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Role of Hairpin-Quadruplex DNA Secondary Structural Conversion in the Promoter of hnRNP K in Gene Transcriptional Regulation

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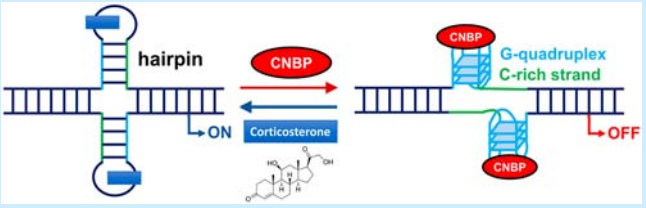
Supporting Information

ABSTRACT: The promoter of hnRNP K oncogene was found to contain a G/C-rich sequence on the same DNA strand, which can form interconvertible G-quadruplex, i-motif, and hairpin structures. Protein CNBP could bind and stabilize the G-quadruplex, inducing transformation of the hairpin into the G-quadruplex, resulting in down-regulation of hnRNP K transcription. In contrast, Corticosterone could bind and stabilize the hairpin, inducing transformation of the G-quadruplex into the hairpin, resulting in up-regulation of hnRNP K gene transcription.



Besides the well-known right-handed double helical structure of B-form DNA (B-DNA), it has been clear that DNA can form various other secondary structures, in particular, sequence motifs, including G-quadruplex, hairpin, and i-motif.[1] These DNA secondary structures have been identified in the promoters of human genomic DNA, and can become recognition binding sites for transcription factors. Recently, we have shown that G-quadruplex in the promoter of heterogeneous ribonucleoprotein K (hnRNP K) can regulate gene transcription.[2] At high concentrations of cellular nucleic acid binding protein (CNBP), it can bind with a G-rich sequence in hnRNP K promoter and induce its formation of G-quadruplex, thus down-regulating hnRNP K transcription. Up-regulation of CNBP induced human fibrosarcoma cell death and suppressed human fibrosarcoma cells motility and invasiveness. In the present study, we found that the G/C-rich sequence in the promoter of hnRNP K oncogene could form interconvertible G-quadruplex, i-motif, and hairpin structures. CNBP could bind and stabilize the G-quadruplex, which induced transformation of the hairpin structure into G-quadruplex, resulting in down-regulation of hnRNP K transcription. To further study the effect of CNBP, we silenced the expression of CNBP in HT1080 tumor cells, which produced only moderate effect on the expression of hnRNP K, tumor cell death and metastasis, as shown in Figure S1A−F. Therefore, it is possible that other factors besides CNBP and G-quadruplex might also be involved in regulating expression of hnRNP K and tumor cell behavior. We noticed that the CNBP binding region G15−C15 is close to a C-rich region. This C-rich region could form an i-motif structure in vitro under acidic condition (Figure S2A,B), indicating that it is likely to form an i-motif structure in vivo with its binding protein or under crowding conditions. Interestingly, these two closely located G-rich and C-rich regions could form an intramolecular hairpin structure (Figure 1).

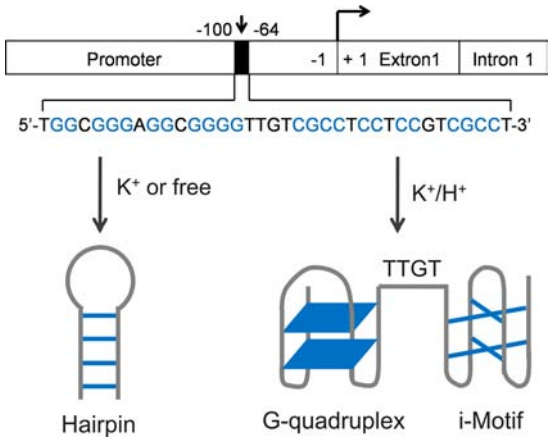


Figure 1. Schematic representation of the hnRNP K promoter and its G/C-rich region.

To study whether these G/C-rich sequences can fold into stable DNA secondary structures, we carried out biophysical experiments on the corresponding synthetic oligonucleotides. The ^{1}H NMR spectrum showed imino proton signals of the G/C-rich sequence at around 13 ppm (Figure 2A), which are characteristics of the Watson−Crick base pairs indicating the existence of a duplex or a hairpin structure.[3] Then, a circular dichroism (CD) experiment was performed for the sample in 10 mM KCl solution at pH 7.0, and the CD spectrum showed

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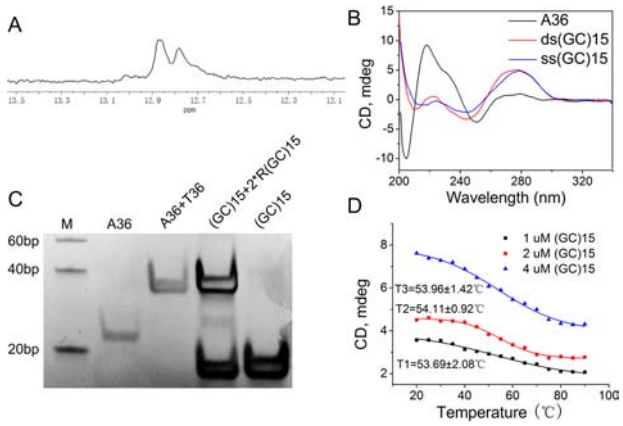

Figure 2. Hairpin structure formed in the promoter of hnRNP K. (A) The ^{1}H NMR spectrum for the imino region of G15−C15 in KCl solution at 25 °C. (B) Circular dichroism spectra of G15−C15. CD analyses were performed on 3 μM G15−C15 in the absence and presence of various concentrations of KCl. (C) Native PAGE images of A36, A36 + T36, (GC)15 + 2*R(GC)15, and (GC)15 in the presence of 10 mM KCl. R(GC)15 was (GC)15's complementary strand. (D) CD-melting curves for increasing concentrations of (GC)15 obtained based on the 280 nm ellipticity.

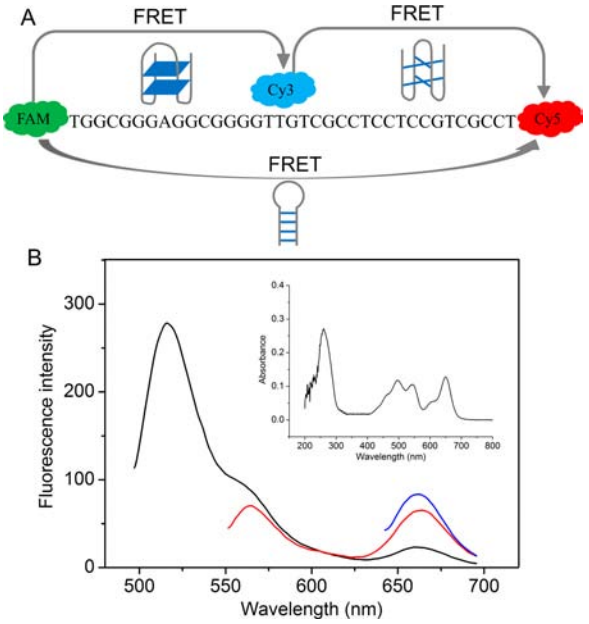

Figure 3. Studies of various DNA secondary structures with triple-FRET system. (A) Schematics of energy transfer for each fluorescence resonance energy transfer pair in the trifluorophore-labeled G15−C15 (F-G15-Cy3-C15-Cy5). (B) Electronic absorption (inset) and fluorescence spectra of F-G15-Cy3-C15-Cy5.

that G15−C15 ((GC)15) displayed peaks of positive molar ellipticity at 280 nm and negative molar ellipticity at 240 nm, which are also characteristics of a duplex or a hairpin structure (Figure 2B).[4] To know whether the DNA secondary structure was intramolecular species or intermolecular species, we carried out nondenaturing PAGE experiment. As shown in Figure 2C, we observed a band migrated over the 20-base oligothymidylate compared to other control groups, indicating the formation of an intramolecular hairpin structure. CD-melting experiment was also carried out, and it was found that the T_m of (GC)15 was not significantly changed when increasing the concentration of (GC)15 (Figure 2D), also indicating the formation of an intramolecular hairpin structure.[5] Taken together, our results all suggested that (GC)15 could form an intramolecular hairpin structure.

To know whether various secondary DNA structures are interconvertible, we studied the stability of hairpin structure and its interconversion with its corresponding single strand DNA and G-quadruplex under various conditions in vitro. In this study, we designed and prepared an innovative Triple FRET (Tri-FRET) model system, as shown in Figure 3A, which can indicate the presence of various secondary DNA structures easily. It should be noted that trifluorophore-labeled biological systems have been well studied previously for detection of complex biological transformations.[2,3,6] In the present study, we labeled G15−C15 with three fluorophores, including FAM, Cy3, and Cy5. FAM acts as the donor for Cy3 and Cy5, Cy3 acts as an acceptor for FAM and a donor for Cy5, while Cy5 acts as an acceptor for both FAM and Cy3. The UV−vis absorption spectrum of the trifluorophore-labeled G15−C15 is shown in the inset of Figure 3B. Four absorption peaks at 260, 495, 550, and 647 nm were observed, indicating the presence of DNA, FAM, Cy3, and Cy5, respectively.

To obtain FRET efficiency among the three fluorophores, G15−C15 was excited at 475, 530, and 620 nm (Figure 3B). The excitation at 475 nm resulted in emissions at 520, 564, and 660 nm (Figure 3B, black curve). The 520 nm peak is from the FAM emission. The 564 nm shoulder is attributable to the emission of FAM, the emission from Cy3, and FRET from FAM to Cy3. The 660 nm peak is mainly a Cy5 emission resulted from energy transfer from both FAM and Cy3, with a small fraction of the emission from both FAM and Cy3. With a 530 nm excitation, the influence of FAM can be ignored. A common Cy3−Cy5 pair FRET spectrum was observed (Figure 3B, red curve). With a 620 nm excitation, only Cy5 emitted at 660 nm (Figure 3B, blue curve).[7]

With our triple FRET model system, we studied the stability of the hairpin structure formed with FAM-Cy5. We found that the unfolding ratio of hairpin structure was increased when increasing amount of various complementary strand C1 was added. However, even in the presence of 8 times of its complementary strand, hairpin structure still existed (Figure S3), indicating significant stability of the hairpin structure.

To know whether the DNA secondary structural conversions in the promoter of hnRNP K gene affect the expression of the protein hnRNP K, we carried out reporter assays as shown in Figure S4A, with our constructed plasmid (psiCheck2-hnRNP K-WT: WT) containing the G15−C15 promoter sequence of hnRNP K. We also constructed three G15−C15 mutants (Mut1, Mut2, and Mut3) interrupting different DNA secondary structures for comparative studies (Figure S4A). We evaluated the efficiency of translation by using the standard luminescence assay for luciferase catalytic activity. Our results indicated that the formation of hairpin structure in the promoter of hnRNP K increased the expression of luciferase (Figure S4B). Our triple-FRET data also showed that hairpin structure is very stable in vitro. These results indicated that hairpin structural formation is important in the mechanism for regulating transcription and expression of hnRNP K in cancer cells.[8]

Since our triple-FRET assay showed that G15−C15 promoter sequence preferred to fold into stable hairpin structure instead of G-quadruplex in vitro, and protein CNBP can induce G-quadruplex formation,[4a] in the present study, triple-FRET experiment was carried out to investigate whether

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CNBP could induce transformation of the hairpin structure into G-quadruplex. After increasing amount of CNBP was added to the hairpin structure, upon excitation at 475 nm, the peak around 650 nm was found to be decreased, while the peak around 550 nm increased, as shown in Figure 4, indicating that

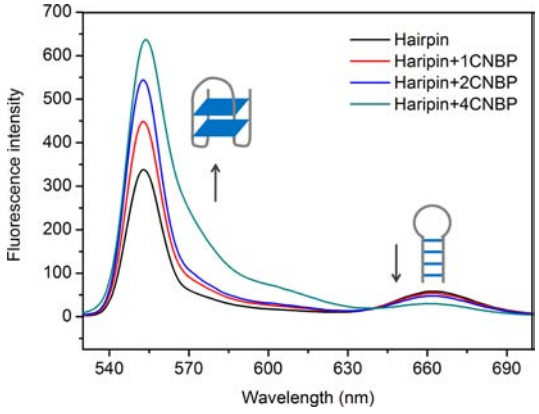

Figure 4. CNBP induced transformation of hairpin to G-quadruplex. After increasing amount of CNBP was added to the hairpin, upon excitation at 475 nm, the peak around 650 nm decreased, while the peak around 550 nm increased, indicating that CNBP induced transformation of hairpin to G-quadruplex.

CNBP induced transformation of the hairpin structure into G-quadruplex. The G15−C15 sequence was then mutated through base substitution, which maintained G-quadruplex, i-motif, and complete complementary hairpin structure. The CNBP induced structural transformation experiment was also carried out for our mutated G15−C15 hairpin with the same triple-FRET assay (Figure S5), which generated less spectral changes, indicating the mutated hairpin is more stable than the wild type.

Next, a reporter assay was performed to evaluate the effect of conformational transformation in the hnRNP K gene promoter on the transcription of hnRNP K gene. After cotransfecting 100 ng of this plasmid with increasing amount of recombinant CNBP overexpression plasmid, we detected decreasing amount of firefly luciferase relative to the renilla luciferase in a dose-dependent manner, compared to the sample transfected without recombinant CNBP overexpression plasmid as a control, as shown in Figure S6A. This indicated that CNBP down-regulated the luciferase catalytic activity by inducing transformation of the promoter hairpin structure to G-quadruplex. Then, we performed mutation reporter assay with our mutated G15−C15 inserted into the promoter of luciferase gene. Compared to the wild-type hairpin, the mutated hairpin structure is more stable, as indicated in the above experiment. Our result showed that the effect of CNBP on the mutated plasmid was less significant than that on the wild-type plasmid (Figure S6B). These results suggested that CNBP decreased the luciferase catalytic activity through inducing transformation of promoter hairpin structure to G-quadruplex.

To better understand the effect of hairpin-quadruplex DNA secondary structural conversion on hnRNP K gene transcription, we screened various types of compounds for small molecule binding ligand that could stabilize the hairpin structure. A CD-melting based screening assay was used to identify potential interactive small molecules. In the present study, after assay test of more than 10 commercially available steroid hormones or their derivatives, Corticosterone (S2, Figure 5A) was found to be able to bind and stabilize the hairpin structure. Corticosterone is a hormone synthesized in

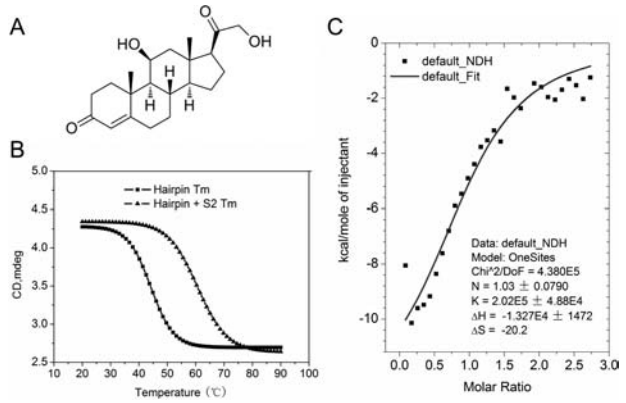

Figure 5. Corticosterone (S2) was found to bind and stabilize the hairpin. (A) Structure of Corticosterone. (B) CD melting curves of hairpin, and hairpin with Corticosterone monitored at 280 nm. (C) ITC calorimetric data for titration of 5 μM hairpin with serial injections of Corticosterone solution (5 μL aliquots of 0.3 mM).

response to stress. It binds and activates MCRs (mineralocorticoid receptors) and GRs (glucocorticoid receptors), and affects ionic conductance through the membrane. CD-melting assay showed that Corticosterone stabilized hairpin, and the ΔT_m value was determined to be around 18 °C (Figure 5B). Isothermal titration calorimetry (ITC) is a sensitive technique for characterizing bimolecular processes.[9] Our ITC data showed binding stoichiometry of 1:1 for Corticosterone and hairpin, with the binding affinity of 2.02×10^5 M^{-1} (Figure 5C). Then, our triple-FRET assay was used to study the effect of Corticosterone on CNBP induced hairpin-quadruplex structural conversion. After increasing amount of Corticosterone was added to the hairpin structure and CNBP was then added to the mixture, upon excitation at 475 nm, less change was detected around 650 and 550 nm, as shown in Figure S7, indicating that CNBP transformed less hairpin structure to G-quadruplex in the presence of Corticosterone, due to its stabilization of the hairpin.

Since Corticosterone could bind and stabilize the hairpin structure affecting hairpin-quadruplex structural conversion, it is interesting to know whether Corticosterone could influence the transcription of hnRNP K gene. We constructed a firefly luciferase expression plasmid with G15−C15 inserted into the promoter of luciferase gene. After cotransfecting 100 ng of this plasmid with increasing amount of Corticosterone, we detected increasing amount of firefly luciferase relative to the renilla luciferase, compared to a control (Figure S8). This result suggested that Corticosterone up-regulated the luciferase catalytic activity by inducing transformation of the promoter G-quadruplex to hairpin structure. This provided a new mechanism for the effect of Corticosterone on gene transcription.

On the basis of our experimental results, a possible mechanism for the hnRNP K transcriptional regulation by hairpin-quadruplex DNA secondary structural conversion is proposed. This alternative DNA secondary structural conversion mechanism influenced by CNBP and Corticosterone may generally affect expressions of various essential genes. In consideration of a previous report that a pregnanol derivative can bind to another hairpin structure,[3] it is likely that other

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steroid hormones could bind to various different hairpin structures and selectively influence gene transcription. This suggested a potentially new mechanism for how steroid hormone affects gene transcription. The present result could increase our understanding of gene transcription and expression, which should shed light on new drug design and development.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b02310.

Material and methods; additional experimantal data (PDF)

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Notes

The authors declare no competing financial interest.

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