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hnRNP A2, a potential ssDNA/RNA molecular adapter at the telomere

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ABSTRACT

The heterogeneous nuclear ribonucleoprotein (hnRNP) A2 is a multi-tasking protein that acts in the cytoplasm and nucleus. We have explored the possibility that this protein is associated with telomeres and participates in their maintenance. Rat brain hnRNP A2 was shown to have two nucleic acid binding sites. In the presence of heparin one site binds single-stranded oligodeoxyribonucleotides irrespective of sequence but not the corresponding oligoribonucleotides. Both the hnRNP A2-binding *cis*-acting element for the cytoplasmic RNA trafficking element, A2RE, and the ssDNA telomere repeat match a consensus sequence for binding to a second sequence-specific site identified by mutational analysis. hnRNP A2 protected the telomeric repeat sequence, but not the complementary sequence, against DNase digestion: the glycine-rich domain was found to be necessary, but not sufficient, for protection. The N-terminal RRM (RNA recognition motif) and tandem RRMs of hnRNP A2 also bind the single-stranded, template-containing segment of telomerase RNA. hnRNP A2 colocalizes with telomeric chromatin in the subset of PML bodies that are a hallmark of ALT cells, reinforcing the evidence for hnRNPs having a role in telomere maintenance. Our results support a model in which hnRNP A2 acts as a molecular adapter between single-stranded telomeric repeats, or telomerase RNA, and another segment of ssDNA.

INTRODUCTION

The ends of linear chromosomes pose a problem for the DNA replication machinery. During replication, the lagging strand is left with a region that cannot be replicated (1,2). In the absence of any compensatory mechanism, successive replications lead to the loss of potentially essential DNA from the ends of the chromosomes. This shortening is minimized by capping the chromosome ends with repetitive sequences, TTAGGG in vertebrates (3,4) of expendable DNA. These telomeric regions are 3–15 kb long in human somatic cells. Progressive telomeric shortening limits the number of divisions a human cell can undergo: telomere attrition is associated with chromosome instability and cellular senescence (5).

In cells of the germ line and in most cancer cells, telomere length is preserved by the action of a reverse transcriptase, telomerase, which consists of a protein catalytic component, other protein subunits and an essential RNA subunit (hTR) that acts as a template for the synthesis of the telomeric repeat DNA (6,7). Some immortalized mammalian cell lines and tumors maintain their telomeres in the absence of telomerase activity by one or more mechanisms referred to as alternative lengthening of telomeres (ALT) that may involve recombination and copy switching (8–10). ALT cell lines are also characterized by the presence of novel nuclear structures, the ALT-associated PML bodies (APBs), which contain the promyelocytic leukemia protein (PML), telomere repeat binding factors 1 and 2 (TRF1 and TRF2), several proteins involved in DNA repair and recombination, and extrachromosomal telomeric DNA (11–13).

The double-stranded segment of telomeres, like the rest of the genome, is wrapped up into nucleosomes formed with histones and non-histones, and additional telomere-specific proteins (14,15). The end portion, which includes a

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30–200 nt single-stranded 3′ overhang, may not be contained within this structure, but rather bound and protected by a different set of proteins. The proteins bound and the conformation of the 3′ overhang may vary depending on whether or not the DNA is being actively extended by telomerase. When not being extended, the DNA is thought to be looped back with the overhang displacing a portion of the double-stranded telomere and thereby forming D- and t-loops. Loop formation is promoted by TRF2 (16–18) which, with TRF1, has been characterized as a negative regulator of telomere length (19,20).

A number of heterogeneous nuclear ribonucleoproteins (hnRNPs), including C1/C2 (21), D (22,23), E (24) and K (25,26), have been reported to bind either the telomere repeat (*in vitro*) or hTR (*in vitro* and *in vivo*). hnRNP A1 and UP1, its proteolytic derivative comprising the tandem RNA recognition motifs (RRMs), bind the telomeric repeat sequence-specifically (24,27) and protect it from nuclease digestion *in vitro* (28,29). Both hnRNP A1 and UP1 also bind hTR *in vitro* (30), and UP1 (but not hnRNP A1) binds the telomerase holoenzyme (28,31). hnRNP A1 and UP1 can bind telomerase RNA and telomeric DNA simultaneously *in vitro* (30), suggesting that hnRNP A1/UP1 not only protects the single-stranded telomeric DNA but also recruits telomerase to telomeres (32).

Mouse and human hnRNP A2 also bind the telomeric repeat sequence (24,33,34), as do their alternatively spliced isoforms, B1 and B0b (35) [the latter renamed A2b (36)]. A2b also promotes telomerase-mediated extension of a template oligonucleotide *in vitro*. It was proposed that because the expression of hnRNP B1 is upregulated in cancer cells (37–42), the unusually high levels of this protein may provide a mechanism by which telomere length could be maintained, and telomerase activity promoted inappropriately, thus allowing rapid and ongoing division of cells (35). Recent experiments in which small interfering RNAs were used to lower the levels of hnRNPs A1 and A2 in several cancer cell lines, transformed cells and normal cells reinforce the concept that these proteins function as telomeric capping factors (43).

We show here, using pull-down and electrophoretic mobility shift assays (EMSAs), that hnRNPs A2 and A3 are the predominant single-stranded telomere repeat binding proteins in the rat brain. hnRNP A2 was found to possess two oligonucleotide binding sites. One site binds ssDNA with little or no nucleotide sequence preference whereas the second binds oligoribonucleotides or oligodeoxyribonucleotides sequence-specifically. Mutational analysis has identified a consensus nucleotide sequence for binding to the latter site that is consistent with the association with the human single-stranded TTAGGG telomere repeat and the A2RE11 cytoplasmic RNA trafficking element (44). Using purified hnRNP A2 domains, we have established that both RRMs are required for association with the telomeric repeat, whereas the RRM1 alone, of hnRNP A1, has been reported to suffice for repeat binding (28). Intact hnRNP A2 protein, but neither individual or concatenated RRMs nor the Gly-rich domain (GRD), is able to protect telomeric DNA from DNase. In contrast, the concatenated RRMs of hnRNP A1 are sufficient for protection from DNase (28). hnRNP A2 and its RRM concatamer also bind the telomerase RNA, with only weak binding to the isolated RRM1, and none to RRM2, in contrast to hnRNP A1. Thus hnRNP A2 can potentially bind the telomeric DNA repeat and the RNA component of telomerase simultaneously, or it may bind ssDNA in both sites thus acting as an intramolecular or intermolecular cross-link. Finally, we show for the first time that hnRNP A2 is colocalized with TRF2 and PML in the subset of APB nuclear bodies. These data are consistent with a role for hnRNP A2 in protecting telomeric DNA *in vivo* against nuclease digestion, and in recruiting hTR to the telomere.

MATERIALS AND METHODS

Protein expression and purification

Human hnRNP A2 (44) and domains of this protein, residues 1–89 (RRM1), 101–179 (RRM2), 1–189 (RRM1+2′) and 189–341 (GRD), were expressed in *Escherichia coli* and purified as previously described (45). The domains were expressed with N-terminal hexahistidine tags that were subsequently cleaved with enterokinase, leaving each with the N-terminal extension Ala-Met-Ala-Ile-Ser from the expression vector. The tag was not removed from GRD. The resultant proteins were isolated by reverse-phase high-performance liquid chromatography (HPLC) on a C18 column using a gradient of acetonitrile in 0.1% trifluoroacetic acid (TFA), lyophilized twice to remove TFA and allowed to refold at low concentration near pH 7. The identity and purity of all proteins was established by PAGE in the presence of SDS and by electrospray mass spectrometry on a PerkinElmer Sciex 165 spectrometer; all were judged to be better than 90% pure.

Brain protein extraction

Rat brain proteins were extracted as previously described (46). Tissues were removed from 21-day-old Wistar rats and placed in ice-cold extraction buffer [20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid), 0.65 M KCl, 2 mM EGTA, 1 mM $MgCl_2$, 2 M glycerol, 14.3 mM β-mercaptoethanol, 8.7 mM NP-40, 12.1 mM deoxycholic acid, 1 mM phenylmethylsulfonyl fluoride and 21 μM leupeptin, pH 7.4] immediately prior to homogenization. The homogenate was centrifuged at 13 000 *g* for 40 min at 4°C and the top two layers were removed and kept at 4°C.

Affinity isolation and analysis of nucleic acid-binding proteins

Oligonucleotides biotinylated on the 3′ nucleotide with the following sequences (here specified only for the oligoribonucleotides) were synthesized by Proligo (Singapore): ZIP, GCG GAC UGU UAC UGA GCU GCG UUU UAC ACC CUU; A2RE, GCC AAG GAG CCA GAG AGC AUG; A2RE11, GCC AAG GAG CC; AURE, GUU UAU AAU UUU UUU AUU ACU G; NS1, CAA GCA CCG AAC CCG CAA CUG; Telo1, TTAGGG; Telo3, $(TTAGGG)_3$; Telo6, $(TTAGGG)_6$; Anti6, $(CCCTAA)_6$. Streptavidin-coated superparamagnetic particles and magnetic particle separators were purchased from Roche (Mannheim, Germany). For each assay 0.5 mg of magnetic particles was incubated on ice for 10 min with 2.5 μg of biotinylated oligonucleotide in a 250 μl solution of 10 mM Tris–HCl, 1 mM EDTA and 100 mM NaCl, pH 7.5. Unbound nucleic acid was washed off with buffer containing 10 mM Tris–HCl, 1 mM EDTA and 1 M NaCl, pH 7.5. About 5 mg of brain protein was added to 0.5 mg of the labeled

magnetic particles. Binding took place for 30 min on ice in 1 ml of binding buffer (10 mM HEPES, 3 mM $MgCl_2$, 40 mM NaCl, and 5% glycerol, pH 7.5) with 10 g/l heparin added to reduce non-specific binding. The particles were washed with binding buffer before protein bound to the particles was released by incubation at 65°C for 10 min in 30 µl of 0.1% SDS, 0.5% β-mercaptoethanol and 0.01% bromophenol blue. The resultant protein solution was electrophoresed on a 12% polyacrylamide gel containing SDS and stained with Coomassie blue, or electroblotted onto polyvinylidene difluoride (Immobilon-P, Millipore, Bedford, MA) membrane using Tris/glycine transfer buffer at 4°C. The antibodies used for westerns have been described previously (47).

DNase digestion

^{32}P-labeled oligonucleotides Telo6, $(TTAGGG)_6$, and Anti6, $(CCCTAA)_6$, were purified for DNase protection assays. Following labeling of the DNA, 10 µl of dye (0.35% Orange G (Sigma), 30% sucrose and 2% w/v SDS) was added and the samples were electrophoresed on a 15 cm 20% polyacrylamide/7 M urea gel. Gels were subsequently exposed to X-ray film for 1 min. The region of gel corresponding to the major signal on the autoradiograph was then excised and placed into 6 vol of buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8, 0.1% SDS), and incubated at 50°C for 30–60 min. The DNA was then purified using a QIAEX II DNA purification kit (Qiagen, Hilden, Germany).

Protein was added to buffer (10 mM HEPES, pH 7.5, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 1 mM DTT and 1g/l heparin) to a final vol of 10 µl prior to the addition of labeled nucleic acid and incubation at 30°C for 30 min. Five units of deoxyribonucleotidase I (DNase I; Sigma) was then added, and the reaction volume was made up to 20 µl with DNase buffer (10× DNase buffer: 400 mM Tris–HCl, pH 8, 100 mM $MgSO_4$, 10 mM $CaCl_2$), and incubated at 37°C for a further 30 min. The reaction was stopped by incubation at 65°C for 10 min. About 10 µl of Orange G/SDS dye was then added, and the samples were electrophoresed through 15 cm 20% polyacrylamide/urea gels and the DNA was detected by autoradiography.

Human telomerase RNA transcription and binding

The hTR/pGEM-T vector containing the cDNA for human telomerase RNA (48) was linearized and residual proteins and salts were removed from the digested vector by ethanol precipitation. The DNA pellet was dissolved in water and quantified. Labeled *in vitro* transcriptions were performed using Promega's Riboprobe *in vitro* Transcription Systems and ^{32}P αUTP (PerkinElmer Life Sciences, Norwalk, CT). Transcripts of the hTR/pGEM-T vector were obtained using SP6 RNA polymerase and the complementary hTR transcript was generated using the T7 RNA polymerase. To ensure the correct size of the generated transcripts, Century Plus RNA markers (Ambion, Austin, TX) were also used, transcribed using the T7 RNA polymerase. Following transcription and DNase treatment (RQ1 RNase-free DNase; Promega), the reactions were stopped using 95% formamide/bromophenol blue (Sigma), before electrophoresis through 5% polyacrylamide/urea gels and detection by autoradiography. Using the autoradiograph and the RNA markers as a guide, slices of the polyacrylamide/urea gel containing the desired transcript were excised and the RNA therein was purified using a modified version of the protocol described by Chabot (49). This entailed placing the excised gel slice into a solution of 0.5 M ammonium acetate, 1 mM EDTA (made up in DEPC-treated water) and incubating it at 4°C overnight on a roller or at 65°C for 1 h. The supernatant was then transferred to a fresh tube, 2 vol of 100% ethanol was added and precipitation was allowed to proceed overnight at −20°C. The precipitate was collected by centrifugation at 16 000 *g* at 4°C and washed with 70% ethanol. The resulting pellet was then air-dried, redissolved in nuclease-free DEPC-treated water and stored at −80°C.

The binding of protein to the transcribed RNA was performed in 10 µl buffer containing 10 mM HEPES, pH 7.6, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 75 mM KCl, 1 mM DTT and 1 g/l heparin. RNA was added prior to the addition of protein. The reaction mixtures were incubated on ice for 5 min, before the addition of 2 µl of RNA/protein gel loading buffer (30 mM Tris–HCl, pH 7.5, 40% sucrose, 0.2% bromophenol blue, made up in DEPC-treated water). Samples were then electrophoresed through non-denaturing 5% polyacrylamide gels and autoradiographed. In some experiments the RNA was denatured by heating to 95°C and then cooled immediately before use in the binding assays.

UV-cross-linking EMSAs

Recombinant proteins were obtained as described previously (45). About 1.5 pmol of each oligonucleotide, ^{32}P-labeled with T4 polynucleotide kinase (New England Biolabs, Beverly, MA), and 10 pmol of each protein were mixed in binding buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 4% w/v glycerol) with a final volume of 15 µl, and incubated for 20 min on ice. The reaction mixtures were then irradiated with 500 mJ of 254 nm light in a Bio-Rad GS Genelinker UV chamber. These samples were electrophoresed on 15% SDS–polyacrylamide gels and autoradiographed. In competition experiments, the cold competitor (75 pmol) and radiolabeled oligonucleotide were added prior to addition of protein.

Mutational analysis of oligonucleotide binding

The consensus sequence for binding to the specific site on hnRNP A2 was obtained from competition UV-cross-linking experiments performed as described above. Oligonucleotides were synthesized, each containing a single nucleotide change from the wild-type A2RE11 sequence. About 10 pmol of recombinant hnRNP A2, ^{32}P-labeled dA2RE11 (the oligodeoxyribonucleotide equivalent of A2RE11), and 50-fold of mutated oligodeoxyribonucleotide (Genset Oligos, Lismore, Australia) sequence were incubated, irradiated and run on 15 cm 15% SDS–polyacrylamide gels. Starting with the A2RE11 sequence, competition with a 50-fold excess of each of the other three deoxynucleotides, one at a time, at each position was examined. Binding to the specific site was distinguished from non-specific binding by comparison of the EMSAs with the A2RE11 mutants and with the non-specific oligodeoxyribonucleotide dNS1.

Colocalization of PML, hTRF2 and hnRNP A2 in cell nuclei

The localization of the three proteins PML, TRF2 and hnRNP A2 was determined in telomerase-negative JFCF-6T.1J/1–4D

cells (SV40-immortalized human fibroblasts) and telomerase-positive JFCF-6T.1J/6B cells. Immunohistochemistry was performed on cells fixed to glass slides with 2% paraformaldehyde and permeabilized with methanol/acetone (1:1 v/v; −20°C for 15 min). The primary antibodies used include a goat polyclonal against PML (sc9862; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse monoclonal against hTFR2 (#05-521; Upstate Biotechnology, Lake Placid, NY) and a rabbit polyclonal against hnRNP A2/B1 (47), which were visualized with Texas Red-, AMCA- and FITC-conjugated donkey secondary antibodies, respectively (Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were mounted with 90% glycerol containing DABCO (Sigma) antifade. Slides were analyzed on a Leica DMLB epifluorescence microscope with appropriate filter sets. Separate images were taken with a cooled CCD camera (SPOT2; SciTech, Preston South, VIC, Australia) and further processed and merged using SPOT software.

RESULTS

hnRNP A2 has a site that binds ssDNA with little or no sequence preference

Pull-down experiments were performed in the presence of heparin with four different oligoribonucleotides and the corresponding oligodeoxyribonucleotides separately immobilized on superparamagnetic beads. The same molar amount of oligonucleotide, and an excess of protein extract sufficient to saturate the binding sites on the particles, was used in each experiment. As anticipated, hnRNP A2 was detected in the A2RE11 and dA2RE11 pull downs, and the three oligoribonucleotides with unrelated sequences did not bind hnRNP A2 in the presence of heparin (44,45). However, the other three oligodeoxyribonucleotides also bound this protein (Figure 1), which taken together with earlier observations indicates that it has a site that binds ssDNA more tightly than the corresponding RNA with little or no nucleotide sequence preference. Such a site was earlier detected on recombinant hnRNP A2 expressed in bacteria, but binding of oligoribonucleotides or oligodeoxyribonucleotides to this non-specific site was eliminated on addition of heparin, which minimizes non-specific interactions (45). Similarly, hnRNP A2 was isolated from rat tissue extracts in pull-down experiments performed in the presence of heparin with immobilized A2RE but not with an oligoribonucleotide with scrambled sequence (44). Thus, some feature of the rat hnRNP A2, which may be a post-translational modification or association with other molecules, greatly strengthens oligodeoxyribonucleotide binding to the non-specific site over that manifested by the recombinant protein.

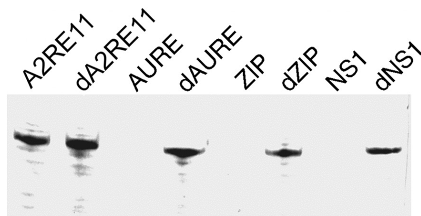

Figure 1. Rat hnRNP A2 has sequence-specific and non-specific sites for binding oligodeoxyribonucleotides. A western blot of protein binding to oligodeoxyribonucleotides and oligoribonucleotides immobilized on superparamagnetic beads in pull-down experiments with brain protein extracts. Heparin was added to the protein extract to suppress non-specific binding. The protein was detected with a polyclonal primary antibody to a peptide from human/mouse hnRNP A2, and an alkaline-phosphatase-conjugated secondary antibody. These oligonucleotides were: the A2RE11 trafficking element (A2RE11 and dA2RE11, the former being the oligoribonucleotide), the AU-rich element (AURE and dAURE), the β-actin mRNA zipcode (ZIP and dZIP) and the non-specific sequence (44,46) (NS1 and dNS1). With the exception of the A2RE11, which binds to a specific site on hnRNP A2, the oligodeoxyribonucleotides but not the oligoribonucleotides bound hnRNP A2.

hnRNP A2 exhibits sequence-specific binding to single-stranded telomeric DNA

Pull-down experiments with the telomere repeat immobilized on superparamagnetic particles isolated several proteins from rat brain protein extracts that were not bound to particles bearing no DNA or an oligonucleotide with scrambled sequence (45,46) (Figure 2A). These proteins were identified from their apparent molecular masses on SDS–polyacrylamide gels and by western blotting (Figure 2B) as hnRNPs A1, a minor component as judged by Coomassie Blue R250 staining, and A2 and their less abundant isoforms, and four isoforms of hnRNP A3 (47). These three proteins share high sequence identity and notably the residues of hnRNP A1/UP1 identified by X-ray crystallography as participating directly in telomeric DNA–protein interactions (27,50) are conserved between

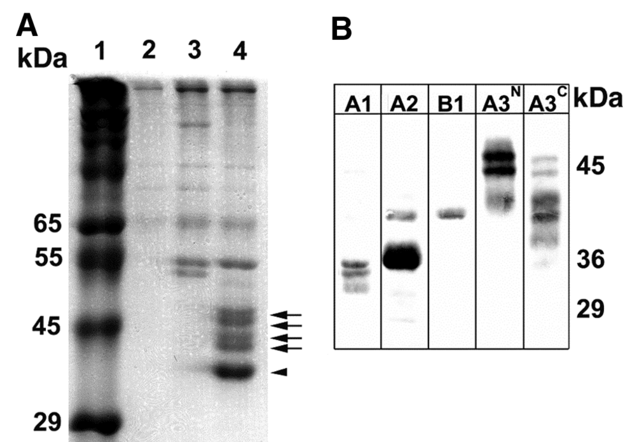

Figure 2. hnRNPs A1, A2, A3 and their isoforms are isolated in pull downs with a telomeric oligonucleotide. (**A**) Rat brain protein extract was incubated with superparamagnetic particles bearing no oligonucleotide (track 2), the scrambled A2RE ribonucleotide NS1 (track 3) or $d(TTAGGG)_4$ (track 4). Bound proteins were eluted using the SDS–gel electrophoresis sample preparation solution. Proteins were separated on a 15 cm 12% SDS–polyacrylamide gel and stained with Coomassie Blue R250. The four arrows on the right indicate the hnRNP A3 isoforms (47) and the arrowhead indicates hnRNP A2. The masses of standard proteins (track 1) are shown in kDa (the standards used in this experiment suggest that the slowest migrating hnRNP A3 band has a molecular mass over 45 kDa; however, it normally migrates with an apparent mass of 42 kDa). (**B**) Proteins isolated as above were separated by SDS–PAGE and electroblotted onto a polyvinylidine difluoride membrane. Antibodies directed against peptides in hnRNPs A1, A2/B1(labeled A2), B1, and the two higher molecular weight isoforms ($A3^N$) and all four isoforms of hnRNP A3 ($A3^C$) were used to demonstrate the presence of these proteins in the eluate (47). Molecular masses, in kDa, are indicated on the right.

hnRNPs A1 and A3 and, with one conservative (basic to basic) change, between hnRNPs A1 and A2. The observed binding of the telomeric DNA repeat to both hnRNPs A1 and A2 (24,33) is consistent with the conservation of the RNA-binding residues on these proteins.

As noted above, hnRNPs A2 (45) and A3 (47) were previously shown to possess two sites for binding a *cis*-acting cytoplasmic RNA trafficking element, A2RE or A2RE11, one specific for this oligonucleotide sequence and the other with very weak sequence dependence. The binding of the deoxyribonucleotide analog of A2RE11 (dA2RE11) and the telomeric DNA repeat to the same sites on hnRNP A2 was demonstrated in UV-cross-linking EMSAs. In these experiments an excess of unlabeled competitor was added with a fixed amount of purified recombinant hnRNP A2 to labeled dA2RE11, dNS1 [a scrambled A2RE11 sequence, CAAGCACCGAA; (46)] or telomeric repeat-containing sequences. An excess of a single telomeric repeat (Telo1; Figure 3A) or a trimer (Telo3, (TTAGGG)$_3$; Figure 3B) abolished binding of both dA2RE11 and dNS1. dNS1 partly competed with dA2RE11, but less effectively with Telo1 or Telo3; and dA2RE11 eliminated dNS1 binding but did not outcompete either telomeric repeat sequence for the specific site. Thus, excess of dA2RE11, Telo1 or Telo3 displace dNS1 from the non-specific site and the telomeric repeats eliminate dA2RE11 association with both the specific and non-specific sites. Although formally it is possible that telomeric repeat binding to hnRNP A2 acts as an allosteric inhibitor of binding, it is likely that they compete directly for the same site with even the single 6-nt Telo1 binding more tightly than the A2RE11 oligodeoxyribonucleotide. Although the single and triple telomeric repeats compete effectively with dA2RE11, the oligoribonucleotide A2RE11 binds more tightly than all three oligodeoxyribonucleotides (Figure 3C).

Mutational analysis identifies a consensus hnRNP A2-binding sequence that matches both A2RE11 and the telomeric repeat

Binding of A2RE11 and the telomeric repeat to what appears to be the same sequence-specific site on hnRNP A2 led us to investigate the molecular requirements for association with this site. Using competition UV-cross-linking EMSAs we examined the ability of a 50-fold excess of unlabeled dA2RE11 mutated in single positions, dA2RE11 and dNS1 to compete with the radiolabeled dA2RE11 probe. Figure 4A shows representative data. Figure 4B presents the consensus sequence for binding. One nucleotide, G6, is absolutely required and a further four may be one of two nucleotides. There is no discrimination between nucleotides for 4 of the 11 positions. Parallel experiments with truncated dA2RE11 molecules had indicated that for this oligodeoxyribonucleotide only one nucleotide could be deleted from either end without weakening binding to hnRNP A2 (data not shown).

The sequence TTAGGGT matches the consensus sequence exactly and the remaining four positions in the 11mer may be filled by any deoxynucleotide or are possibly not bound to the protein. Given the multi-functional nature of hnRNP A2—e.g. in RNA packaging, alternative RNA splicing, telomere maintenance, RNA transport and translation—it is anticipated that

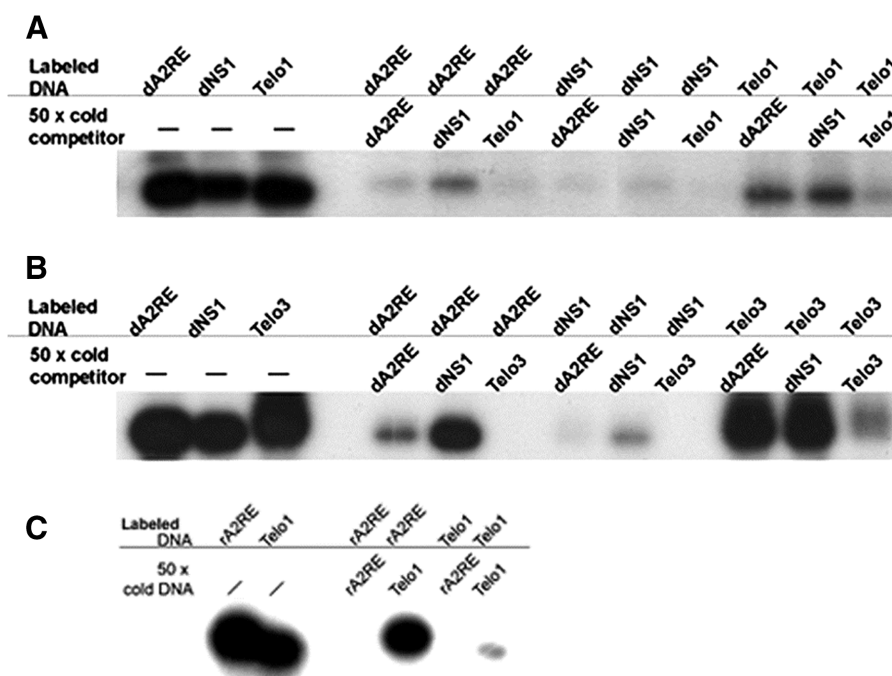

Figure 3. The telomeric repeat competes for the hnRNP A2 dA2RE11 binding site. Competition UV-cross-linking EMSAs in which 1.5 pmol ^{32}P-labeled dA2RE11, dNS1 or telomeric sequences (Telo1 and Telo3) were incubated with 10 pmol of purified recombinant hnRNP A2 in the presence or absence of 75 pmol unlabeled competitor, irradiated, electrophoresed on a 15% SDS–polyacrylamide gel and autoradiographed. Telo1 (**A**) contains a single telomeric repeat and Telo3 (**B**) oligonucleotide has three repeats of the telomeric sequence [i.e. (TTAGGG)$_3$]. (**C**) Competition UV-cross-linking EMSAs performed by incubating ^{32}P end-labeled rA2RE11 and Telo1 with recombinant hnRNP A2 (10 pmol) in the presence or absence of unlabeled competitor, as above. The telomeric DNA repeat sequence competes with dA2RE11, but weakly with A2RE11, for the binding site on recombinant hnRNP A2.

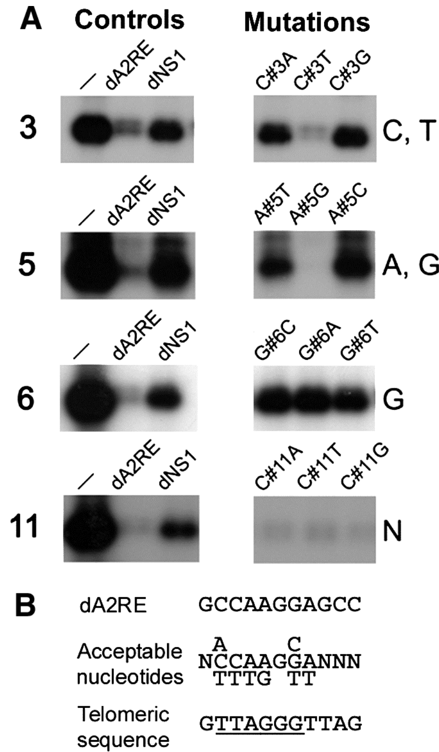

Figure 4. A consensus oligonucleotide sequence for binding to hnRNP A2. (**A**) Point mutations were introduced into the dA2RE11 sequence at each position by replacement of the native nucleotide with the other three, separately generating all four oligodeoxyribonucleotides for each position. The ability of the modified oligonucleotides to compete with the unmutated dA2RE11 was assessed by using competition UV-cross-linking EMSAs with recombinant hnRNP A2 in the absence of heparin. Representative results are shown. Controls are shown on the left and the results for the mutations at the indicated positions of the A2RE11 are shown on the right. Competing oligodeoxynucleotides have the point mutations indicated above the autoradiographs. (**B**) The wild-type dA2RE11 sequence and single-nucleotide changes that still allow specific binding are shown along with the deduced consensus sequence. In deriving the consensus sequence it has been assumed that substitution at each position is without direct effect on the binding of the non-mutated nucleotides.

it will bind other ssDNA and ssRNA motifs specified by this consensus sequence.

Telomeric DNA repeat binds the concatenated RRMs

Previous experiments showed that A2RE binding to hnRNP A2 was recapitulated with the tandem RRMs with an additional 10 C-terminal residues (i.e. residues 1–189 of the rat or human protein, RRM1+2′), with little binding to the isolated RRMs (45). A parallel result was obtained with Telo3: no binding of the radiolabeled oligonucleotide to RRM1 was observed, with some binding to RRM2 and much stronger association with the concatenated RRMs and the whole protein (Figure 5). This reinforces the conclusion that the telomeric repeat oligonucleotides and A2RE11 most likely recognize the same sites, both specific and non-specific, on the protein and that the C-terminal glycine-rich region is not needed for tight (nM) binding. These results for the individual RRMs differ from previous observations with hnRNP A1, which showed strong binding of a decameric telomere repeat to RRM1, weaker binding to RRM2 and the strongest association with the RRM concatamer (UP1) (28).

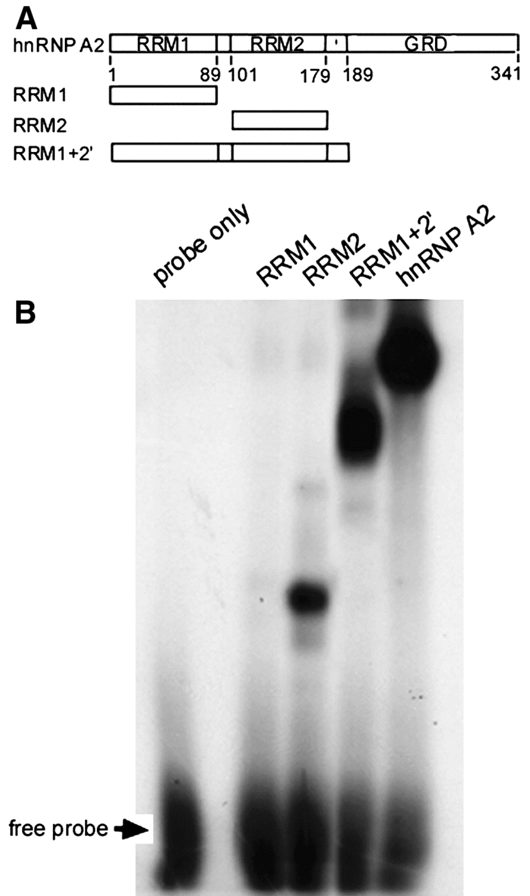

Figure 5. Both RRM domains of recombinant hnRNP A2 are required for binding the telomeric sequence. (**A**) Schematic representation of the recombinant proteins used. RRM, RNA recognition motif; GRD, glycine-rich domain. The prime indicates the presence of the additional 10 residues (179–189) beyond the C-terminal end of RRM2 that markedly increase the affinity of the concatenated RRMs for A2RE and A2RE11. (**B**) UV-cross-linking EMSA autoradiograph generated by incubating ^{32}P end-labeled Telo3 and equimolar concentrations of the recombinant proteins, irradiating the mixtures and separating the proteins on a 15% SDS–polyacrylamide gel. Free Telo3 probe is indicated at bottom left. Although the telomeric sequence does not appear to bind RRM1, it binds RRM2 and associates more tightly with the concatenated RRMs and the whole protein.

hnRNP A2 protects the telomeric DNA repeat against endonuclease digestion

The ^{32}P-labeled telomeric repeat DNA and an oligonucleotide with the complementary sequence were added to hnRNP A2 at several different protein concentrations before digestion with the endonuclease DNase I. The effects of the added protein on the oligonucleotide stability were monitored by gel electrophoresis. In the absence of the hnRNP A2 both oligonucleotides were largely degraded, but with increasing protein concentrations Telo6, but not the complementary oligonucleotide (Anti6), showed increasing levels of protection from DNase action (Figure 6A). The protection of this 36-nt molecule suggests that either multiple copies of hnRNP A2 bind or the DNA structure is such that all potential cleavage sites are obscured by one protein molecule.

Experiments with different protein modules suggest that the role played by hnRNP A2 in DNase protection is more complex than simple binding of the oligonucleotide. The

RRM1+2′ concatamer appears to bind almost as effectively as the whole protein (45) and might therefore be expected to confer protection against degradation. However, protection was afforded only by the intact protein and not by this concatamer or the individual RRMs (Figure 6B), thus implicating the glycine-rich region as a factor promoting the stability of the oligonucleotide. But the GRD alone appeared not to protect the oligonucleotide (Figure 6C).

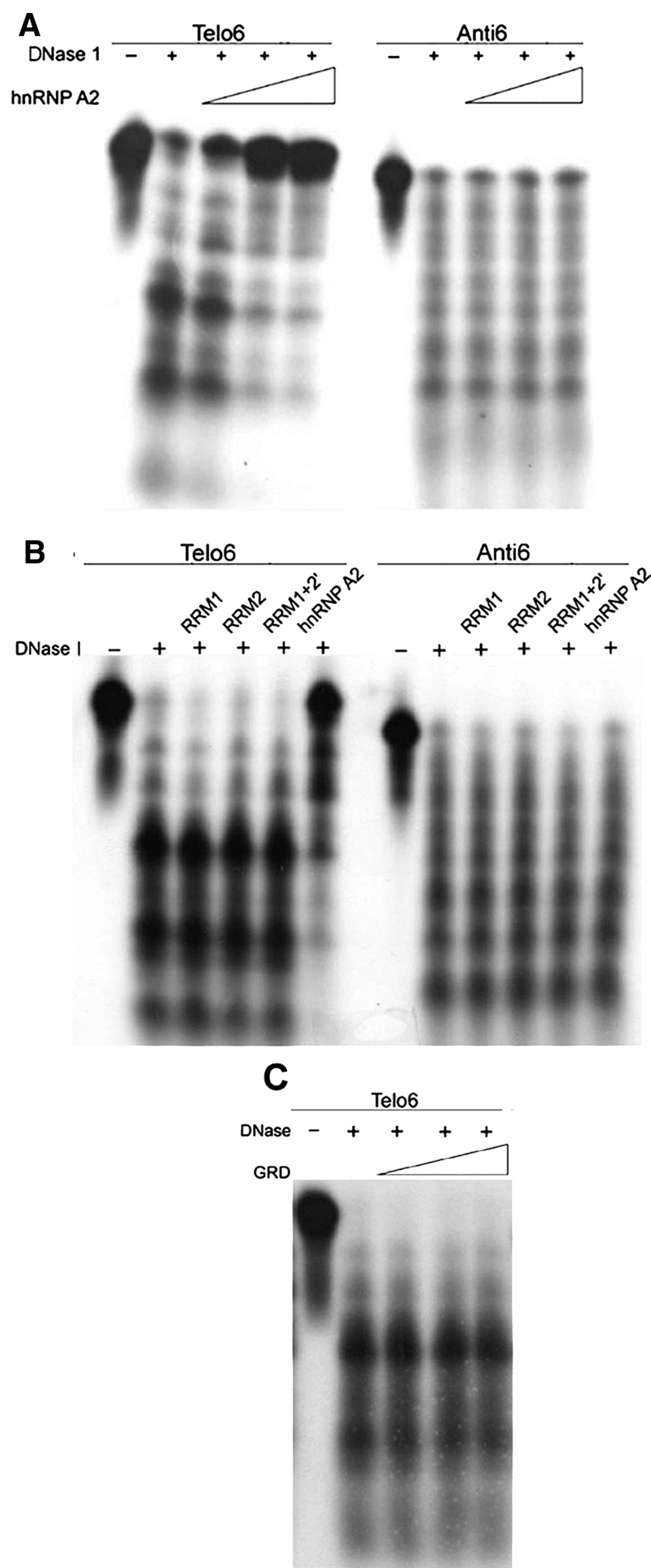

Figure 6. Recombinant hnRNP A2 protects telomeric DNA against DNase digestion. (**A**) Increasing concentrations of recombinant hnRNP A2 (1, 4 and 10 μM) were incubated with the ^{32}P end-labeled hexameric telomeric DNA repeat [Telo6, $(TTAGGG)_6$], or the complementary sequence [Anti6, $(CCCTAA)_6$], before adding the endonuclease DNase I. Breakdown products of the oligonucleotides were separated on a 20% polyacrylamide/7 M urea gel. Both oligonucleotides are extensively degraded in the absence of hnRNP A2. In the presence of this protein the telomeric sequence (left panel), but not the complementary sequence (right), is resistant to endonuclease digestion. (**B**) The GRD of hnRNP A2 is necessary but not sufficient for DNase I protection. Each recombinant protein (10 μM of RRM1, RRM2, RRM1+2′ or hnRNP A2) was assessed for its ability to protect the telomeric DNA. The proteins were incubated with ^{32}P end-labeled Telo6 or Anti6 before adding DNase I. Breakdown products of the oligonucleotides were separated on a 20% polyacrylamide/7 M urea gel and visualized by autoradiography. The individual or concatenated RRMs provided no protection: the intact protein was required, suggesting that the glycine-rich region is involved in protecting the telomeric sequence against enzymatic degradation. The telomeric sequence, but not its complement, is protected. (**C**) DNase I protection assay performed as described above, using 1, 4 and 10 μM of the recombinant GRD protein. This domain appears to be necessary but not sufficient for RNA protection.

hnRNP A2 binds the RNA template of telomerase

In addition to binding telomeric ssDNA repeats, hnRNP A2 also binds the first 71 nt of human telomerase RNA, the segment known to bind hnRNP A1 (30). This 5′ segment of hTR was ^{32}P-labeled, incubated with increasing amounts of recombinant hnRNP A2 and separated by PAGE. In the absence of hnRNP A2 the 71-nt RNA segment migrates in two bands with apparent sizes of ~139 nt (the anticipated size, including the vector T7 sequence) and ~280 nt (possibly arising through dimerization or formation of secondary structures): the former is unchanged but the latter is absent at high hnRNP A2 concentrations, suggesting that the more slowly migrating form of the RNA interacts preferentially with the protein. At higher protein concentrations, the RNA was retarded, indicating interaction of the protein with the RNA (Figure 7A, arrow on the right). The marked difference in RNA binding between the samples with 2 and 5 pmol of hnRNP A2 suggests cooperative binding: a similarly abrupt shift in binding with increasing protein concentration has been observed for the binding of hnRNP A1 RRM1 to the $(TTAGGG)_{10}$ DNA oligonucleotide (30).

The specificity of the protein–RNA interaction was demonstrated by the lack of protein binding to the heat-denatured RNA (Figure 7B). The denatured RNA was almost entirely the faster, possibly monomeric, form shown not to bind to the protein. The small amount of the slower migrating RNA remaining after denaturation was retarded by hnRNP A2: this is faintly visible in the right-hand track of Figure 7B (arrow on the left). Little RNA binding to RRM2 was evident in UV-cross-linking EMSAs performed with the expressed modules of hnRNP A2, but binding to RRM1 matched that of the tandem RRMs (Figure 7C).

hnRNP A2 is preferentially localized with PML and TRF2 in APBs

To date, there have been no reports of colocalization *in vivo* of hnRNP proteins and telomeric DNA although hnRNP C1/C2

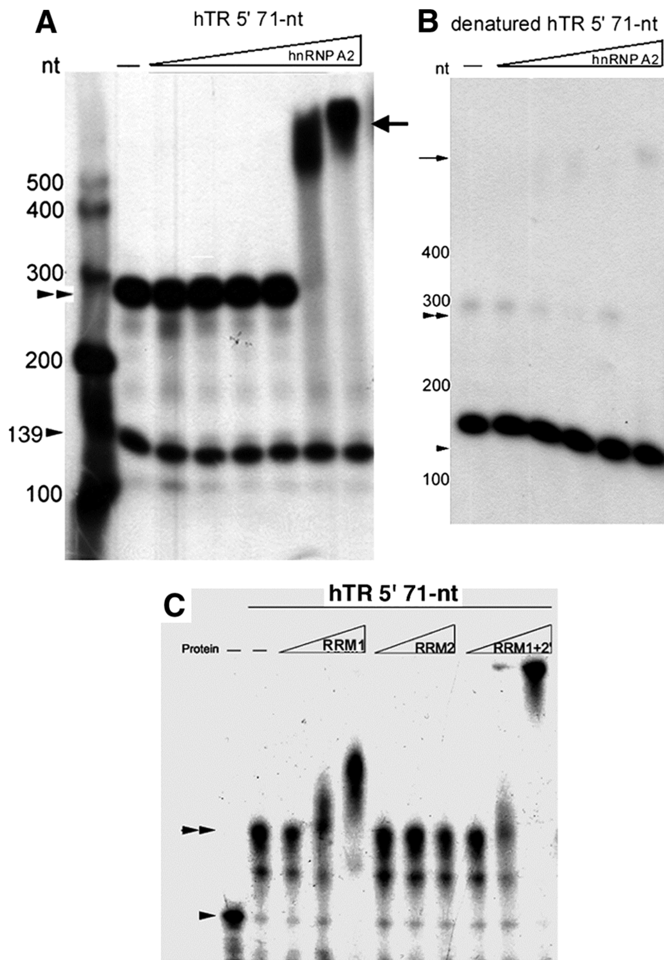

Figure 7. hnRNP A2 binds the first 71 nt of human telomerase RNA. (**A**) ^{32}P-labeled hTR 5′ 71-nt was incubated with increasing amounts (0, 0.1, 0.5, 1, 2, 5 and 10 pmol) of recombinant hnRNP A2, and separated on a 15-cm 5% polyacrylamide gel. These assays were performed in the presence of 1 µg/µl heparin. The arrow on the right marks the retarded RNA–protein complex. (**B**) Assay was performed as above, except that the RNA used was first denatured by heating to 95°C for 5 min, cooled by placing immediately on ice and then incubated with protein. (**C**) ^{32}P-labeled hTR 5′ 71-nt RNA was incubated with 1, 10 and 100 µM of each recombinant protein, and separated on a 15-cm 5% native polyacrylamide gel. The single and double arrowheads point to the monomer and dimer forms of the RNA, respectively.

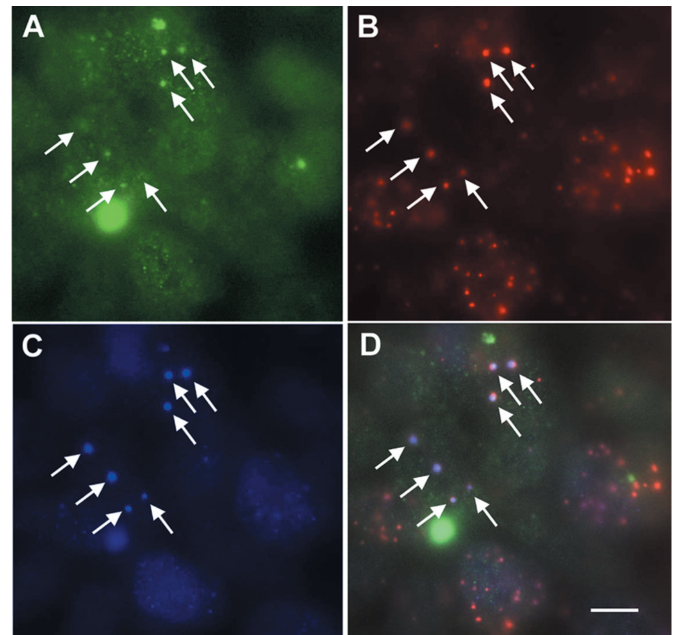

Figure 8. hnRNP A2/B1, PML and TRF2 are colocalized in nuclear bodies. Colocalization of hnRNP A2/B1, PML and hTRF2 at APBs in the nuclei of telomerase-negative, immortalized human fibroblasts (JFCF-6T.1J/1–4D cells). (**A**) hnRNP A2/B1 (FITC, green). (**B**) PML (Texas Red, red). (**C**) TRF2 (AMCA, blue). (**D**) Colocalization is indicated by the arrows in the merged image. Scale bar: 10 µm.

has been demonstrated to colocalize with TRF1 and TRF2, both of which are primarily telomere-associated proteins (21). Examination of metaphase spreads of telomerase-positive and telomerase-negative human fibroblast lines by immunostaining with antibodies to hnRNP A2 followed by fluorescence *in situ* hybridization for telomeric DNA frequently showed localization of hnRNP A2 at telomeres (data not shown), but the protein could not be detected consistently, possibly indicating that the amount of protein is at the limit of detection with these antibodies. Immunofluorescence staining of human fibroblasts with antibodies to hnRNP A2, TRF2 and PML showed that these proteins colocalized in APBs (Figure 8). These structures are found only in telomerase-negative cell lines and tumors that use the ALT mechanism of telomere maintenance, and are a subset of the PML nuclear bodies that are characterized by the presence of extrachromosomal telomeric DNA, telomere binding proteins, such as TRF1 and TRF2, and other proteins involved in recombination and DNA repair (11). These *in vivo* observations thus support our *in vitro* demonstration of specific interactions between hnRNP A2 and the telomeric DNA repeat sequence.

DISCUSSION

Many proteins are involved in the synthesis and maintenance of telomeres. They include Pot1, which binds the G-rich telomere end (51), and TRF1 and TRF2, which bind double-stranded telomeric DNA (16). These proteins are negative regulators of telomere extension. Loss of TRF2 leads to cell-cycle arrest, apoptosis (52) and end-to-end ligation of telomeres (53). Other proteins associated with TRF1, TRF2 or the ssDNA repeats have been identified, including several members of the hnRNP family. The roles of the hnRNPs in telomere biology are not well established but if, as proposed, some recruit telomerase to the chromosome ends they would be expected to support telomere extension, with diminished protein expression resulting in telomere shortening.

The hnRNP A/B proteins are abundant in many cell types. They are localized primarily to the nucleus where they may bind the telomeric DNA repeat (24,33–35) and participate in telomere maintenance. They are also involved in packaging nascent mRNA, in alternative splicing (24,54–56), and in cytoplasmic RNA trafficking (44,46), translation (57) and stabilization (58,59). In each role they presumably interact with sets of molecules that incorporate different RNA or DNA elements.

All the major proteins in the hnRNP A/B family, A1, A2 and A3, and their isoforms, were isolated in our pull-down experiments with the telomeric repeat. In the rat brain, hnRNPs A2 and A3 are the predominant binding proteins. These proteins share high sequence identity, with hnRNPs A3 and A1 being more closely related than hnRNPs A2 and A1. Over the tandem RRM region they have ~80% sequence identity and hence hnRNPs A2 and A3 are likely to share the typical RRM βαββαβ fold of hnRNP A1 (60). The crystal structure of the UP1 proteolytic fragment of hnRNP A1 (residues 1–196 of the human protein) complexed to the single-stranded telomeric repeat [d(TTAGGG)$_2$] (50) shows that the amino acid residues of hnRNP A1 that interact with the oligodeoxyribonucleotide are present in hnRNP A2, with the exception of a single Lys to Arg substitution. Because only the main chain atoms of this residue, K183, interact with G11, it is unlikely that substitution with arginine would interfere with the interaction between hnRNP A2 and the telomeric repeat. Despite this conservation of interacting residues, there are differences in the binding of the telomeric repeats by hnRNPs A1 and A2, as discussed below.

In an earlier study we presented evidence from EMSA and biosensor data that recombinant hnRNP A2 has two sites that bind oligoribonucleotides or oligodeoxyribonucleotides, but with this protein only the sequence-specific site was occupied in the presence of heparin (45). In contrast, we discovered in this study that rat hnRNP A2 also has two sites but binds oligodeoxyribonucleotides at the non-specific site even in the presence of heparin (Figure 1). Possession of two sites raises the possibility of the protein acting as a non-covalent cross-linker of nucleic acids. DNA or RNA molecules (containing e.g. the telomere repeat or A2RE11) occupying the specific site may be linked to any other ssDNA. The ability to bind the telomeric repeat at both sites may also be important for protection of the ssDNA from nuclease activity.

Although the telomere repeat binds the isolated RRM1 of hnRNP A2 weakly (if at all) and isolated RRM2 somewhat more strongly, the tandem extended RRMs (residues 1–189) are needed for binding that matches the whole protein. This binding pattern deviates from that for hnRNP A1, the first RRM of which was found to bind more tightly than RRM2 (28). RRM1 is sufficient for A1/UP1binding to a telomeric DNA oligonucleotide (30). This difference points to the divergence in the mode of interaction for hnRNPs A1 and A2, with the latter possibly not mimicking the anti-parallel dimeric molecular arrangement observed in the crystalline state, in which oligonucleotides bind RRM1 of one hnRNP A1 molecule and RRM2 of the other.

The requirements in the nucleic acid for binding to the sequence-specific site of hnRNP A2 were established by mutational analysis, starting with A2RE11. This led to a consensus sequence that includes the telomeric sequence (Figure 4) and the hnRNP A1-binding d(GGCAG) tandem repeats of the mouse hypervariable minisatelite (29). Changes in a single nucleotide have been shown to markedly alter oligonucleotide binding to hnRNP A2 (Figure 4) and UP1 (27). As a consequence of the redundancy at several positions in the consensus sequence, it can give rise to many different oligonucleotides. hnRNP A2, a recognized multi-tasking protein (61), may therefore potentially interact with numerous different RNA or DNA elements, thereby influencing many metabolic or signaling pathways. Moreover, it could act as the link between pathways utilizing nucleic acids that compete for binding to its RRMs.

UP1 binds the telomeric repeat sequence specifically *in vitro* (28), protecting it from degradation by nucleases, and demonstrates helix-unwinding ability (62). UP1 also binds the telomerase holoenzyme *in vitro*, promoting telomere extension at low concentrations (31), but inhibiting it at higher concentrations (28). In addition, both hnRNP A1 and UP1 bind telomerase RNA and telomeric DNA simultaneously *in vitro* (30), and may thus be capable of recruiting telomerase to form part of the protective complex on telomere ends.

The segment of hnRNP A2 equivalent to UP1, UP1-B (which is contained within RRM1+2′), is necessary and sufficient to bind the telomeric sequence *in vitro* but it did not mirror the behavior of UP1: in our experiments it did not protect the telomeric DNA (Figure 6A). Protection against endonuclease-catalyzed degradation required the whole protein, suggesting that the glycine-rich C-terminal domain of hnRNP A2 plays a critical role in this action (Figure 6B). Protection *in vivo* is also provided by other telomere-associated molecules that bind to multiple sites, such as Pot1 (51).

hnRNP A2 retarded the 5′ 71-nt segment of hTR in an EMSA (Figure 7A). This includes the template region which is within the single-stranded region (63) that is most likely to bind hnRNP A2. The apparent binding of hnRNP A2 to dimers, rather than monomers, of the 5′ 71-nt hTR segment (Figure 7) may not faithfully recapitulate the molecular interactions *in vivo*, but functional telomerase is dimeric and includes two RNA molecules that cooperate functionally (64,65).

Fiset and Chabot (30) earlier proposed that hnRNP A1 could simultaneously bind telomerase, through interaction with its RNA, and the telomeric DNA repeat, thus providing a mechanism for recruitment of telomerase to the chromosome ends (32). Our observation that hnRNP A2 has two binding sites for single-stranded oligonucleotides leads to a parallel conclusion but, based on current evidence, the molecular mechanisms differ for these two proteins. The sites on hnRNP A1 for the telomeric DNA repeat and telomerase RNA were identified as residing on RRM1 and RRM2, respectively, whereas both RRM modules of hnRNP A2 are needed for tight binding to the repeat. RRM1 and the tandem RRMs of hnRNP A2 bind the 71-nt RNA segment, whereas no binding of RRM2 was apparent (Figure 7C). The telomerase RNA is therefore unlikely to be bound to the non-specific hnRNP A2 site, which appears not to bind oligoribonucleotides strongly (Figure 1), nor does it bind the entire sequence-specific site, which spans the tandem RRMs. Further investigation is needed to establish whether its association with RRM1 makes use of part of the sequence-specific site.

In the mouse erythroleukemic cell line CB3 (66), there is no measurable hnRNP A1 expression and the terminal repeat DNA fragments are markedly shorter than those in the similar cell line, CB7, which expresses hnRNP A1 (31). But even after multiple passages of CB3, the mean terminal repeat fragment length did not change significantly, indicating that although hnRNP A1 may be required for full telomerase-mediated extension, in its absence another protein (or proteins) can prevent degradation of the telomere. On the basis of the results

reported here, it is possible that the closely related hnRNP A2 protein can fulfill some or all of these functions.

Interestingly, hnRNP A2 and TRF2 are both present at higher than background levels in APBs. These intranuclear domains, which contain extrachromosomal telomeric DNA and telomere-specific binding proteins together with PML proteins, are specific for ALT-positive cell lines (11). Their function is unknown, but it has been proposed that they might be depots of telomeric DNA and associated proteins required for ALT, or function as platforms for the ALT process. Telomeric DNA is not associated with the PML bodies of telomerase-positive cell lines and it is absent from many PML bodies within ALT cell nuclei, i.e. APBs are a subset of the PML bodies in ALT cells. The presence of hnRNP A2 in APBs (but not in PML bodies that do not contain telomeric DNA) suggests that telomeric chromatin is sufficient for the localization of hnRNP A2, and that hTR and telomerase activity are not required. hnRNP A2 was not present in every APB, consistent with the possibility that some aspect of the state of the telomeric chromatin is responsible for hnRNP A2 localization. It will be of interest to determine whether there are interactions among the hnRNPs and other proteins, such as Pot1 (51), that protect the telomere ends from degradation.

In conclusion, from our studies it is evident that hnRNP A2 binds in a complex fashion to ssDNA and ssRNA. We found that rat brain hnRNP A2 has two sites for binding oligonucleotides. One binds oligodeoxyribonucleotides in preference to oligoribonucleotides and shows little discrimination between diverse sequences. The second, which requires both RRMs, has been shown to bind to sequences defined by the consensus sequence N(A,C,T)(C,T)(A,T)(A,G)G(C,G,T)(A,T)NNN: this sequence encompasses the telomeric repeat sequence. In addition, the template-containing segment of telomerase RNA binds to RRM1, raising the possibility that it competes with the ssDNA telomeric repeat for the hnRNP A2 sequence-specific site. As the G-rich overhanging strand lengthens, it may compete more effectively with telomerase RNA for binding to RRM1, displacing the RNA and thus limiting the telomerase-mediated strand extension. Although hnRNP A2 binds telomerase RNA, the preferential localization of hnRNP A2 with TRF2 in APBs suggests that telomerase is not needed for association with telomeric DNA. Combined with our *in vitro* binding data, the localization in APBs suggests an important role for this protein in telomere maintenance.

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REFERENCES

1. Watson,J.D. (1972) Origin of concatemeric T7 DNA. *Nature New Biol.*, **239**, 197–201.
2. Olovnikov,A.M. (1973) A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. *J. Theoret. Biol.*, **41**, 181–190.
3. Moyzis,R.K., Buckingham,J.M., Cram,L.S., Dani,M., Deaven,L.L., Jones,M.D., Meyne,J., Ratliff,R.L. and Wu,J.R. (1988) A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. *Proc. Natl Acad. Sci. USA*, **85**, 6622–6626.
4. Meyne,J., Ratliff,R.L. and Moyzis,R.K. (1989) Conservation of the human telomere sequence (TTAGGG)n among vertebrates. *Proc. Natl Acad. Sci. USA*, **86**, 7049–7053.
5. Harley,C.B. (1991) Telomere loss: mitotic clock or genetic time bomb?. *Mutation Res.*, **256**, 271–282.
6. Bryan,T.M. and Cech,T.R. (1999) Telomerase and the maintenance of chromosome ends. *Curr. Opin. Cell Biol.*, **11**, 318–324.
7. Neumann,A.A. and Reddel,R.R. (2002) Telomere maintenance and cancer–look, no telomerase. *Nature Rev. Cancer*, **2**, 879–884.
8. Murnane,J.P., Sabatier,L., Marder,B.A. and Morgan,W.F. (1994) Telomere dynamics in an immortal human cell line. *EMBO J.*, **13**, 4953–4962.
9. Bryan,T.M., Englezou,A., Gupta,J., Bacchetti,S. and Reddel,R.R. (1995) Telomere elongation in immortal cells without detectable telomerase activity. *EMBO J.*, **14**, 4240–4248.
10. Dunham,M.A., Neumann,A.A., Fasching,C.L. and Reddel,R.R. (2000) Telomere maintenance by recombination in human cells. *Nature Genet.*, **26**, 447–450.
11. Yeager,T.R., Neumann,A.A., Englezou,A., Huschtscha,L.I., Noble,J.R. and Reddel,R.R. (1999) Telomerase-negative immortalized cells contain a novel type of promyelocytic (PML) body. *Cancer Res.*, **59**, 4175–4179.
12. Grobelny,J.V., Godwin,A.K. and Broccoli,D. (2000) ALT-associated PML bodies are present in viable cells and are enriched in cells in the G2/M phase of the cell cycle. *J. Cell Sci.*, **113**, 4577–4585.
13. Wu,G., Lee,W.H. and Chen,P.L. (2000) NBS1 and TRF1 colocalize at promyelocytic leukemia bodies during late S/G2 phases in immortalized telomerase-negative cells. Implications of NBS1 in alternative lengthening of telomeres. *J. Biol. Chem.*, **275**, 30618–30622.
14. Rossetti,L., Cacchione,S., Fua,M. and Savino,M. (1998) Nucleosome assembly on telomeric sequences. *Biochemistry*, **37**, 6727–6737.
15. Fajkus,J. and Trifonov,E. (2001) Columnar packing of telomeric nucleosomes. *Biochem. Biophys. Res. Commun.*, **280**, 961–963.
16. Griffith,J., Comeau,L., Rosenfield,S., Stansel,R., Bianchi,A., Moss,H. and de Lange,T. (1999) Mammalian telomeres end in a large duplex loop. *Cell*, **97**, 503–514.
17. Collins,K. (2000) Mammalian telomeres and telomerase. *Curr. Opin. Cell Biol.*, **12**, 378–383.
18. Blackburn,E.H. (2001) Switching and signalling at the telomere. *Cell*, **106**, 661–673.
19. Smogorzewska,A., van Steensel,B., Bianchi,A., Oelmann,S., Schaefer,M.R., Schnapp,G. and de Lange,T. (2000) Control of human telomere length by TRF1 and TRF2. *Mol. Cell. Biol.*, **20**, 1659–1668.
20. de Lange,T. (2002) Protection of mammalian telomeres. *Oncogene*, **21**, 532–540.
21. Ford,L., Suh,J.M., Wright,W. and Shay,J. (2000) Heterogeneous nuclear ribonucleoproteins C1 and C2 associate with the RNA component of human telomerase. *Mol. Cell. Biol.*, **20**, 9084–9091.
22. Eversole,A. and Maizels,N. (2000) *In vitro* properties of the conserved mammalian protein hnRNP D suggest a role in telomere maintenance. *Mol. Cell. Biol.*, **20**, 5425–5432.
23. Bandiera,A., Tell,G., Marsich,E., Scaloni,A., Pocsfalvi,G., Akindahunsi,A.A., Cesaratto,L. and Manzini,G. (2003) Cytosine-block telomeric type DNA-binding activity of hnRNP proteins from human cell lines. *Arch. Biochem. Biophys.*, **409**, 305–314.
24. Ishikawa,F., Matunis,M.J., Dreyfuss,G. and Cech,T.R. (1993) Nuclear proteins that bind the pre-mRNA 3′ splice site sequence r(UUAG/G) and the human telomeric DNA sequence d(TTAGGG)n. *Mol. Cell. Biol.*, **13**, 4301–4310.
25. Lacroix,L., Lienard,H., Labourier,E., Djavaheri-Mergny,M., Lacoste,J., Leffers,H., Tazi,J., Helene,C. and Mergny,J.-L. (2000) Identification of two human nuclear proteins that recognize the cytosine-rich strand of human telomeres *in vitro*. *Nucleic Acids Res.*, **28**, 1564–1575.
26. Denisenko,O. and Bomsztyk,K. (2002) Yeast hnRNP K-like genes are involved in regulation of the telomeric position effect and telomere length. *Mol. Cell. Biol.*, **22**, 286–297.

27. Myers,J.C. and Shamoo,Y. (2004) Human UP1 as a model for understanding purine recognition in the family of proteins containing the RNA recognition motif (RRM). *J. Mol. Biol.*, **342**, 743–756.
28. Dallaire,F., Dupuis,S., Fiset,S. and Chabot,B. (2000) Heterogeneous nuclear ribonucleoprotein A1 and UP1 protect mammalian telomeric repeats and modulate telomere replication *in vitro*. *J. Biol. Chem.*, **275**, 14509–14516.
29. Fukuda,H., Katahira,M., Tsuchiya,N., Enokizono,Y., Sugimura,T., Nagao,M. and Nakagama,H. (2002) Unfolding of quadruplex structure in the G-rich strand of the minisatellite repeat by the binding protein UP1. *Proc. Natl Acad. Sci. USA*, **99**, 12685–12690.
30. Fiset,S. and Chabot,B. (2001) hnRNP A1 may interact simultaneously with telomeric DNA and the human telomerase RNA *in vitro*. *Nucleic Acids Res.*, **29**, 2268–2275.
31. LaBranche,H., Dupuis,S., Ben-David,Y., Bani,M.-R., Welliger,R.J. and Chabot,B. (1998) Telomere elongation by hnRNP A1 and a derivative that interacts with telomeric repeats and telomerase. *Nature Genet.*, **19**, 199–202.
32. Ford,L.P., Wright,W.E. and Shay,J.W. (2002) A model for heterogeneous nuclear ribonucleoproteins in telomere and telomerase regulation. *Oncogene*, **21**, 580–583.
33. McKay,S.J. and Cooke,H. (1992) hnRNP A2/B1 binds specifically to single-stranded vertebrate telomeric repeat TTAGGGn. *Nucleic Acids Res.*, **20**, 6461–6464.
34. McKay,S.J. and Cooke,H. (1992) A protein which specifically binds to single stranded TTAGGGn repeats. *Nucleic Acids Res.*, **20**, 1387–1391.
35. Kamma,H., Fujimoto,M., Fujiwara,M., Matsui,M., Horiguchi,H., Hamasaki,M. and Satoh,H. (2001) Interaction of hnRNP A2/B1 isoforms with telomeric ssDNA and the *in vitro* function. *Biochem. Biophys. Res. Commun.*, **280**, 625–630.
36. Hatfield,J., Rothnagel,J.A. and Smith,R. (2002) Characterisation of the mouse hnRNP A2/B1/B0 gene and identification of processed pseudogenes. *Gene*, **295**, 33–42.
37. Goto,Y., Sueoka,E., Chiba,H. and Fujiki,H. (1999) Significance of heterogeneous nuclear ribonucleoprotein B1 as a new early detection marker for oral squamous cell carcinoma. *Jpn. J. Cancer Res.*, **90**, 1358–1363.
38. Sueoka,E., Goto,Y., Sueoka,N., Kai,Y., Kozu,T. and Fujiki,H. (1999) Heterogeneous nuclear ribonucleoprotein B1 as a new marker of early detection for human lung cancers. *Cancer Res.*, **59**, 1404–1407.
39. Matsuyama,S., Goto,Y., Sueoka,N., Ohkura,Y., Tanaka,Y., Nakachi,K. and Sueoka,E. (2000) Heterogeneous nuclear ribonucleoprotein B1 expressed in esophageal squamous cell carcinomas as a new biomarker for diagnosis. *Jpn. J. Cancer Res.*, **91**, 658–663.
40. Fleischhacker,M., Beinert,T., Ermitsch,M., Seferi,D., Possinger,K., Engelmann,C. and Jandrig,B. (2001) Detection of amplifiable messenger RNA in the serum of patients with lung cancer. *Ann. NY Acad. Sci.*, **945**, 179–188.
41. Hamasaki,M., Kamma,H., Wu,W., Kaneko,S., Fujiwara,M., Satoh,H., Haraoka,S., Kikuchi,M. and Shirakusa,T. (2001) Expression of hnRNP B1 in four major histological types of lung cancers. *Anticancer Res.*, **21**, 979–984.
42. Sueoka,E., Sueoka,N., Goto,Y., Matsuyama,S., Nishimura,H., Sato,M., Fujimura,S., Chiba,H. and Fujiki,H. (2001) Heterogeneous nuclear ribonucleoprotein B1 as early cancer biomarker for occult cancer of human lungs and bronchial dysplasia. *Cancer Res.*, **61**, 1896–1902.
43. Patry,C., Bouchard,L., Labrecque,P., Gendron,D., Lemieux,B., Toutant,J., Lapointe,E., Wellinger,R. and Chabot,B. (2003) Small interfering RNA-mediated reduction in heterogeneous nuclear ribonucleoparticule A1/A2 proteins induces apoptosis in human cancer cells but not in normal mortal cell lines. *Cancer Res.*, **63**, 7679–7688.
44. Munro,T.P., Magee,R.J., Kidd,G.J., Carson,J.H., Barbarese,L., Smith,L.M. and Smith,R. (1999) Mutational analysis of a heterogeneous nuclear ribonucleoprotein A2 response element for RNA trafficking. *J. Biol. Chem.*, **274**, 34389–34395.
45. Shan,J., Moran-Jones,K., Munro,T.P., Kidd,G.J., Winzor,D.J., Hoek,K.S. and Smith,R. (2000) Binding of an RNA trafficking response element to heterogeneous nuclear ribonucleoproteins A1 and A2. *J. Biol. Chem.*, **275**, 38286–38295.
46. Hoek,K.S., Kidd,G.J., Carson,J.H. and Smith,R. (1998) hnRNP A2 selectively binds the cytoplasmic transport sequence of myelin basic protein mRNA. *Biochemistry*, **37**, 7021–7029.
47. Ma,A.S.W., Moran-Jones,K., Shan,J., Munro,T.P., Snee,M.J., Hoek,K.S. and Smith,R. (2002) hnRNP A3, a novel RNA trafficking response element binding protein. *J. Biol. Chem.*, **277**, 18010–18020.
48. Bryan,T.M., Marusic,L., Bacchetti,S., Namba,M. and Reddel,R.R. (1997) The telomere lengthening mechanism in telomerase-negative immortal human cells does not involve the telomerase RNA subunit. *Hum. Mol. Genet.*, **6**, 921–926.
49. Chabot, B. (1995) Synthesis and purification of RNA substrates. In Higgins,S.J. and Hames,B.D. (eds.), *RNA Processing. A Practical Approach.* IRL Press at Oxford University Press, New York **Vol. 1**, 1–28.
50. Ding,J., Hayashi,M.K., Zhang,Y., Manche,L., Krainer,A.R. and Xu,R.-M. (1999) Crystal structure of the two-RRM domain of hnRNP A1 (UP1) complexed with single-stranded telomeric DNA. *Genes Dev.*, **13**, 1102–1115.
51. Baumann,P. and Cech,T.R. (2001) Pot1, the putative telomere end-binding protein in fission yeast and humans. *Science*, **292**, 1171–1175.
52. Karlseder,J., Broccoli,D., Dai,Y., Hardy,S. and de Lange,T. (1999) p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. *Science*, **283**, 1321–1325.
53. van Steensel,B., Smogorzewska,A. and de Lange,T. (1998) TRF2 protects human telomeres from end-to-end fusions. *Cell*, **92**, 401–413.
54. Mayeda,A., Munroe,S., Caceres,J. and Krainer,A. (1994) Function of conserved domains of hnRNP A1 and other hnRNP A/B proteins. *EMBO J.*, **13**, 5483–5495.
55. Abdul-Manan,N. and Williams,K. (1996) hnRNP A1 binds promiscuously to oligoribonucleotides: utilization of random and homo-oligonucleotides to discriminate sequence from base-specific binding. *Nucleic Acids Res.*, **24**, 4063–4070.
56. Hutchison,S., LeBel,C., Blanchette,M. and Chabot,B. (2002) Distinct sets of adjacent hnRNP A1/A2 binding sites control 5′ splice site selection in the hnRNP A1 pre-mRNA. *J. Biol. Chem.*, **277**, 29745–29752.
57. Kwon,S., Barbarese,E. and Carson,J.H. (1999) The cis-acting RNA trafficking signal from myelin basic protein mRNA and its cognate trans-acting ligand hnRNP A2 enhance cap-dependent translation. *J. Cell Biol.*, **147**, 247–256.
58. Hamilton,B.J., Nagy,E., Malterg,J.S., Arrick,B.A. and Rigby,W.F.C. (1993) Association of heterogeneous nuclear ribonucleoprotein A1 and C proteins with reiterated AUUUA sequences. *J. Biol. Chem.*, **268**, 8881–8887.
59. Hamilton,B.J., Burns,C.M., Nichols,R.C. and Rigby,W.F.C. (1997) Modulation of AUUUA response element binding by heterogeneous nuclear ribonucleoprotein A1 in human T lymphocytes. *J. Biol. Chem.*, **272**, 28732–28741.
60. Xu,R.-M., Jokhan,L., Cheng,X., Mayeda,A. and Krainer,A.M. (1997) Crystal structure of UP1, the domain of hnRNP A1 that contains two RNA-recognition motifs. *Structure*, **5**, 559–570.
61. Shyu,A.-B. and Wilkinson,M.F. (2000) The double lives of shuttling mRNA binding proteins. *Cell*, **102**, 135–138.
62. Herrick,G. and Alberts,B. (1976) Nucleic acid helix-coil transitions mediated by helix-unwinding proteins from calf thymus. *J. Biol. Chem.*, **251**, 2133–2141.
63. Antal,M., Boros,E., Solymosy,F. and Kiss,T. (2002) Analysis of the structure of human telomerase RNA *in vivo*. *Nucleic Acids Res.*, **30**, 912–920.
64. Beattie,T.L., Zhou,W., Robinson,M.O. and Harrington,L. (2001) Functional multimerization of the human telomerase reverse transcriptase. *Mol. Cell. Biol.*, **21**, 6151–6160.
65. Wenz,C., Enenkel,B., Amacker,M., Kelleher,C., Damm,K. and Lingner,J. (2001) Human telomerase contains two cooperating telomerase RNA molecules. *EMBO J.*, **20**, 3526–3534.
66. Ben-David,Y., Bani,M.-R., Chabot,B., de Koven,A. and Bernstein,A. (1992) Retroviral insertions downstream of the heterogeneous nuclear ribonucleoprotein A1 gene in erythroleukemia cells: evidence that A1 is not essential for cell growth. *Mol. Cell. Biol.*, **12**, 4449–4455.