Identification of G-quadruplex structures that possess transcriptional regulating functions in the *Dele* and *Cdc6* CpG islands

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**Abstract**

**Background:** G-quadruplex is a DNA secondary structure that has been shown to play an important role in biological systems. In a previous study, we identified 1998 G-quadruplex-forming sequences using a mouse CpG islands DNA microarray with a fluorescent-labeled G-quadruplex ligand. Among these putative G-quadruplex-forming sequences, G-quadruplex formation was verified for 10 randomly selected sequences by CD spectroscopy and DMS footprinting analysis. In this study, the biological function of the 10 G-quadruplex-forming sequences in the transcriptional regulation has been analyzed using a reporter assay.

**Results:** When G-quadruplex-forming sequences from the *Dele* and *Cdc6* genes have been cloned in reporter vectors carrying a minimal promoter and the luciferase gene, luciferase expression is activated. This has also been detected in experiments applying a promoterless reporter vector. Mutational analysis reveals that guanine bases, which form the G-tetrads, are important in the activation. In addition, the activation has been found to decrease by the telomestatin derivative L1H1-7OTD which can bind to the G-quadruplex DNA. When *Dele* and *Cdc6* CpG islands, containing the G-quadruplex-forming sequence, have been cloned in the promoterless reporter vector, the luciferase expression is activated. Mutational analysis reveals that the expression level is decreased by mutation on *Dele* G-quadruplex; however, increased by mutation on *Cdc6* G-quadruplex.

**Conclusion:** *Dele* and *Cdc6* G-quadruplex formation is significant in the transcriptional regulation. *Dele* and *Cdc6* G-quadruplex DNA alone possess enhancer and promotor function. When studied in more complex CpG islands *Dele* G-quadruplex also demonstrates promotor activity, whereas *Cdc6* G-quadruplex may possess a dual function of transcriptional regulation.

**Keywords:** *Cdc6*, *Dele*, G-quadruplex, Transcriptional regulation, 7OTD

**Background**

Although genomic DNA usually adopts the canonical double helix structure [1–3], formation of non-canonical DNA structures, such as G-quadruplex (G4) DNA and i-motif, have also been found in human genomic DNA [2, 4, 5]. G4 is a DNA secondary structure that consists of two or more planar guanine tetrads and is stabilized by Hoogsteen hydrogen bonds along with a monovalent cation [3, 6–10]. DNA strand orientation and length of the loop sequences connecting the guanine runs provide a structural diversity to G4 forms, such as parallel, antiparallel, or a mixed structure [2, 9–12]. The characteristic topologies and existence of G4 in significant locations, such as in the telomeres and several promoters, suggest potential functions of G4 structures in gene regulation [13].

G4-forming sequences in promoter regions have been found to play a functional role in the suppression of proto-oncogenes [2, 14], such as *c-MYC* [15–18], *BCL-2*
[19], VEGF [20], and RET [21]. Several studies on the G4-forming sequence within the nuclease hypersensitivity element III1 (NHEIII1) of the c-MYC promoter have reported that mutating the G4-forming sequence destabilizes the G4 structure, resulting in a three-fold increase in basal transcriptional activity of the c-MYC promoter [15, 16]. Binding of the c-MYC G4 to the cationic porphyrin TMPyP4 resulted in repression of the promoter activity [17]. Nucleolin has been identified as the c-MYC G4 binding protein that represses c-MYC expression [18]. The BCL-2 gene contains a GC-rich region upstream of the P1 promoter that has been shown to be critically involved in the regulation of the BCL-2 gene expression. It has been demonstrated that three individual G4 structures can be formed in this GC-rich region [19, 22]. As for VEGF, the stabilization of G4 DNA by quinoline derivatives represses gene transcription and consequently causes angiogenesis inhibition [20]. In addition, G4 ligands, such as TMPyP4 and telomestatin, have been shown to stabilize the G4 structure of the RET proto-oncogene promoter and lead to the repression of gene expression [21].

On the other hand, there are few examples of the transcriptional activation by G4 formation. A recent study of p32 G4 structure that is located in the P1 promoter of the Bcl-2 gene has demonstrated that reduced transcription activity in the mutated vectors compared to the native vectors [23]. This result implicated a transcriptional activation by the G4 structure. Furthermore, in the insulin-linked polymorphic region (ILPR), G4 DNA is formed by a two-repeats of consensus sequence [24, 25], and the G-quartet formation was observed to activate transcription, where single/double mutation in the sequence has reduced promoter activity [26–28]. In addition, the G4-forming c-myb GGA repeat region has shown to play a contrast role of both a transcriptional repressor and an activator, where one or two deletions of (GGA)4 motifs have increased c-myb promoter activity, while the deletion of all three regions has eliminated the promoter activity [29]. The involvment of G4 structures in regulating the transcription process suggests that they may hold the key to new therapeutic approaches in numerous areas of human disease, including cancer [30].

A genome-wide in silico analysis has demonstrated that sequences with the potential to form G4 motifs are enriched near transcription start sites, in the telomeres, ribosomal DNA, immunoglobulin heavy-chain switch regions, and CpG islands (CGIs) [31–33], suggesting widespread regulatory influence of the G4 motifs [34]. In a previous study, we identified 1998 G4-forming sequences in mouse CGIs using a mouse CGI microarray with a fluorescent-labeled G4 ligand L1Cy5-7OTD [35]. Among the identified G4-forming sequences, CD spectroscopy and DMS footprinting analysis were performed on 10 randomly selected G4-forming sequences and the G4 structure formation was confirmed. In this study, our aim was to analyze the biological function of the 10 G4 DNAs as the in transcriptional regulation.

**Results**

**Dele and Cdc6 G4-forming sequences activate reporter gene expression**

The 10 G4 DNA sequences from Jard2, Foxa2, Med4, Chd4, Ntpcr, Bmi1, Wt1, Sp130, Cdc6, and Dele genes (Table 1; Additional file 1) have been cloned into the luciferase reporter vector with a minimal promoter containing a TATA-box promoter element. The sequences were 42–50 nucleotides long. The successful plasmid construction has been verified by gel shift experiments and sequencing of the reporter vector.

The _Dele_ G4-forming sequence was found on two divergently overlapping genes, _Dele_ and 1700086O06Rik, located on the mouse genome; therefore, _Dele_ G4 has been cloned into the luciferase vector in the forward and reverse direction (Dele-F G4 and Dele-R G4). The reporter vectors have been transfected into NIH3T3 cells and expressed luciferase activity is measured after 48 h of cultivation. In order to reduce experimental variability the measured enzyme activity has been normalized with respect to the pGL4.74 vector which is coding for Renilla luciferase. The relative luciferase activity with and without G-quadruplex forming sequences is evaluated for a minimum of three transfections. The results of this set of

| Name | Sequences (5’-3’) |
|------|------------------|
| Jard2 | GTGAGGCTAGCGGGGAGGTCTGGGGGGTTGGAGGAAGGGAAAATG |
| Dele | ATAGCCGCGATGGGGGGTTAGATCTGGGAAAGGGGGCAAGAGG |
| Foxa2 | GTCCAGGATGCTAGAGGGGTTGGGGGGAGGGTACCGGTAGAAGG |
| Chd4 | TAAAGAGGAGGGGACGGTATGAGGAGGGGGGGTTGGAGGT |
| Ntpcr | CTTGTGATGCTAGGGGGAGGGGAGGGGAGGGGAGGGGAGGG |
| Med4 | ACTTGCTGGGCTGGTGGGAGGGTACCCGGAAGGGGAGGG |
| Bmi1 | CATCCTTTTTTGGGGTGACGAGGGGAGGGGAGGGAGGG |
| Wt1 | AGGGAGATGGGGGCTGGCAATGAGGGGAGGGGAGGGGAGGG |
| Sp130 | AGGGAGATGGGGGCTGGCAATGAGGGGAGGGGAGGGGAGGG |
| Cdc6 | TGGGAGGGGGGGGGAGGGGAGGGGAGGGGAGGGGAGGG |

Guanine runs, which form the G-quadruplex structures, are shown in bold and mutation sites are underlined.
experiments are compiled in Fig. 1 and show rather high expression values for Cdc6, Dele-F and Dele-R.

Mutant vectors have also been constructed and investigated in order to verify that really the G4-forming sequence affects the transcriptional activity. Mutant-type DNAs have been designed by replacing one of the guanine triplets to thymines (Additional file 1). These guanines were reported to be strongly involved in G4 formation [35], which can be verified by CD measurements in this study (see below).

A comparative analysis of expression levels with the G4-forming sequences and the mutant sequences demonstrates a noticeable differences for Foxa2, Med4, Chd4, Ntpcr, Cdc6, Dele-F and, Dele-R, but no significant difference in Jard2, Bmi1, Wt1, and Sp130 (Fig. 1; Additional file 2). These results indicate that these DNA sequences possess an enhancer activity on the luciferase reporter vector, which is connected to the possibility to form specific G4 secondary structures. We focused on Cdc6 and Dele G4 DNAs because the G4 DNAs showed a remarkable high activation in wild-type G4 DNAs. Moreover in regards to the statistical analysis, the t-test between wild-type and mutant-type demonstrated that Cdc6 and Dele showed more significant difference (P < 0.0001) compared to Foxa2, Med2 (P < 0.01) and Chd4, Ntpcr (P < 0.001); therefore, we performed detail analysis of the Cdc6 and Dele G4 DNAs.

It has been reported that enhancers frequently do not only interact with promoters but also promoter–promoter interaction is feasible, indicating that promoter can also work as enhancer for other gene promoters [36]. To investigate whether Dele and Cdc6 G4 DNAs also possess a promoter activity, they have been cloned into a promoterless vector and then a reporter assay is performed. It is found that Dele-F, Dele-R and Cdc6 G4 DNAs activate luciferase expression in this system. Furthermore, the activation of protein expression is clearly decreased by thymine mutations in the G4 region (Fig. 2; Additional file 3). These results indicate that Dele and Cdc6 G4 DNAs have a role in transcriptional activation, both as a promoter and enhancer. In order to perform this function, the formation of secondary structures seems to be essential.

In order to evaluate the function of Dele and Cdc6 G4 DNAs in CpG islands (CGIs) that may contain more regulatory elements, the 696-bp DNA fragment containing 475-bp Cdc6 CGI and 555-bp DNA fragment containing 477-bp Dele CGI have been cloned into the promoterless vector to analyze the transcriptional activities. The results demonstrate that the luciferase activities have increased more than one order of magnitude by the cloning of Cdc6, Dele-F and Dele-R CGI DNAs (Fig. 3; Additional file 4). The luciferase activities are higher than that of G4 alone cloning vectors, indicating that the CGIs contain cis-regulatory elements outside of the G4 forming regions.

The transcriptional activity of the mutant-type of Cdc6 CGI DNA has been found higher than that of the wild-type. This result suggests that Cdc6 G4 may possess a dual function of transcriptional regulation, such as c-myc G4 [16], swinging in both ways as an activator and as a suppressor under the effect of transcriptional factors.

The transcriptional activity of the mutant-type of Dele-F CGI DNA has decreased compared to the wild-type.
In contrast to Dele-F, the transcriptional activity of the Dele-R CGI mutant-type remains similar to that of the wild-type. Two divergently overlapping genes, Dele and 1700086O06Rik, are located on the Dele CGI, suggesting that the Dele CGI may contain at least two regulatory sequences. Our results indicate that the Dele CGI contains a promoter for 1700086O06Rik, an insulator, and a promoter for Dele (that also possesses enhancer activity). The insulator sequence may have a blocking activity that prevents promoter-enhancer interaction [37]; therefore, the enhancer activity of the Dele G4 for 1700086O06Rik promoter has not been detected.

G4 ligand suppresses the transcriptional activation of Dele and Cdc6 G4 DNAs

Ligands which can bind to G-quadruplex structures can have different effect on the secondary DNA structure. Distortion or stabilization is often found depending on the binding mode of the ligand. Here the effect of a telomestatin derivative L1H1-7OTD has been investigated with respect to the effect of the G4 ligand on the transcriptional activity of the G4 DNA. This ligand can bind to the top G-tetrad structure through π-stacking and electrostatic interaction [38, 39].

In a first set of experiments, reporter assays with Dele-F, Dele-R and Cdc6 G4 DNA reporter vectors carrying a minimal promoter have been performed in the presence of L1H1-7OTD. The vectors have been transfected into NIH3T3 cells and then the medium has been changed to the medium containing the G4 ligand 1 day after the transfection. After the 1 day cultivation, the luciferase activity has been measured. In this assay, results with L1H1-7OTD-treated and -untreated controls, respectively. As a result, there has been no significant difference of luciferase activity in the presence and the absence of the ligand (Additional file 5). One reason for this could be that the G4 ligand can not easily enter the cells. Therefore in a second set of experiments, the vectors have been mixed with the G4 ligand first and then transfected into NIH3T3 cells in the presence of the G4 ligand in the medium. The results show an inhibition of the luciferase expression with the wild-types of the G4 ligand-treated Dele-F and Cdc6 G4 DNAs, whereas no significant changes have been detected with all the mutant vectors (Fig. 4; Additional file 6). Although CD measurements show a stabilizing effect of the L1H1-7OTD binding to the G quadruplex (see below), this has no beneficial effect on the activation of protein expression. In this moment one can only hypothesize that the ligand binding to the top of the G4 structure modifies the interaction of the DNA structure with respective transcription factors by influencing the dynamics, the charge and the steric conditions.

CD spectroscopy analyses of Dele and Cdc6 G4 structures

CD spectra analysis has been performed to confirm the secondary structure formation of the wild- and mutant-type of Dele and Cdc6 G4 DNA (Fig. 5; Additional file 7). The CD spectra of the wild-types show a positive cotton effect at around 264 nm for Dele G4 DNA, and at around 262 nm for Cdc6 G4 DNA, with a negative effect at around 242 nm for Dele G4 DNA, and at around 240 nm for Cdc6 G4 DNA. Thus, the G quadruplex formation can be confirmed under these experimental conditions.
In the presence of the G4 ligand (L1H1-7OTD), these spectra do not change much, indicating that the formation of parallel-type G4 structures in the wild-types of Dele and Cdc6 G4 DNAs is not disturbed by the presence of the ligand (Additional files 8, 9) [40]. CD melting analysis reveals that the $T_m$ values are 70 °C for Dele G4 DNA and 77 °C for Cdc6 G4 DNA in the absence of the G4 ligand, while the $T_m$ values increase to 72 °C for Dele G4 DNA, and to 81 °C for Cdc6 G4 DNA by addition of the G4 ligand. These are clear arguments that the ligand binding to the G4 structure of the Dele and Cdc6 increases the stability of the G4s at least under the chosen experimental conditions (Fig. 6; Additional file 10).

The findings are in agreement with an NMR study on a structurally similar macrocyclic compound L2H2-6M(2) OTD, which has been found to bind to the top G-tetrad structure through π-stacking and electrostatic interaction. Here the folding topology did not change upon ligand binding [41].

CD spectra of the mutant-type sequences (mutating one G-run to T-run) that may form transient secondary structures, show only low molar ellipticity at around 260 nm and a shoulder between 280 and 300 nm, which are not related to the characteristic G4 structures (Fig. 5; Additional files 8, 9). Additionally in the presence of the G4 ligand, the CD spectra of the mutant-type sequences are not affected. These results strongly support the G4 formation of the two wild-type sequences of Dele and Cdc6 and also verify the disability of the selected mutants to form such defined secondary structures. In consequence, the conclusions, which have been taken from the comparative analysis of mutated and wild type sequences, seem to be valid.

**Discussion**

Recently, the death ligand signal enhancer (DELE) has been identified as a binding protein for the death-associated protein 3 (DAP 3), which is induced by various stimuli to regulate cell apoptosis. Stable expression of DELE induces apoptosis, whereas the knockdown of DELE has rescued HeLa cells from apoptosis induction [42]. It has also been reported that the cell-division-cycle 6 (CDC6) protein is essential for DNA replication, and the down-regulation of the Cdc6 gene causes inhibition in cell growth accompanied by an increase in cell apoptosis [43]. Abnormal apoptosis is related to many diseases involving...
atrophy [44], such as Parkinson’s disease [45], with excessive cell death leading to tissue and organ damage. In contrast, some oncogenic mutations disrupt apoptosis, leading to tumor progression [46] or metastasis. Therefore, further analysis of Dele and Cdc6 G4 DNAs may contribute to elucidate the apoptosis mechanism.

**Conclusions**

The reporter assay for G4-forming DNA sequences has demonstrated that Dele and Cdc6 G4 DNA may have the role of promoter and enhancer i.e., activating the transcription. This can be concluded from comparative experiments with mutant DNA structures. CD spectra verify the G4 formation of the wild type sequences and the disability of the studied mutants to form this secondary structure. The activation of transcription is inhibited by the telomestatin derivative L1H1-7OTD. CD spectra analysis demonstrates that binding of L1H1-7OTD stabilizes the G4 structures, but will also influence interaction with transcription factors. While for Dele-F G4 DNA transcriptional activation has also been verified in experiments with this sequence in CpG islands (CGIs), the Cdc6 G4 DNA results here in suppression of protein expression. This indicates that Cdc6 G4 may perform a dual functional role in the transcriptional regulation. In summary the results obtained suggest that Dele and Cdc6 G4 structures are formed under physiological conditions in the cell and play a role in regulating transcription. Consequently, the study contributes to the elucidation of mechanisms of Dele and Cdc6 gene regulation.

**Methods**

**Plasmid construction**

The G4 DNAs and mutant-type DNAs (Additional file 1) were cloned in the SfiI site of the pGL4.23 [luc2/miP] or pGL4.10 [luc2] (Promega Corporation, Madison, WI, USA) and then transformed into E. coli DH5α (TOYOBO, Osaka, Japan). The plasmids were purified by PureYield Plasmid Miniprep System (Promega Corporation, Madison, WI, USA). E. coli HST04 dam−/dcm− (Takara, Tokyo, Japan) was transformed by the plasmids to prepare DNA methylation-free plasmids. All plasmids were sequenced using a 3730xl DNA analyzer (Thermo Fisher Scientific, Waltham, MA, USA). The G4 ligands were amplified from C57BL/6 mouse genomic DNA by PCR. The PCR primers are shown in Additional file 1. The PCR products were purified by Wizard SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA) and then digested by SfiI (NEB, Ipswich, MA, USA). The products were cloned in the SfiI site of pGL4.10 [luc2] and the plasmids were prepared as described above. To construct the mutant-types vectors, the site-directed mutagenesis was performed using KOD plus- mutagenesis kit (TOYOBO, Osaka, Japan) according to manufacturer’s protocol (Additional file 11).

**Cell culture**

NIH3T3 cells (RCB1862, RIKEN BRC) were cultured in dulbecco modified eagle medium (DMEM) medium (Sigma-Aldrich, St. Louis, Missouri, USA) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, Missouri, USA), 1× penicillin–streptomycin–1-glutamine solution (Wako, Tokyo, Japan) at 37°C in 5% CO₂.

**Reporter assay**

The NIH3T3 cells were transfected with 100 ng of the firefly luciferase reporter vector and 100 ng of the Renilla luciferase control vector by Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s protocol. After 48 h, cells were harvested, and luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA) and SPARK 10 M microplate reader (TECAN, Männedorf, Switzerland). The ratio of firefly luciferase activity to Renilla luciferase activity was utilized to calculate the firefly luciferase expression level in each cell and then these results were normalized with that of the pGL4.10 or pGL4.23 luciferase reporter vector. All reporter assays were performed in triplicate.

In the experiment to analyze the G4 ligand effect, two methods were used. In the first method, the 100 ng of the firefly luciferase reporter vector, and 100 ng of the Renilla control vector were transfected into NIH3T3 cells by Lipofectamine 3000. After 24 h, the medium was changed to one with 10 μM G4 ligand L1H1-7OTD. After 24 h, the luciferase activity was measured as described above. In the second method, the culture medium was changed to one with 10 μM G4 ligand L1H1-7OTD prior to transfection. The NIH3T3 cells were transfected with 100 ng of the firefly luciferase reporter vector, 100 ng of the Renilla control vector, and 10 μM of the G4 ligand L1H1-7OTD by Lipofectamine 3000, after which the luciferase activity was measured as described above. The luciferase expression level in the presence or absence of L1H1-7OTD was normalized with that of the pGL4.23 luciferase reporter vector in the presence or absence of L1H1-7OTD, respectively. All reporter assays were performed in triplicate.

**CD spectra measurement**

Wild-types and mutant-types of the Dele and Cdc6 G4 oligonucleotides were purchased from Macrogen, South Korea (Additional file 7). The oligonucleotides were dissolved as to create stock solutions (100 μM) in distilled water. Prior to use, all oligonucleotides were diluted to 15 μM in TK buffer (50 mM Tris–HCl, 100 mM KCl,
The oligonucleotides were denatured at 95 °C for 3 min and then allowed to cool to room temperature for 30 min. After heat-treatment, the oligonucleotides were diluted to 10 μM in the presence or absence of 10 μM L1H1-7OTD, and then incubated for 10 min before measurement. The CD spectra were measured with a J-1500 CD Spectrometer (JASCO, Tokyo, Japan) at 220 to 320 nm using a 1 mm path-length cuvette at every 5 °C from 20 to 95 °C. The baseline of each spectrum was corrected for signal contributions by the buffer with and without the G4 ligand. In the CD melting analysis, molar ellipticities were measured concomitantly at 1 °C intervals for wild-types of Dele and Cdc6 G4 DNAs, at 264 nm and 262 nm, respectively. To determine $T_m$ values, the molar ellipticity at 25 °C was set as 100%, and the molar ellipticity at 95 °C was set as 0%. Using GraPh-Pad Prism7 software, curve fitting was performed to yield normalized molar ellipticities. $T_m$ values were recorded as temperatures equivalent to 50% of the normalized molar ellipticity.

**Additional files**

- **Additional file 1.** G4 DNA sequences used in the reporter assay.
- **Additional file 2.** Luciferase reporter assay results of G4 DNA sequences cloned in pGL4.23 vector.
- **Additional file 3.** Luciferase reporter assay results of G4 DNA sequences cloned in pGL4.10 vector.
- **Additional file 4.** Luciferase reporter assay results of CGI DNA sequences cloned in pGL4.23 vector.
- **Additional file 5.** Luciferase reporter assay results with or without L1H1-7OTD for G4 DNA sequences cloned in pGL4.23 vector.
- **Additional file 6.** Luciferase reporter assay results with or without L1H1-7OTD for G4 DNA sequences cloned in pGL4.23 vector.
- **Additional file 7.** G4 DNA sequences used in the CD Spectroscopy.
- **Additional file 8.** CD spectra results of Dele G4 DNA wild or mutant-type sequences with or without L1H1-7OTD.
- **Additional file 9.** CD spectra results of Cdc6 G4 DNA wild-type sequence with or without L1H1-7OTD.
- **Additional file 10.** Detailed molar ellipticity of wild-types of Dele and Cdc6 G4 DNAs in the absence and the presence of L1H1-7OTD.
- **Additional file 11.** Primer sequences used for CGI vectors construction and mutagenesis.

**Abbreviations**

7OTD: 7 oxazole telomestatin derivative; CD: circular dichroism; Cdc6: cell-division-cycle 6; CpG: cytosine-phosphate-guanine; Dele: death ligand signal enhancer; G4: G-quadruplex.

**Authors’ contributions**

WY conceived and designed the experiments. DHB and AB performed the experiments. DHB, AB, FL, KI, KIk, KN, IK, and WY analyzed the data. DHB, FL and WY wrote the manuscript. All authors read and approved the final manuscript.

**Acknowledgements**

We thank Dr. Tomohiko Yamazaki (National Institute for Materials Science, Japan) for the kind gift of NIH3T3 cells.

**Competing interests**

The authors declare that they have no competing interests.

**Availability of data and materials**

The datasets supporting the conclusions of this article are included within the additional files.

**Funding**

This study was supported by Tokyo University of Technology. The funding body had no role in the design of the study, collection, analysis, interpretation of data and in writing the manuscript.

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Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Received: 19 May 2017 Accepted: 23 June 2017**

**Published online:** 27 June 2017

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