at 30 μM strand concentration was distinctly biphasic, having the Tm values of 50 °C (lower transition) and 73 °C (upper transition). The lower transition corresponds to the bimolecular structure, whereas the upper melting transition was attributed to the hairpin structure. The heating as well as cooling curves of APOE22C were monitored at 260 nm to establish the existence of thermodynamically stable species. The heating and cooling curves depicted in Figure 7c,d are found to be superimposed on each other (absence of hysteresis), which
show that the structures formed by APOE22C sequence are thermodynamically stable.

**Oligomer Concentration Dependence of APOE22T.** To confirm the molecularity and structures of the secondary structures, oligomer concentration dependence was also performed in UV-thermal melting experiments. Because the melting of bimolecular structure could not be detected at low strand concentrations, so an oligomer concentration dependence study was carried out on APOE22T sequence in 20 mM sodium cacodylate buffer (pH 7.4), 100 mM NaCl, and 0.1 mM EDTA solution. Figure 6b shows the thermal melting profiles of APOE22T at 260 nm. At low oligomer concentration (4 μM), a monophasic curve is obtained at 260 nm with a melting temperature of 73 °C. With an increase in oligomer concentration from 4 to 10, 20, and 30 μM, biphasic curves were observed, indicating the presence of two different structures. It signifies that APOE22T forms a unimolecular (hairpin) structure at low oligomer concentration and the sequence folds to form hairpin as well as bimolecular structures as the concentration is increased further. The melting
temperature of the hairpin structure (upper transition) does not change with increasing oligomer concentration. On the contrary, the melting temperature (T_M) of the bimolecular structure increased from 45 to 58 °C with an increase in oligomer concentration from 10 to 30 μM, respectively.

Also, most significantly, all of the melting profiles of APOE22T were found to be inverted at 295 nm, indicating the formation of a G-quadruplex structure at higher oligomer concentration (Figure 6c). On correlating all of the experimental results, it was concluded that the APOE22T adapted a bimolecular antiparallel G-quadruplex structure at a high oligomer concentration. Here also, the heating as well as cooling curves of APOE22T were monitored at 260 nm wavelength and are shown in Figure 7a,b. The phenomenon of hysteresis was detected in APOE22T, which shows that the G-quadruplex structure formed by APOE22T at a higher oligomer concentration is thermodynamically unstable. At 295 nm, the inverted melting profiles (heating and cooling) are not superimposed, supporting the presence of hysteresis (data not shown).

**Thermal Difference Spectra (TDS).** Thermal Difference Spectra (TDS) were investigated to obtain an insight into the structure adopted by the studied DNA sequences. The difference in the absorbance spectra at high and low temperatures gives a specific signature for DNA secondary structure. Figure 8 shows the TDS spectra of APOE22T and APOE22C at low (4 μM) and high (30 μM) oligomer concentrations. The positive peaks around 275 and 240 nm are obtained in case of APOE22T at 4 μM concentration, which is a specific TDS signature for the DNA duplex with 100% GC content.16 The TDS spectra of APOE22T (4 μM) correlates well with other biophysical and biochemical studies. On the basis of that finding, it was concluded that the sequence forms a hairpin structure at low oligomer concentration. However, at high oligomer concentration (30 μM) of APOE22T, positive peaks around 275 and 243 nm are obtained, matching with the
TDS signature of G-quadruplex. The TDS profile of APOE22T at a higher oligomer concentration does not show a negative peak around 295 nm, which might be due to the long loops of the quadruplex formed. Similarly, Mergny et al. (2010) demonstrated that a sequence containing T15 in the loop (E3E) showed a very small negative peak at 295 nm in the TDS spectra. G-quadruplex structure formed by E3E sequence contains a large number of residues in the loop, causing a very low magnitude of the negative peak at 295 nm in TDS spectra.

Apart from this, CD and T_{M} studies show that the stability of bimolecular G-quadruplex structure (minor species) is very low at low oligomer concentration. The proposed structural model (Figure 9) shows that the bimolecular G-quadruplex structure involves only two G-tetrads, which are also attributed to its lower stability. We conclude that due to low concentrations and less thermal stability of G-quadruplex structure, detecting the significant TDS spectra for G-quadruplex structure was not possible.

The TDS spectra of APOE22C at low (4 μM) and high (30 μM) oligomer concentrations give positive peaks around 275 and 237 nm (Figure 8), exhibiting the presence of a DNA duplex-like structure. The TDS spectra of APOE22C correlate well with other experimental results demonstrating the formation of hairpin as well as antiparallel duplex structures.

Proposed Structures for the Sequences. A significant correlation had been noticed in the gel electrophoresis, circular dichroism, and thermal melting studies of the sequences under study. Structural models of hairpin and duplex structures for APOE22T and APOE22C sequences are illustrated in Figure 9. APOE22T oligomer folds back to form a hairpin structure containing six G−C base pairs in the stem and the tetranucleotide −TGTG− in the loop. The loop region containing four to five unpaired bases has been reported to increase the stability as compared to smaller or larger loops. DNA hairpins containing four to five nucleotides in the loop region have the potential to increase the stacking interactions in addition to decreased steric hindrance.

The presence of a G−C base pair adjacent to the loop contributes to the overall stability of the hairpin structure containing two or three bases in the loop region. Kaushik et al. in 2003 have even reported the formation of a hairpin structure with one base loop by a quasi-palindromic sequence. In addition to the hairpin structure, APOE22T formed an antiparallel bimolecular G-quadruplex structure, which has two guanine quartets, at a high oligomer concentration (Figure 9). It is already reported that the G-quadruplex structures with three G-tetrads are more favorable as compared to G-quadruplex containing two G-tetrads, but several reports have investigated the formation of G-quadruplex structures involving even only two G-tetrads.
attributed to six Watson–Crick hydrogen bonds and two guanine–guanine interactions.

On the other hand, in the SNP version, APOE22C exists in two structural forms, hairpin and intermolecular duplex. The hairpin structure obtained for APOE22C comprises a tetranucleotide loop having TGGC bases. The single base change (from T to C) does not affect the stability of the hairpin structure to a large extent, but an additional antiparallel duplex structure is formed in the APOE22C sequence, which has cytosine in place of thymine. The C-counterpart of the sequence results in an increase in the G−C base pairs, leading to the formation of a duplex containing fourteen G−C base pairs and six mismatched base pairs.

**CONCLUSIONS**

In the present report, ε3 and ε4 isoforms of APOE gene at the SNP site were investigated to see if the structural difference at gene level is also reflected at nucleotide level. The knowledge may give insight into the occurrence of late-onset Alzheimer’s disease. With the help of several biophysical and biochemical techniques such as gel electrophoresis, circular dichroism, and thermal melting studies, it was concluded that APOE22T forms a stable hairpin structure at low oligomer concentration, whereas it adapts an antiparallel quadruplex at higher concentrations. In contrast, its C-counterpart, APOE22C, where the formation of hairpin structure and do not show the significant signature of G-quadruplex at a high oligomer concentration. The proposed structural models correlate well with the biochemical and biophysical studies performed in this paper.

**MATERIALS AND METHOD**

The DNA oligonucleotides were purchased from Bio Basic Inc., Canada, as PAGE purified lyophilized powder and synthesized on the 1 μmol scale. These DNA oligonucleotides were stored at −20 °C. The nearest-neighbor method was employed to calculate the extinction coefficient (ε) followed by the determination of the concentration of oligonucleotides spectrophotometrically by measuring the absorbance at 260 nm. To prepare the stock solutions of the oligomers used, lyophilized powder was directly dissolved in the MilliQ water. All of the other chemicals were of analytical grade and purchased from Sigma Chemicals. The DNA oligomers used under the present study and their complementary sequences along with their extinction coefficients are listed in Table 1.

**Nondenaturating Gel Electrophoresis**. Nondenaturing gel electrophoresis was performed with the oligonucleotide samples in 20 mM sodium cacodylate buffer (pH 7.4) and 0.1 mM EDTA containing 100 mM NaCl. The final volume of the sample was 20 μL. Furthermore, to check the purity of these oligomers, a 20% denaturating PAGE using 7 M urea was made to run before carrying out the native gel studies. All of the studied DNA oligonucleotides migrated as single-stranded species in denaturing PAGE, confirming the 100% purity of the sample. To carry out the nondenaturating gel experiments, the samples were heated at 95 °C for 5 min and slowly cooled to room temperature. The oligonucleotide (at 10 μM strand concentration) samples were incubated at 4 °C overnight before loading onto 15% polyacrylamide gel. The gel was pre-equilibrated at 4 °C for about 1 h. The gel comprises 20 mM sodium cacodylate buffer (pH 7.4) with 100 mM NaCl and 0.1 mM EDTA. On the other hand, the running buffer contained 1× TBE with the same concentration of NaCl and EDTA. The tracking dye consisted of an equimolar mixture of orange G and glycerol. The gels were run at a constant voltage of 65 V in a cold room (~4 °C). After electrophoresis, the gel was stained with stains-all solution and visualized under white light and photographed by Alphalager 2200 (Alpha InfotechCorpn.).

**Circular Dichroism**. To gather information about conformational status of APOE22T and APOE22C oligonucleotides, circular dichroism (CD) spectroscopy was used. The CD spectra were recorded on a JASCO-815 spectropolarimeter. An average of three scans was taken over a wavelength range of 200−320 nm at a scanning rate of 100 nm/min. The buffer solution was scanned alone at room temperature and subtracted from the average scans for each oligonucleotide sample. The

| s. no. | DNA sequences | ex. coef. (M−1 cm−1) |
|-------|---------------|----------------------|
| 1     | (APOE22T); d-TGGAGGACGTCGTCGCACTTCCTCA | 202 000 |
| 2     | (APOE22Tc); d-AGGCGGCCGCAACGTCGCTCCCA | 201 200 |
| 3     | (APOE22C); d-TGGAGGACGTCGTCGCACTTCCTCA | 199 900 |
| 4     | (APOE22Cc); d-AGGCGGCCGCAACGTCGCTCCCA | 198 300 |
| 5     | (PAL12); d-CTTGAGCTCAAG | 113 700 |
| 6     | (PAL24); d-CTTGAGCTCAAG | 226 100 |
| 7     | (M35); d-GACTGACTTAAGCGCATAGCTAGCTAGCTCGATAGCTGA | 342 600 |
| 8     | (M35c); d-TCACTCTACGACTGACTTAAGCGCATAGCTAGCTAGCTCGATAGCTGA | 333 100 |

“Signifies for control size markers in gel electrophoresis experiments.

Table 1. Various DNA Oligonucleotide Sequences Used in the Present Study

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samples were scanned in a 1 cm path length quartz cuvette of 1 mL volume capacity. The data were collected in units of millidegrees versus wavelength and normalized to the total DNA strand concentration. The CD spectroscopy was also used to investigate the melting temperature of the GC-rich sequence. The temperature versus ellipticity profiles were monitored by heating the samples from 5 to 95 °C at a rate of 0.5 °C/min.

**UV-Thermal Denaturation.** The UV-thermal denaturation experiments of DNA oligonucleotide sequences were performed on a UV-2450PC Shimadzu UV–visible spectrophotometer equipped with a Peltier thermo-programmer, TMS PC-8(E) 200, and interfaced with a Pentium IV computer for data collection and analysis. The stopped quartz cuvettes of 1 and 0.1 cm optical pathlength with 110 and 35 μL volume, respectively, were used for the measurements. The samples were prepared by taking a desired strand concentration. After that they were heated at 95 °C for 5 min followed by slow cooling and then kept overnight at 4 °C. The temperature versus the absorbance profiles were monitored at 265 nm by a stopped flow technique. The temperature was monitored at 265 nm by a stopped flow technique. The temperature was monitored at 265 nm by a stopped flow technique.

**Thermal Difference Spectra (TDS).** The TDS were recorded on a UV-2450PC Shimadzu UV–visible spectrophotometer. The stopped quartz cuvette of 35 μL volume and 0.1 cm path length was used for the experiment. The samples of desired concentrations were prepared by using 20 mM sodium cacodylate buffer (pH 7.4) containing 100 mM NaCl and 0.1 mM EDTA solution. After that, they were heated at 95 °C for 5 min followed by slow cooling and then kept overnight at 4 °C. The wavelength versus absorbance curves were monitored at 20 °C as well as at 90 °C. To obtain the TDS, the spectrum at 20 °C was subtracted from that at 90 °C.

**AUTHOR INFORMATION**

Corresponding Author
*E-mail: skukreti@chemistry.du.ac.in, shrikantrukukreti6@gmail.com. Tel: +91-11-27666726.*

ORCID

Swati Chaudhary: 0000-0003-0238-1217

**Notes**

The authors declare no competing financial interest.

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