Structure of hnRNP D Complexed with Single-stranded Telomere DNA and Unfolding of the Quadruplex by Heterogeneous Nuclear Ribonucleoprotein D*

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Heterogeneous nuclear ribonucleoprotein D, also known as AUFI, has two DNA/RNA-binding domains, each of which can specifically bind to single-stranded d(TTAGGG)n, the human telomeric repeat. Here, the structure of the C-terminal-binding domain (BD2) complexed with single-stranded d(TTAGGG) determined by NMR is presented. The structure has revealed that each residue of the d(TAG) segment is recognized by BD2 in a base-specific manner. The interactions deduced from the structure have been confirmed by gel retardation experiments with series of mutants BD2 and DNA. It is known that single-stranded DNA with the telomeric repeat tends to form a quadruplex and that the quadruplex has an inhibitory effect on telomere elongation by telomerase. This time it is revealed that BD2 unfolds the quadruplex of such DNA upon binding. Moreover, the effect of BD2 on the elongation by telomerase was examined in vitro. These results suggest the possible involvement of heterogeneous nuclear ribonucleoprotein D in maintenance of the telomere 3’-overhang either through protection of a single-stranded DNA or destabilization of the potentially deleterious quadruplex structure for the elongation by telomerase.

1 The abbreviations used are: hnRNP, heterogeneous nuclear ribonucleoprotein; BD, binding domain; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; HSQC, heteronuclear single quantum-coherence spectroscopy; r.m.s.d., root mean square deviation.

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hnRNP D Telomere Structure and Quadruplex Unfolding

Preparation of DNA Oligomers—Non-labeled DNA oligomers, synthesized with a DNA synthesizer and purified by reverse phase HPLC, were purchased (Nippon Seihun) and further treated on a Dowex 50 cation exchange column as described previously (12). 13C,15N-labeled d(TTAGGG) and d(GTTAGGGT) were synthesized by the hairpin extension method, using a Klenow fragment (3’-5’ exo-) (Daichi Kagaku) with 13C,15N-labeled dNTPs (Nippon Sanso), and purified following the reported method (13).

NMR and CD Spectroscopies—For NMR experiments, BD2 (2.0 mM) and d(TTAGGG) (2.4 mM) were dissolved in a solution comprising 50 mM Tris-HCl (pH 7.8), 1 mM MgCl2, 100 mM NaCl, 1 mM EDTA, and 5% glycerol. Fluorescence of the gel was recorded with a FLA 2000 (Fuji Film).

RESULTS

Resonance Assignments and Structure Determination of the Complex—Sequential assignments of the main chain and side chain 1H, 13C, and 15N resonances of hnRNP D BD2 complexed with d(TTAGGG) were made in the same way as reported for BD1 (10) and BD2 (11) in a free state. The assignments of the main chain Hδ2 and N resonances, together with those of Hδ2 and Nδ2 of Asn residues, are presented in red in Fig. 1; the assignments in the free state are also shown in black for reference. The BD2 residues that exhibited large chemical shift perturbation on complex formation and intermolecular NOEs to d(TTAGGG) are labeled. NOESY spectra indicating intermolecular NOEs for T2H3 (B), A3H2 (C), and G4H1 (D) are shown.

dTTP at 30 °C for 90 min. [d(GGGATTGGGATTGGGATTGGGTT)] was used as a primer (25). The extension products were purified by phenol-chloroform extraction and ethanol precipitation. The products were separated on a 10% polyacrylamide gel containing 8 M urea and detected with a FLA 2000.

N-terminal were expressed and purified with a Ni affinity column (Qiagen).

Human telomerase was reconstituted as described previously (22). Human telomerase reverse transcriptase (hTERT) was prepared with a T7-coupled transcription/translation system (Promega). Human telomerase RNA (hTR) was transcribed from template DNA. hTERT and hTR were mixed to yield the human telomerase. Primer extension reaction with the reconstituted telomerase was carried out as described previously (23, 24) with and without hnRNP D BD2 in a solution comprising 50 mM Tris-HCl (pH 7.8), 1 mM MgCl2, either 0 or 100 mM KCl, 5 mM β-mercaptoethanol, 1 mM spermidine, 1 mM primer, 1.25 μM [α-32P]dGTP (800 Ci/mmol), 1 mM dATP, and 1 mM

Fig. 1. NMR spectra of the hnRNP D BD2-d(TTAGGG) complex. A, 1H,15N HSQC spectra of hnRNP D BD2 in a free state (black) and a complex state with d(TTAGGG) (red) at 15 °C. The BD2 residues that exhibited large chemical shift perturbation on complex formation and intermolecular NOEs to d(TTAGGG) are labeled. NOESY spectra indicating intermolecular NOEs for T2H3 (B), A3H2 (C), and G4H1 (D) are shown.
exhibits a typical RNP-type fold, i.e. the same as that in the free state. BD2 in the complex shows the restrained energy-minimized mean structure of the superposition of the 20 final structures of the complex. Fig. 2 hydrogen bonds, and 127 dihedral angle constraints were used for structure calculation.

Val208-Ser210 region of Phe225-Phe230; and surface of BD2, with no interaction with BD2. In fact, no observed for BD2 in the free state (10). Additionally, a short 18864/H9252 0.81 DNA (2–5) 0.81 ± 0.30 BD2 excluding loop 3 (181–214, 225–259) and DNA (2–5) 0.55 ± 0.21a * For backbone atoms of BD2 and all heavy atoms of DNA. Sequential assignments of the resonances of d(TTAGGG) in the complex were made with the standard method as reported for other DNA (26) and RNA (27). Because of the 10% excess of d(TTAGGG) over BD2 in the solution, exchange cross-peaks between resonances corresponding to the free and complex states were observed for d(TTAGGG) in NOESY and ROESY spectra (data not shown). The resonance assignments of d(TTAGGG) in the complex were confirmed by these exchange cross-peaks, the resonance assignments of free d(TTAGGG) being accomplished beforehand. On the basis of resonance assignments, many intermolecular NOEs were identified (Fig. 1, A–D). The structure of the complex was calculated on the basis of distance and dihedral angle constraints. The structural statistics are shown in Table I. The r.m.s.d. of the 20 final structures versus the mean structure as to the backbone atoms of BD2, excluding loop 3 (181–214, 225–259), and all heavy atoms of DNA (T2-G5) was 0.55 ± 0.21 Å. Local r.m.s.d. as to backbone atoms of BD2 excluding loop 3 (181–214, 225–259) was 0.19 ± 0.03 Å and that as to all heavy atoms of DNA (T2-G5) was 0.81 ± 0.30 Å. Structure of the Complex—Fig. 2A shows a stereo view of superposition of the 20 final structures of the complex. Fig. 2B shows the restrained energy-minimized mean structure of the complex. The overall structure of BD2 in the complex is essentially the same as that in the free state. BD2 in the complex exhibits a typical RNP-type fold, i.e. a four-stranded antiparallel β-sheet (β1, Lys183-Gly188; β2, Val208-Pro214; β3, Phe225-Phe230; and β4, Ser250-Lys255) packed against two α-helices (α1, Gln195-Phe205; and α2, Glu233-Glu241), as observed for BD2 in the free state (10). Additionally, a short β-strand (β4′, Tyr244-Val247) was identified in loop 5 located between α2 and β4. A β-hulge structure was found in the Val208-Ser210 region of β2. These features were found in the free state as well. The backbone r.m.s.d. between the structures in the free and complex states of BD2 for the Val181-Ser259 region was 2.4 Å.

The complex structure reveals that the Thy2-Gua4 segment of single-stranded d(TTAGGG) is specifically recognized by BD2 (Fig. 3). On the other hand, the Thy1, Gua5, and Gua6 residues are located away from the interactive β-sheet surface of BD2, with no interaction with BD2. In fact, no intermolecular NOE to BD2 was observed for these residues. Thy-2 is recognized by three hydrogen bonds with BD2, T2O2-R255H3, T2H3-E253O1-ε2, and T2O4-Y224H1 (Fig. 3A). Of the 2 Thy residues, an H3 resonance was observed only for the Thy-2 residue, which is consistent with the formation of the hydrogen bond. Ade-3 is recognized through the stacking interaction with Phe-185, and by the hydrophobic interaction of A3H2 with A257CβH3 and M258CγH4 (Fig. 3B). Gua-4 is recognized through the stacking interaction with Phe-227 and by two hydrogen bonds, G4H1-M258O′ and G4-O6-Lys183H5 (Fig. 3C). Among the 3 Gua residues, an H1 resonance was observed only for the Gua-4 residue, which is consistent with the formation of the hydrogen bond. It is notable that to make the interactions possible, Gua-4 takes on the syn conformation, which was directly supported by the experimental observation of a very strong G4H8-G4H1 NOE. The other DNA residues take on the anti conformation. Additionally, the electrostatic interaction between the 5-phosphate group of Gua-4 and the guanidinium group of Arg223 was found.

Table I

| Structural statistics of hnRNPD BD2 complexed with d(TTAGGG) |
|---------------------------------------------------------------|
| 1474 distance constraints (112 intraresidue, 386 sequential, 233 medium-range, and 561 long-range distance ones for BD2, 62 intraresidue and 25 sequential distance ones for d(TTAGGG), and 95 BD2-d(TTAGGG) intermolecular distance ones), together with 48 distance constraints for 24 hydrogen bonds, and 127 dihedral angle constraints were used for structure calculation. |
| Number of structures | 20 |
| R.m.s.d. from idealized geometry | |
| Bonds (Å) | 0.0041 ± 0.0001 |
| Angles (degrees) | 0.602 ± 0.008 |
| Impropers (degrees) | 0.489 ± 0.015 |
| R.m.s.d. from experimental constraints | |
| Distances (Å) | 0.0425 ± 0.0005 |
| Dihedral angles (degrees) | 0.791 ± 0.062 |
| NOE violations | |
| Number of violations greater than 0.4 Å | 0 ± 0 |
| Dihedral angle violations | 0 ± 0 |
| Number of violations greater than 5.0 degrees | |
| Ramachandran analysis for BD2 (181–259) | |
| In most favored regions (%) | 82 |
| In additionally favored regions (%) | 15 |
| In generously allowed regions (%) | 3 |
| In disallowed regions (%) | 0 |
| R.m.s.d. to the average structure (Å) | |
| Backbone | 0.19 ± 0.03 |
| All heavy atom | 0.73 ± 0.05 |
| BD2 excluding loop 3 (181–214, 225–259) and DNA (2–5) | 0.81 ± 0.30 |
| DNA (2–5) | 0.55 ± 0.21a |

* For backbone atoms of BD2 and all heavy atoms of DNA.

Confirmation of the Interactions through Gel Retardation Experiments and Chemical Shift Perturbation—The interactions identified in the complex were biochemically confirmed through gel retardation experiments using mutant BD2s. First, we recorded either a CD or 1H-15N HSQC spectrum of each mutant BD2 and then confirmed that all mutant BD2s retain similar overall folding to that of the wild type BD2. A drastic decrease in the intensity of the band corresponding to the protein-DNA complex was observed for the F185A and F227A mutants (Fig. 4), which confirms the importance of the Ade3-Phe185 and Gua4-Phe227 stacking interactions. Similarly, the large decreases observed for the E253A, A257C, and R223A mutants are consistent with the importance of the hydrogen bonding interaction between Thy2 and Glu253, the hydrophobic interaction between Ade3 and Ala257, and the electrostatic interaction between Gua4 and Arg223. The involvement of Met258, Lys183, Tyr244, and Lys255 in the interactions was also supported by the decreases observed for their mutants. As a control, Asn217, which is not involved in the interaction in the complex structure, was mutated to an Ala residue. As expected, no decrease in the intensity was observed for N217A (Fig. 4).

Gel retardation experiments were also carried out with mutant DNAs and wild type BD2. It was expected that replacement of the third Ade residue in the d(TTAGGG) unit of the
DNA with a Gua residue would abolish the hydrophobic interactions with A257C\text/H9252\text/H3 and M258C\text/H9253\text/H2 and that replacement of the fourth Gua residue of the unit with an Ade residue would abolish the hydrogen bonding interactions with M258O\text/H11032\text/H3 and Lys-183\text/H9256. In fact, a decrease of 40–50% in the intensity of the complex band was observed for these mutant DNAs. The results confirm that the Ade-3 and Gua-4 residues are recognized through base-specific interactions, in addition to a more general stacking interaction with a Phe residue. In summary, the interactions identified in the complex structure were all supported by the gel retardation experiments. The vital role of the interactions involving the Thy-2-Ade-3-Gua-4 segment in the complex formation was further supported by chemical shift perturbation data. Large chemical shift perturbations were induced for aromatic resonances of BD2 on complex formation with d(TTAGGG). The spectral pat-
tern of the aromatic resonances in the complex state was distinct from that in the free state. Then, each residue of d(TTAGGG) was replaced with a Cyt residue, and the spectral pattern of BD2 complexed with the mutant DNA was analyzed. When d(TCAGGG), in which Thy-2 is replaced with a Cyt residue, was added to BD2, a spectral pattern corresponding to free BD2 was observed in addition to one corresponding to complex BD2, the molar ratio of free:complex being ~1:2 (data not shown). This indicates that some fraction of BD2 exists in a free state, due to the decrease in the affinity caused by the replacement of Thy-2 with a Cyt residue. The same phenomenon was observed when either Ade-3 or Gua-4 was replaced with a Cyt residue. Thus, the importance of the interactions involving Thy-2, Ade-3, and Gua-4 residues was confirmed. When Thy-1, Gua-5, or Gua-6 was replaced with a Cyt residue, in contrast, the spectral pattern of BD2 complexed with a mutant DNA was the same as that of BD2 complexed with the wild type DNA, no trace of the spectral pattern of free BD2 being detected (data not shown). This indicates that the interactions involving Thy-1, Gua-5, and Gua-6 are dispensable for the complex formation, if any.

**Unfolding of the Telomeric DNA Quadruplex on Binding of hnRNP D**—It is known that DNA with the telomeric sequence tends to form a quadruplex under physiological ionic conditions. We have reported that hnRNP D has the ability to inhibit the formation of an RNA quadruplex (10). Here, we examined whether hnRNP D unfolds a preformed DNA quadruplex with the telomeric sequence or not. Under physiological ionic conditions, three DNAs, d(TTAGGG) (6-mer), d(TTAGGTTAGGG) (12-mer), and d(TTAGGTTAGGGTTAGGGTTAGGG) (24-mer), gave a positive CD peak at either 260–265 or 290 nm (Fig. 5, A–C), both of which are established marker peaks for a quadruplex (28, 29). It was supposed that 6-, 12-, and 24-mer form a tetrameric parallel quadruplex, a dimeric antiparallel quadruplex, and a monomeric antiparallel quadruplex, respectively. Recently, the formation of a dimeric parallel quadruplex (30, 31) and a monomeric parallel quadruplex (12, 26, 32, 33) has been reported for the related sequences. These findings imply the possibility that 12-mer may form a dimeric parallel quadruplex as well and that 24-mer may form a monomeric parallel quadruplex to some extent. Nonetheless, the possible structures are all quadruplexes, and a positive CD peak at either 260–265 or 290 nm can still be used as a marker peak for quadruplexes. When hnRNP D BD2 was added to the DNA solution (15–60 μM), the intensity of these peaks decreased (Fig. 5, A–C). These results reveal that hnRNP D unfolds the preformed quadruplex of telomeric DNA on binding.

For the 6-mer, the intensity of the marker peak at 260–265 nm decreases to half when the molar ratio of BD2 to DNA is four (Fig. 5A). For the 12-mer, the intensity of the marker peak at 260–265 nm decreases to half when the molar ratio of BD2 to DNA is eight (Fig. 5B). The 12-mer comprises two d(TTAGGG) sequences, so when the quadruplex is unfolded, two d(TTAGGG) sequences/12-mer DNA are released for binding of BD2. If this is taken into account, the extent of unfolding of the quadruplex can be regarded as comparable for the 6- and the 12-mer. For the 24-mer, the intensity of the marker peak at 290 nm decreases to half when the molar ratio of BD2 to DNA is 16:32 (Fig. 5C). Because the 24-mer comprises 4 d(TTAGGG) sequences, the extent of unfolding of the 24-mer quadruplex can also be regarded as roughly comparable with that of the 6- and 12-mer quadruplexes. Thus, the unfolding of the quadruplex is observed to the same extent for longer DNA.

Unfolding of a telomeric DNA quadruplex by hnRNP D was revealed in other experiments. The chemical shift perturbations of hnRNP D BD2 caused by the addition of d(TTAGGG) in a single-stranded form and a quadruplex form were compared. In the first experiment, the d(TTAGGG) to be added was kept in a solution containing neither Na⁺ nor K⁺ ions, both of which are known to stabilize a quadruplex. The absence of imino proton resonances of the 3 Gua residues confirmed that d(TTAGGG) takes on a single-stranded form (data not shown). BD2 was also kept in a solution lacking Na⁺ and K⁺. In the second experiment, d(TTAGGG) to be added was kept in a solution containing 150 mM K⁺. Formation of the quadruplex under the conditions was confirmed by the observation of imino proton resonances of the 3 Gua residues at 10.5–11.5 ppm (data not shown), which is characteristic of the quadruplex. BD2 was kept in a solution containing 150 mM K⁺ in the second experiment. The HSQC spectrum of BD2 obtained after the addition of a single-stranded form of d(TTAGGG) in the first experiment...
Fig. 6. $^1$H-$^{15}$N HSQC spectra of hnRNP D BD2. Spectra are shown in a free state (A), after the addition of d(TTAGGG) in a single-stranded form (B), and a preformed quadruplex form (C) and after the addition of d(TTAGGTTAGGGTTAGGGTTAGGG) in a single-stranded form (D) and a preformed quadruplex form (E). Panels A, B, and D were recorded in 0 mM KCl and panels C and E in 150 mM KCl.

(Fig. 6B) was almost identical to that obtained after the addition of a quadruplex form of d(TTAGGG) in the second experiment (Fig. 6C), both HSQC spectra being clearly different from the HSQC spectrum of free BD2 (Fig. 6A). If BD2 bound to the quadruplex form of d(TTAGGG) in the second experiment, the magnetic environment of BD2 must be to some extent different from that bound to the single-stranded d(TTAGGG) in the first experiment. Then, the corresponding HSQC spectra should be different from each other. Thus, the observation of the nearly identical HSQC spectra in the two experiments reveals that the preformed quadruplex DNA with the telomeric sequence is unfolded into a single-stranded form for interaction with BD2 under physiological ionic conditions. Similarly, when the 24-mer in either a single-stranded or quadruplex form was added to BD2, many common spectral features were observed in the two resultant HSQC spectra (Fig. 6, D and E). This indicates that the quadruplex of the 24-mer is also unfolded to some extent on interaction with BD2. In this way, unfolding of the
preformed quadruplex of telomeric DNA by hnRNP D was directly demonstrated for the first time with structural experimental methods, i.e. CD and NMR.

It is noticed that in Fig. 6, panels D and E are not completely identical. The difference partly arises from disappearance of peaks for Fig. 6E, although some of the missing peaks can be detected when the level of the plot for Fig. 6E is lowered. Except for the disappearance, the spectral difference is still present between panels D and E. This may suggest the possibility that a small fraction of BD2 binds to the quadruplex of 24-mer, although further certification is needed.

**Primer Extension Assay of the Effect of hnRNP D on Telomerase Activity**—Primer extension reaction with the reconstituted telomerase was carried out (Fig. 7A). The primer was successfully extended in the absence of K+ (lane 1), whereas the extension was inhibited in the presence of K+ (lane 2). The formation of the quadruplex in the presence of K+ is supposed to be responsible for the inhibition. Then hnRNP D BD2, which can unfold the quadruplex, was added to the solution, but the extension was still inhibited (lane 3).

Next, the effect of hnRNP D BD2 on the extension was examined in the absence of K+ (Fig. 7B). It was found that the addition of hnRNP D BD2 led to a reduction in the extension (lane 2).

**DISCUSSION**

**Comparison with Other Telomere-binding Proteins**—The complex structures of proteins that bind to a single-stranded DNA with the telomeric sequence have been reported for TEBP of *Oxytricha nova* (34), Cdc13 of *Saccharomyces cerevisiae* (35), Pot1 of *Schizosaccharomyces pombe* (36), and human hnRNP A1 (37). The complex structure of hnRNP D BD2 was compared with these four structures. The five complexes share the common feature that single-stranded telomeric DNA binds to the β-sheet surface of each protein with either an OB fold or RNP (or RRM) fold. Aromatic rings are characteristically exposed outwards from the β-sheet surface of OB and RNP folds, and the rings undergo stacking interactions with DNA bases in all cases. However, great diversity was seen in the mode of specific recognition of telomeric DNA. In the case of TEBP, three OB folds cooperatively recognize DNA. In the case of Cdc13, a greatly expanded interface with the involvement of a large loop is utilized for the recognition. In the case of Pot1, an unusual DNA structure itself plays a critical role in the recognition. In the cases of hnRNP A1 and hnRNP D, the relatively restricted interface of a single binding domain can independently recognize a single d(TTAGGG) unit.

The target sequences of TEBP and Cdc13 in the complexes contain only Gua and Thy residues. Although an Ade residue is present in the target sequence of Pot1, this Ade residue is not recognized by a protein residue but by other DNA residues (36). Thus, the hnRNP D complex and the hnRNP A1 complex are the only cases where the recognition of the Ade residue of DNA by a protein has been demonstrated. In both complexes, there is a stacking interaction between the Ade residue and the Phe residue. The difference is that the Ade residue of DNA is recognized through the hydrophobic interaction in the hnRNP D complex as mentioned above, whereas it is recognized mainly through hydrogen bonds in the hnRNP A1 complex (37).

An interesting difference between hnRNP A1 and hnRNP D is that only the Thy-2-Gua-4 segment of the d(TTAGGG) unit is recognized by hnRNP D, whereas either the Thy-2-Gua-5 or Thy-1-Gua-5 segment is recognized by hnRNP A1 (37). Exclusive recognition of the Thy-2, Ade-3 and Gua-4 residues by hnRNP D was established by three different lines of evidence: 1) The chemical shift perturbation on complex formation with hnRNP D BD2 was remarkable for the 3 residues, whereas it was moderate for the other residues, if any; 2) almost all intermolecular NOEs with BD2 were detected for the 3 residues (24 NOEs for Thy-2, 33 NOEs for Ade-3, and 34 NOEs for Gua-4), whereas almost no NOEs were observed for the others (only 5 NOEs for Thy-1); and 3) a decrease in the binding affinity was found through the detection of free BD2 on NMR when each of the 3 residues was mutated to a Cyt residue, whereas such a decrease was not found when the others were mutated. It should be noted that the non-recognition of Gua-5 is not due to the use of a short DNA, a 6-mer. In fact, when a longer DNA, d(GTTAGGTGTTA), was titrated with BD2, the chemical shift perturbation was again remarkable for the TAG segment, whereas it was rather moderate for the Gua residue following this segment (data not shown), as was observed for the 6-mer. Moreover, we have already reported that the chemical shift perturbation of hnRNP D on complex formation with a 24-mer comprising four repeating units is essentially the same as that with a 6-mer (10), which indicated that the length of the 6-mer is enough to examine the interactions.

The difference in recognition found in the complex structures of hnRNP A1 and hnRNP D is consistent with the biochemical finding with dimethyl sulfate (DMS) and P1 nuclease footprints that Gua-4 and Gua-5 of the d(TTAGGG) unit are protected by hnRNP A1 from methylation by DMS, whereas only Gua-4 is protected by hnRNP D from methylation by DMS and cleavage by P1 nuclease (even Gua-4 is not protected under some conditions) (3).

The difference in recognition between hnRNP A1 and hnRNP D may be rationalized on the basis of the difference in the primary sequence. The 5’-phosphate of the fifth Gua residue of the second d(TTAGGG) unit of d(TTAGGGTTAGGG) is electrostatically recognized by Arg-140 of hnRNP A1 BD2 (37). The corresponding residue in the primary sequence of hnRNP D BD2 is Asn-217, which cannot undergo the electrostatic interaction. This may account for the difference in the recognition found between the hnRNP A1 and hnRNP D complexes. Moreover, the fifth Gua residue of the first d(TTAGGG) unit of d(TTAGGGTTAGGG) is recognized through the hydrogen bond with Arg-92 of hnRNP A1 BD1 (37). The corresponding residue of hnRNP D BD1 is Met-175, which cannot undergo a similar hydrogen bonding interaction. This may explain the difference in the degree of protection between hnRNP A1 and hnRNP D found in the dimethyl sulfate footprint experiment.
The fact that Gua-5 of the d(TTAGGG) unit, in addition to Gua-6, is not covered by hnRNP D but exposed to the solvent may suggest that the access of telomerase RNA and/or other telomere-binding proteins to telomeric DNA is comparatively easier for the hnRNP D-bound form than the hnRNP A1-bound form. This idea may imply diversity in the details of the involvement in telomere maintenance of homologous proteins hnRNP D and hnRNP A1.

**Biological Implications of Unfolding of the Telomeric DNA Quadruplex by hnRNP D—A Gua-rich strand of minisatellite DNA Pc-1 consists of tandem repeats of d(CAGGG).** We found that the Gua-rich strand forms a quadruplex and causes the arrest of DNA synthesis under physiological conditions (38). The link was implied between the arrest and hypermutable features of Pc-1 and other minisatellite DNA with similar repetitive units. We also found that UP1, a proteolytic product of hnRNP A1, unfolds the quadruplex of Pc-1 and abrogates the arrest of DNA synthesis at the d(GGCAG) repeats (39). Thus, the biological significance of unfolding of the quadruplex in DNA synthesis was revealed. Now, it is known that DNA with the telomeric sequence easily forms a quadruplex under physiological ionic conditions and that the formation of the quadruplex interferes with the elongation of telomere DNA by telomerase (40, 41). Here, we have found that hnRNP D BD2 unfolds the quadruplex of telomeric DNA. These facts suggest the possible involvement of hnRNP D in maintenance of the telomere DNA. In analogy with the role of UP1 as a destabilizer of the deleterious quadruplex in DNA synthesis, hnRNP D may abrogate the interference with the elongation of telomere DNA through destabilization of the telomere quadruplex. By unfolding the quadruplex into a single-strand, hnRNP D may provide the environment for telomerase to function. In this case, hnRNP D functions as a chaperon for DNA, a DNA chaperon that transforms “improperly folded” DNA into “properly folded” DNA for successful elongation by telomerase.

The effect of hnRNP D BD2 on telomerase activity was experimentally examined by means of a primer extension assay in vitro. Consistent with previous reports (40, 41), the quadruplex formation in the presence of K+ caused interference with the elongation by telomerase (Fig. 7A, lane 2). We expected hnRNP D BD2 would abrogate the interference by unfolding the quadruplex, but this was not the case (Fig. 7A, lane 3). The extent of unfolding might not be large enough to abrogate the interference under the current assay conditions. Another possibility is that BD2 bound to an unfolded single-stranded form might hinder the access of telomerase to DNA. To examine this point, the effect of hnRNP D BD2 was studied in the absence of K+ (Fig. 7B). The quadruplex is not formed under these conditions. When BD2 was added, reduction of the elongation was observed (Fig. 7B, lane 2). This suggests that BD2 bound to single-stranded DNA expelled the telomerase from the DNA and/or prevented the telomerase from gaining access to and proceeding along the DNA, resulting in reduction of the elongation in vitro.

It is instructive to refer to the situation for hnRNP A1. We revealed that UP1 unfolds the quadruplex of telomeric DNA (39), which was later supported by another group (42). It was also found that hnRNP A1 interferes with the telomere elongation by telomerase in vitro (24). Thus, hnRNP D and hnRNP A1 (UP1) share two key features. It has been reported that a mouse cell line deficient in hnRNP A1 expression harbors telomeres that are shorter than those of a related cell line expressing normal levels of hnRNP A1. Restoration of hnRNP A1 expression in hnRNP A1-deficient cells increased the telomere length. Telomere elongation was also observed upon the introduction of exogenous UP1 (43). This report clearly indicated the positive effect of hnRNP A1 on telomere elongation in vivo. The apparent discrepancy between the in vitro and in vivo results may partially be explained by the fact that binding of hnRNP A1 to single-stranded telomere DNA provided protection against nucleolytic degradation (24). This protection may contribute to the positive effect on telomere elongation. The same thing can be assumed for hnRNP D. Another possible interpretation of the apparent discrepancy for hnRNP A1 is that a not yet identified cellular factor, which is not present in the in vitro system, may be required for hnRNP A1 to exert the positive effect on telomere elongation in vivo. For example, a factor that promotes the dissociation of hnRNP A1 from DNA after unfolding of the quadruplex might be present in cells to guarantee the access of telomerase to DNA. This kind of factor may also help hnRNP D to exert the positive effect on telomere elongation in vivo as a DNA chaperon.

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