Identification of a new RNA-RNA interaction site for human telomerase RNA (hTR): structural implications for hTR accumulation and a dyskeratosis congenita point mutation

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ABSTRACT
The enzyme telomerase is a ribonucleoprotein that has a critical role in the maintenance of stable telomeres in organisms that possess linear chromosomes. Using a recently developed single molecule fluorescence coincidence method, we have studied the RNA component of telomerase (hTR) and directly observed multimerisation of hTR in solution. RNA mutagenesis and blocking oligonucleotides were employed to identify the single-stranded internal loop J7b/8a as an important and specific hTR-hTR interaction site. This observation was confirmed by studies on a model RNA fragment (hTR380-444), comprising part of the H/ACA domain, the internal loop J7b/8a and the CR7 domain, that was found to dimerise. Substitution mutagenesis within the proposed RNA-RNA interaction site of hTR380-444 resulted in a loss of dimerisation potential and insertion of the dyskeratosis congenita mutation C408G led to a significant reduction in dimer formation. Together, these results suggest that this RNA-RNA interaction site may be functionally relevant.

INTRODUCTION
Telomeres are dynamic nucleoprotein complexes that cap the termini of eukaryotic chromosomes. Telomeres protect the chromosome ends from degradation and fusion and provide a means for the complete replication of the genome (1,2). Telomere maintenance is performed by the enzyme telomerase, which uses part of its intrinsic RNA to template the synthesis of telomeric DNA repeats (GGTTAG in humans) (3,4). Emerging data from various organisms, including ciliates, yeast and vertebrates, indicate that telomerase may act as an interdependent multimer, most probably a dimer, comprising at least two active sites (14-16). Prescott et al. showed that yeast telomerase contains at least two functional RNA moieties both capable of templating DNA synthesis (14). More recently, Wenz et al. demonstrated that human telomerase is active in a dimeric form with two RNA molecules per telomerase complex (15). A heterodimer of human telomerase reconstituted from wild-type and template mutant RNAs is weakly active compared to the wild-type homodimer, suggesting that both hTRs in the human telomerase ribonucleoprotein complex are interdependent and functionally interact with one another (15). During the course of the studies reported in this manuscript, Blackburn and co-workers reported that hTR can dimerise in vitro, using native PAGE, and proposed that the dimeric form is functionally significant for telomerase catalytic activity (17).

Here we report the direct observation of hTR dimer and trimer in solution, in the absence of protein(s) or other accessories, using a new single molecule method. Studies involving deletion analysis and oligonucleotide mapping have identified the internal loop J7b/8a as a specific hTR-hTR interaction site. Deletions and mutations within this region have been previously shown to alter levels of hTR accumulation in cell-based studies (18,19). The experiments described here demonstrate that specific modifications within the J7b/8a region
spermidine, 8 mM MgCl₂, 50 mM NaCl), 10 mM dithio-
template, 13 mM threitol, 2.5 mM each NTP, 200 U RNAguard and 100 U T7 RNA polymerase (Stratagene). Following incubation at 37 °C for 2 h, the DNA template was digested with 2.5 U/g DNA of hTR (hTR208) was generated using PpuMI for plasmid linearisation as described previously (20). Full-length hTR (nucleotides 1–208 (hTR208)) were synthesized from plasmid pUC18-hTR+1 (13)

internal loop J7b/8a is also indicated with its sequence shown in the inset. The self-complementary sequence is indicated in italic. 3¢-Terminal deletion (hTR208) was generated using PpuMI for plasmid linearisation as indicated.

MATERIALS AND METHODS
RNA synthesis and purification
Full-length and truncated telomerase RNAs were prepared by run-off in vitro transcription using T7 RNA polymerase as described previously (19). Full-length hTR (nucleotides 1–451) and hTR fragments spanning nucleotides 1–208 (hTR208) were synthesized from plasmid pUC18-hTR+1 previously digested with either BamHI or PpuMI, respectively (as shown in Fig. 1). The DNA templates (detailed sequences given in Supplementary Material) for the production of hTR380–444, CM-hTR380–444 and DC-hTR380–444 were obtained from Invitrogen. Briefly, in vitro transcription was carried out in a 100 μl reaction mixture containing 2 μg DNA template, 1X T7 buffer (40 mM Tris–HCl, pH 8.0, 2 mM spermidine, 8 mM MgCl₂, 50 mM NaCl), 10 mM dithiothreitol, 2.5 mM each NTP, 200 U RNAguard and 100 U T7 RNA polymerase (Stratagene). Following incubation at 37°C for 2 h, the DNA template was digested with 2.5 U/μg DNA of RNase-free DNase I (Amersham) for 15 min at 37°C. 

In vitro transcribed RNAs were phenol/chloroform extracted and purified by size exclusion chromatography (NAP-5 column; Amersham). After ethanol precipitation, the RNA transcripts were resuspended in water. The integrity and size of the RNA transcripts were determined by agarose gel electrophoresis and staining with ethidium bromide. For the labelling of hTR, GTP was substituted by GTPγS (Roche) to generate hTR transcripts functionalised at the 5¢-end with a thiol-reactive group (hereafter referred to as 5¢-PS-hTR). In vitro transcription was performed as described above.

RNA labelling
The general procedure used to fluorescently label 5¢-PS-hTR was as follows. An aliquot of 1.5 μl of Alexa Fluor 488 or Alexa Fluor 647 maleimide derivatives (Molecular Probes) (10.7 mM, 69.7 μg dissolved in 5 μl of DMSO) was added to 40 μl of 5¢-PS-hTR (1.0 μg/μl, dissolved in Tris–HCl, pH 7.4). The reaction mixture was incubated at 30°C for 2 h and purified by size exclusion chromatography (AutoSeq™G-50 column; Amersham). The labelling efficiency for hTR was determined using a solution of synthetic 100% labelled 40mer DNA at known concentration as a reference. The labelled DNA reference and hTR solutions were analysed by single molecule detection and the number of fluorescent bursts compared directly. The labelling efficiency of hTR was >90% for both Alexa Fluor 488 and Alexa Fluor 647.

Two colour single molecule fluorescence coincidence spectroscopy apparatus
The apparatus used to achieve dual colour single molecule fluorescence coincidence detection has been described previously (21). Briefly, two overlapping laser beams (488 nm, argon ion, model 35LAP321-230 and 633 nm model 25LHP151 HeNe laser; both Melles Griot) were directed through a dichroic mirror and oil immersion objective (Apochromat 60×, NA 1.40; Nikon). The beams were focused 5 μm into a 1 ml sample solution contained in a Lab-TeK chambered coverglass (Scientific Laboratory Suppliers Ltd, UK). The size and divergence of the beams were independently tuned to achieve optimal overlapping confocal volume. Fluorescence was collected by the same objective and imaged onto a 50 μm pinhole (Melles Griot) to reject out-of-focus fluorescence and other background. Green and red fluorescence were then separated using a second dichroic mirror (585DRLP; Omega Optical Filters). Green fluorescence was then filtered by long-pass and band-pass filters (535AF45; Omega Optical Filters) before being focused onto an avalanche photodiode (APD) (SPCM AQA-161; EG&G, Canada). Red fluorescence was also filtered by long-pass and band-pass filters (565ALP and 665AF55; Omega Optical Filters) before being focused onto a second APD (SPCM AQR-141; EG&G, Canada). Outputs from the APDs were coupled to two PC implemented multi-channel scalar cards (MCS-Plus; EG&G, Canada). The maximum overlap of the laser focal volumes (probe volume) was found to be ~30% of the total confocal volume excited by the red and blue lasers.

Single molecule experiment
A freshly prepared stock solution containing equimolar amounts of both Alexa Fluor 488- and Alexa Fluor 647-labelled hTRs (2 nM each) was prepared in TKM buffer (10 mM Tris–HCl, pH 7.4, 100 mM KCl and 10 mM MgCl₂).
The stock solution was incubated at 37°C for 1 h and then diluted to 100 pM total hTR concentration in TKM buffer containing 0.01% Tween-20 prior to the measurements. The diluted sample was mixed and incubated at 20°C for 10 min before measurements. For all the single molecule experiments, data were collected for 90 min at 20°C with a 1 ms bin time on both MCS cards. For oligonucleotide blocking experiments, a labelled hTR transcript was first mixed with a 100-fold molar excess of complementary oligonucleotides (Invitrogen) (Table 1), immediately followed by the addition of the second dye-labelled hTR. The reaction mixture was incubated at 37°C for 1 h before diluting to 100 pM total hTR concentration prior to single molecule analysis as described before. The specificity of the hTR-hTR interaction was verified by mixing one dye-labelled hTR with a 100-fold molar excess of tRNA (Sigma) as a competitor, prior to the addition of the second dye-labelled hTR. The mixture was then incubated at 37°C for 1 h and used for single molecule analysis as described before.

### Coincidence data analysis

Coincidence events were selected by applying a threshold of 15 counts/ms for both channels, while total events were selected by applying the same threshold for either the blue or red channel. The set-up was calibrated with a 40mer dual labelled DNA under identical experimental conditions. In the 30% overlapped confocal volume, the coincidence detection efficiency was measured to be 20% (>15 counts) (21). Assuming 100% labelling efficiency of our set-up (21). An advantage of SmFCS is that the interactions between biomolecules can be investigated in dilute solution under equilibrium conditions. Full-length hTR samples (3 µM) were prepared in the presence or absence of a 100-fold molar excess of blocking oligonucleotide (Table 1), heated for 2 min at 90°C in water and subsequently quenched on ice for 2 min. After the addition of 2-fold concentrated TKM buffer, the sample was allowed to equilibrate for 1 h at 37°C. The sample was loaded onto a 1.0% native agarose gel. Electrophoresis was carried out for 1 h at 4°C and 10 V/cm in TBM buffer (45 mM Tris-borate, pH 8.3, 0.1 mM MgCl₂). RNA bands were visualised by staining with ethidium bromide. Control experiments using tRNA (Sigma) were also performed as described above.

## RESULTS

### Full-length hTR forms multimers in vitro

The multimerisation of hTR was studied using a new technique called single molecule fluorescence coincidence spectroscopy (SmFCS), specifically designed to detect dual labelled species using a two colour excitation/two colour detection set-up (21). An advantage of SmFCS is that the fluorescence bursts of blocking oligonucleotide and tRNA (Sigma) were also performed as described above.

![Image](https://example.com/image.png)

**Table 1. Deoxyoligonucleotide probes used in this study**

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Sequence</th>
<th>Target site</th>
</tr>
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<tbody>
<tr>
<td>c57b/58 10mer</td>
<td>CGCGCCCGGG</td>
<td>J7b/58 internal loop</td>
</tr>
<tr>
<td>c57b/58 22mer</td>
<td>ATCGCCGCGCGCCGCCTCAGG</td>
<td>J5/6 loop</td>
</tr>
<tr>
<td>c56/5 19mer</td>
<td>CGCGCCCGCGCCGCCTCAGG</td>
<td>J6/5 loop</td>
</tr>
<tr>
<td>c56/5 14mer</td>
<td>GACAGGCGCCCAACT</td>
<td>J6/5 loop</td>
</tr>
<tr>
<td>c56/5 22mer</td>
<td>CGCGCTGACAGAGCCAACCTTT</td>
<td>J6 loop</td>
</tr>
<tr>
<td>cL6 14mer</td>
<td>GCCTCCCGAGAAGGC</td>
<td>Hypervariable region</td>
</tr>
<tr>
<td>cL6 26mer</td>
<td>AGTTGGTGCTCCCGAGAAGGCCGG</td>
<td>Hypervariable region</td>
</tr>
<tr>
<td>chTR212–224 14mer</td>
<td>CGACCAGCGCCCAGG</td>
<td>Hypervariable region</td>
</tr>
<tr>
<td>chTR323–349 27mer</td>
<td>ACCTCCGCTCGCCCGGCTGTGTCCCG</td>
<td>Hypervariable region</td>
</tr>
</tbody>
</table>

*The 5’→3’ sequence is indicated from left to right.*
was plotted as a function of $R$ and the histogram was used to analyse the oligomerisation state of full-length hTR$_{451}$ (Fig. 3a). The histogram was fitted by three Gaussian functions. The major peak centred at an $R$ value of 1 (blue line) indicates the presence of hTR$_{451}$ dimers whilst the peaks centred at $R$ values of 0.5 and 2 each indicate the presence of hTR$_{451}$ trimers. Quantitative analysis suggests that at 100 pM total hTR$_{451}$ concentration, 5.5 ± 0.4% is present as dimer and 3.4 ± 0.3% is trimer.

Varying the concentration of hTR$_{451}$ in the initial incubation mixture from 4 nM (total hTR concentration) to 60 nM prior to diluting the sample to 100 pM did not change the level of multimers (i.e. total fluorescence coincidence bursts) or the ratio of dimer to trimer (data not shown), suggesting that single molecule measurements were carried out at equilibrium. Co-incubation of the two labelled hTR$_{451}$ with a 100-fold molar excess of unlabelled tRNA did not change the level of hTR dimer and trimer observed, suggesting that the hTR-hTR interactions are specific (data not shown). Replacing potassium with sodium in the buffer, or the inclusion of 10 mM Mg$^{2+}$, had no significant effect on either the total level of multimerisation or on the dimer to trimer ratio (data not shown). The single molecule experiments were corroborated by classical gel electrophoresis. hTR$_{451}$ (3 μM) was incubated at 37°C for 1 h then analysed by non-denaturing agarose electrophoresis (Fig. 4). Under these conditions, three species of RNA can be detected consistent in size with a monomer, a dimer and a trimer (Fig. 4, lane 3). In a control experiment, hTR$_{451}$ was thermally melted by heating to 90°C and then rapidly quenched on ice, giving only monomeric species (Fig. 4, lane 2), consistent with observations made for other interacting RNA systems (22). The results of the gel experiments are in good qualitative agreement with the single molecule experiments and indicate that hTR$_{451}$ can form dimer and trimer in vitro. However, it has been noted by ourselves and others (23) that the relative intensities of bands in gel experiments can vary with electrophoresis conditions, suggesting that agarose gel electrophoresis may not be the best approach for quantitative analysis of equilibrium distributions.

Whilst one hTR-hTR interaction site has already been proposed (17), the observation of trimers suggests the presence of at least one more interaction site. Two-site interacting RNA-RNA systems are known to exist and play diverse roles, for example in the genome of HIV-2 (24), the

![Figure 2. Principle of the detection of hTR multimers by single molecule coincidence spectroscopy. Individual multimers carrying both labels that enter the probe volume will give rise to coincident bursts of red and blue photons. Individual monomers will produce only red or blue photons.](https://academic.oup.com/nar/article-abstract/31/22/6509/2375980)

![Figure 3. Coincident burst histograms representing the two colour ratio $R$ ($R = I_{red}/I_{blue}$) for full-length hTR (a) and fragment hTR$_{380-444}$ (b). The samples were diluted to 100 pM (total hTR concentration) in TKM buffer (10 mM Tris- HCl, pH 7.4, 100 mM KCl and 10 mM MgCl$_2$) and allowed to stand for 10 min at 20°C. The data were collected for 90 min with a 1 ms bin time on both MCS cards. For histogram (a), the ratio distributions were fitted to three Gaussian functions with means of 1 (hTR dimers), 0.5 and 2 (trimers). For histogram (b), the ratio distributions could be fitted to a single Gaussian function with a peak value of 1. For all experiments, the total number of events was comparable (35 000 ± 4600).](https://academic.oup.com/nar/article-abstract/31/22/6509/2375980)

![Figure 4. In vitro hTR-hTR interaction of hTR$_{451}$ studied by agarose gel electrophoresis. For each experiment, hTR$_{451}$ was first heat denatured in water for 2 min and quench-cooled on ice for 2 min. Following the addition of TKM buffer (10 mM Tris- HCl, pH 7.4, 100 mM KCl, 10 mM MgCl$_2$), the samples were incubated at 37°C for 1 h and analysed by electrophoresis on a 1.0% non-denaturing agarose gel in TBM buffer (45 mM Tris- HCl, pH 8.3, 0.1 mM MgCl$_2$). Lane 1, RNAs molecular weight marker; lane 2, monomeric hTR$_{451}$ control (ice quenched); lane 3, hTR$_{451}$; lanes 4 and 5, hTR$_{451}$ in the presence of a 100-fold molar excess of c77b8a 10mer and cL6 26mer, respectively. Arrows indicate hTR monomer, dimer and trimer.](https://academic.oup.com/nar/article-abstract/31/22/6509/2375980)

bacterial virus ϕ29 RNA (25) and the 3′-UTR of bicoid mRNA (26). The next series of studies were aimed at localising a second hTR interaction site.

A region in the 3′-end of hTR is important for hTR-hTR interaction

To localize a second site for hTR-hTR interaction, the multimerisation potential of hTR$_{451}$ was first compared with that of the truncated RNA fragment spanning nucleotides 1–208 (hTR$_{208}$). hTR$_{208}$ was generated by in vitro transcription of plasmid pUC18-hTR+1 linearised with PpuMI (Fig. 1) and was then 5′-end-labelled with either Alexa Fluor 488 or
events for hTR 208 did not permit resolution of relative close to background levels. The low number of coincidence 209±451. At significantly higher RNA concentrations (3
amounts of dimer and trimer. This experiment suggested the
in nucleotides 209±451 may be mediated via specific
nucleotides 1±208 (17).

Figure 5A presents the relative effect of truncation on
the proportion of multimer formed, normalised to the level
normalised with respect to hTR 380±444. The data have been corrected for
background and the y-axis error bars represent the standard deviation of at
least duplicate experiments. (Inset) Predicted hTR380−444 secondary struc-
ture. Residues 380−444 contain a partial HACA domain and a full CR7
domain. Red italic denotes the two mutations studied.

Figure 5. (A) hTR-hTR interactions as detected by SmFCS. hTR451, full-length hTR; hTR208, truncated hTR transcripts; hTR451+CJ7b/8a-10 mer, hTR451 in the presence of a 100-fold excess of CJ7b/8a 10mer; hTR451+ CL6-26 mer, hTR451 in the presence of a 100-fold excess of CL6 26mer. The relative extent of multimer formation was normalised with respect to hTR451. The data has been corrected for background and the y-axis error bars represent the standard deviation of at least duplicate experiments. (B) Effect of DKC-associated mutation and control mutation on dimerisa-
tion of the model. RNA fragment hTR380−444, hTR fragment containing nucleotides 380−444; DKC-hTR380−444, hTR380−444 with the single point mutation (C408G) in the CR7 domain; CM-hTR380−444, hTR380−444 with substitution of 395GCGC398 by 395UUUU398. The sequence was numbered according to full-length hTR. The relative extent of dimer formation was normalised with respect to hTR380−444. The data have been corrected for background and the y-axis error bars represent the standard deviation of at
least duplicate experiments. (Inset) Predicted hTR380−444 secondary struc-
ture. Residues 380−444 contain a partial HACA domain and a full CR7
domain. Red italic denotes the two mutations studied.

Alexa Fluor 647 as described before. SmFCS studies indicate that hTR208 multimerises to a substantially lower extent than hTR451. Figure 5A presents the relative effect of truncation on the proportion of multimer formed, normalised to the level found for hTR451. Under the conditions of this experiment (i.e. 100 pM RNA concentration) hTR208 multimerisation was close to background levels. The low number of coincidence events for hTR208 did not permit resolution of relative amounts of dimer and trimer. This experiment suggested the presence of an important hTR-hTR interaction at nucleotides 209−451. At significantly higher RNA concentrations (3 μM) hTR208 does exhibit dimer formation by gel electrophoresis (data not shown), consistent with the reported interaction site in nucleotides 1−208 (17).

We hypothesised that a hTR-hTR interaction within nucleotides 209−451 may be mediated via specific RNA-RNA base pairing interactions. Oligonucleotide probes that selectively base pair with hTR have the potential to block specific interactions and were therefore employed to identify a potential recognition element(s). This approach has been used to locate RNA recognition elements in other RNAs, for example HIV genomic RNA (24,27) and tRNA (28). Blocking experiments were focused on targeting single-stranded RNA loops and bulges present between nucleotides 209 and 451 of hTR. Oligonucleotides (Table 1) complementary to the L6 loop, the J5/J6 loop, the J6/J5 loop and the hypervariable paired region of hTR451 (Fig. 1) were found to have no effect on the level of hTR multimer formed nor on the dimer to trimer ratio, by SmFCS analysis, suggesting no detectable recognition sites in these targeted regions. As an example, the data for blocking access to apical loop L6 using the complementary 26mer oligonucleotide cL6 26mer is given in Figure 5A. However, a pair of oligonucleotides (cJ7b/8a 22mer and cJ7b/8a 10mer) (Table 1), each targeting the J7b/8a internal loop, significantly reduced the formation of hTR multimer. For example the oligonucleotide cJ7b/8a 10mer exerted a significant inhibitory effect, reducing the level of hTR multimerisation down to just 12% compared to hTR451 (Fig. 5A). At this reduced level of multimerisation (i.e. low number of fluorescence coincidence events), it was not possible to resolve the ratio of dimer to trimer. Gel electrophoresis experiments corroborate these results, showing a significant reduction in dimer formation and disappearance of trimer in the presence of cJ7b/8a 10mer (Fig. 4, Lane 4) or cJ7b/8a 22mer (data not shown) and no observed effect with other oligonucleotide probes that effectively serve as controls (see for example Fig. 4, Lane 5).

Dimerisation of hTR380−444 and reduction in dimerisation by a DKC mutation

The hTR fragment comprising nucleotides 380−444 (hTR380−444) is a functionally important domain that is required for the in vivo accumulation of hTR (18,19,29) and, furthermore, is the site for a DKC-specific point mutation (8). Since hTR380−444 contains the J7b/8a loop RNA interaction site, the fragment was employed as a minimal model system to explore a possible structure–function relationship. Structural prediction by MFOLD (30) suggested that the fragment hTR380−444 would adopt the same secondary structure as that proposed for full-length hTR451 (13). hTR380−444 was prepared by in vitro transcription using synthetic DNA template, and 5′-end-labelled with either Alexa fluor 488 or Alexa fluor 647, before subjecting a 1:1 mixture of the fluorescently labelled fragments to SmFCS analysis. The plot of the number of coincidence events as a function of R is shown in Figure 3b. The only major peak centred at an R value of 1 (blue line) indicates the presence of dimer. The absence of peaks at R values of 0.5 and 2 indicated that no trimer was formed, confirming the presence of just one significant hTR-hTR interaction site within this fragment. At 100 pM concentration 8.0 ± 0.3% of hTR380−444 is present as dimer. The histogram in Figure 5B shows the effect of specific nucleotide changes on dimerisation of this RNA fragment. Substitution of 395GCGC398 in the J7b/8a loop of hTR380−444 with 395UUUU398 (CM-hTR380−444) (Fig. 5B, inset) reduced dimer formation to near background levels (Fig. 5B). CM-hTR380−444 was predicted by MFOLD (30) to have the same secondary structure as wild-type hTR380−444, further supporting the J7b/8a loop as a specific recognition element for hTR-hTR interaction. The C408G point mutation associated with DKC
Here, we have identified the J7b/8a internal loop as a distinct hTR-hTR interaction site. Whilst the main results were confirmed by non-denaturing gel electrophoresis, the additional sensitivity of single molecule measurements at relatively low concentrations has enabled some discrimination of RNA-RNA interactions. For example, the pseudoknot-mediated interaction observed for fragment hTR208 by gel electrophoresis (at 3 μM RNA) is not detectable at 100 pM concentration in solution, whereas interaction via the J7b/8a loop (and modulation of this interaction) is easily detectable at 100 pM concentration in either hTR451 or hTR380-444. This suggests that the interaction at the J7b/8a loop is stronger than that in hTR208.

Loop–loop or ‘kissing loop’ interactions, via specific base pairing, are a recurrent theme in RNA recognition (32,33), suggesting that they may have been selected as well-adapted structural motifs for promoting initial RNA-RNA recognition. The J7b/8a loop of hTR contains the self-complementary sequence 5’-392CGCGCGCG399-3’. Experiments that either blocked or substituted these RNA bases all resulted in a significant reduction in RNA-RNA interaction, confirming that this loop engages in sequence-specific recognition as part of the structural mechanism for hTR oligomerisation. Comparison with HIV-1 shows that the identical recognition sequence is used in an RNA loop for dimersisation of the genomic RNA (32–34). It would therefore appear reasonable that the self-recognition provided by this RNA sequence is sufficiently stable to support the proposed RNA-RNA interaction in vivo. Based on the resemblance with dimerisation of HIV-1 genomic RNA (32,33), we propose a similar model for the dimerisation of hTR in which annealing of the self-complementary sequence (5’-392CGCGCGCG399-3’) of internal loop J7b/8a through a kissing loop interaction triggers hTR oligomerisation (Fig. 6).

Full-length hTR (Fig. 1) can be broadly divided into two regions based on functionality. The 5’-portion of hTR (domains CR1–CR5; Fig. 1) is important for catalytic activity (13,35). The 3’-portion of hTR comprising Box H/ACA, loop J7b/8a and CR7 is important for controlling the stability and accumulation of hTR in vivo (18,19,29,36). The presence of a sequence-specific hTR-hTR interaction site in the J7b/8a loop raises the possibility that dimerisation via this domain may be linked with the associated function. There are a number of lines of evidence that suggest this hypothesis. Our results have demonstrated that substitution of nucleotides 395–398 in the RNA fragment hTR380-444 with UUUU abolishes dimerisation to 35 ± 8% of wild-type levels. Four conformations for DKC-hTR380-444 were predicted by Mfold (30) (see Supplementary Material), of which only one preserves the single-stranded self-complementary recognition site.

**DISCUSSION AND CONCLUSIONS**

We have employed a novel single molecule fluorescence coincidence method to detect and measure the multimerisation of hTR in solution. At 100 pM concentration in free solution nearly 10% of hTR molecules exist as dimers and trimers. hTR has been estimated to be present at levels of ~10⁴ molecules/cell in telomerase-negative cells and ~10⁵ molecules/cell in cancer cells (31). Assuming linear cell dimensions of ~10 μm, cellular concentrations of hTR are likely to be in the range 10–100 nM, thus suggesting that hTR has the potential to exist substantially in its multimeric state in vivo. The observation of hTR trimers has suggested at least two sites for hTR-hTR recognition. Blackburn and co-workers recently identified the pseudoknot domain as one site for hTR-hTR recognition (17).
that the C408G substitution induces a structural change of the isolated hTR 3’-hairpin (nucleotides 410–429), leading to multiple conformational states (37). Our studies on the hTR380–444 fragment have shown that the C408G mutation leads to a significant reduction in dimerisation capacity. We predicted four alternative structures for DKC-hTR380–444 by MFOLD (30), of which only one retains the capacity to form the loop–loop interaction (see Supplementary Material). This provides a structural consequence that may be linked with the corresponding DKC-related telomerase dysfunction.

In summary, we propose a specific hTR–hTR interaction site in the J7b/8a loop of hTR. Our experimental results, taken together with published cell biological studies (18,19), have led us to propose that this hTR–hTR interaction may be functionally important for hTR accumulation and the assembly of telomerase ribonucleoprotein in vivo. Accordingly, a structural consequence of the DKC C408G mutation is reduced hTR–hTR interaction.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

ACKNOWLEDGEMENTS

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REFERENCES

19. Supplementary Material is available at NAR Online.