Crystal structure of the two-RRM domain of hnRNP A1 (UP1) complexed with single-stranded telomeric DNA

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Human hnRNP A1 is a versatile single-stranded nucleic acid-binding protein that functions in various aspects of mRNA maturation and in telomere length regulation. The crystal structure of UP1, the amino-terminal domain of human hnRNP A1 containing two RNA-recognition motifs (RRMs), bound to a 12-nucleotide single-stranded telomeric DNA has been determined at 2.1 Å resolution. The structure of the complex reveals the basis for sequence-specific recognition of the single-stranded overhangs of human telomeres by hnRNP A1. It also provides insights into the basis for high-affinity binding of hnRNP A1 to certain RNA sequences, and for nucleic acid binding and functional synergy between the RRMs. In the crystal structure, a UP1 dimer binds to two strands of DNA, and each strand contacts RRM1 of one monomer and RRM2 of the other. The two DNA strands are antiparallel, and regions of the protein flanking each RRM make important contacts with DNA. The extensive protein–protein interface seen in the crystal structure of the protein–DNA complex and the evolutionary conservation of the interface residues suggest the importance of specific protein–protein interactions for the sequence-specific recognition of single-stranded nucleic acids. Models for regular packaging of telomere 3’ overhangs and for juxtaposition of alternative 5’ splice sites are proposed.

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A variety of studies have investigated the telomeric repeats, which are double-stranded G-rich structures. These repeats consist of tandem repeats of the hexamer TTAGGG, with varying overall lengths in different species. The 3' ends of chromosomes extend beyond the complementary C-rich strand. In vertebrates, the 3' overhang consists of tandem repeats of the hexamer TTAGGG, with varying overall lengths in different species. Although the telomeric repeats can form G-quartet high-order structures in vitro, it is not clear whether such structures exist in vivo. The single-stranded telomere overhangs are recognized as the substrate for elongation by telomerase.

Proteins that interact specifically with single-stranded telomeric DNA have been isolated and characterized in several organisms, including *Oxytricha nova* (Price and Cech 1987), *Euplotes crassus* (Wang et al. 1992), *Tetrahymena thermophila* (Sheng et al. 1995), *Stylonychia mytiliss* (Fang and Cech 1991), and *Xenopus laevis* (Cardenas et al. 1993), and *Saccharomyces cerevisiae* (Lin and Zakian 1996; Nugent et al. 1996; Viria-Pearlman et al. 1996). The crystal structure of a heterodimeric telomere-binding protein from *Oxytricha nova* (OnTEBP) with single-stranded DNA (ssDNA) has been determined recently (Horvath et al. 1998). The overall structure of the UP1–TR2 complex is shown in Figure 1B. The crystals belong to space group P4_3_2_1, and have unit-cell dimensions of a = b = 51.20 Å, and c = 171.09 Å. There is one protein and one ssDNA molecule per asymmetric unit. The structure was determined by a combination of multiple isomorphous replacement with anomalous scattering (MIRAS) and molecular replacement methods (Table 1). The refined model consists of 183 amino acids (residues 8–190), 11 nucleotides, and 144 ordered water molecules. The final model has an R-factor of 19.5% (free R-factor = 24.9%) and excellent stereochemistry. The Ramachandran plot of the main chain parameters shows 95.1% (154 residues) of the nonglycine, nonproline residues in the most favored region, and none in the disallowed regions (Laskowski et al. 1993).

**Description of the structure**

The overall structure of the UP1–TR2 complex is shown in Figure 1B. Two strands of TR2 bind to two protein molecules. The two protein and two TR2 molecules are related by a crystallographic twofold rotation. The two ssDNA molecules are antiparallel, with each 5' terminus located near RRM1 of one of the protein monomers, and the 3' terminus located near RRM2 of the symmetry-related protein monomer.

Significant conformational changes of UP1 are observed on DNA binding. First, there is a ~15° change in the relative position of the two RRMs, compared with the protein-only form (Shamoo et al. 1997; Xu et al. 1998).
1997), bringing the two β-sheet surfaces closer together (Fig. 2A). This rotation is hinged around the two conserved pairs of Asp–Arg salt-bridges (Arg-75–Asp-155 and Arg-88–Asp-157), which were found in the protein-only structure and are preserved in the structure of the UP1–TR2 complex (Fig. 2B). In spite of the large domain movement, there are only minor changes within each RRM. The root-mean-square (r.m.s.) deviations of Cα positions are 0.386 Å for RRM1 (residues 15–89) and 0.685 Å for RRM2 (residues 106–180), when each RRM is individually aligned to compare the complex and the protein-only forms. Second, the linker connecting the two RRMs becomes ordered on TR2 binding (Fig. 2A). Third, the region near the carboxyl terminus of UP1 (Lys-183–Ser-190), which was disordered in the absence of DNA, also becomes ordered in the presence of DNA and forms an α-helix (Fig. 2A). Both regions make important contacts with DNA (see below).

The telomeric DNA repeats bind to UP1 in a genuinely single-stranded form. The two strands are antiparallel and the interstrand backbone distance ranges from ~25–50 Å, that is, the two strands never come into contact. The path of each strand of TR2 follows two arcs, with each arc traversing one RRM module (Figs. 1B and 3A). The transition from one arc to the other occurs over a single nucleotide, Gua-6. This abrupt change of direction forces the guanine base to loop out. This base has weak electron density (Fig. 3A), presumably because it is highly mobile when exposed to solvent. The upstream arc consists of five nucleotides, Thy-2–Gua-6, and runs across the amino-terminal RRM1 of one UP1 monomer (Figs. 1B and 3B). The 5’-most nucleotide, Thy-1, cannot be modeled reliably because of weak electron density (Fig. 3). There are few interactions between the bases in this arc. The downstream arc consists of six nucleotides, Thy-7–Gua-12, and runs across the carboxy-terminal RRM2 of a symmetry-related neighboring UP1 monomer (Fig. 1B). As with the upstream arc, there are few interactions among the first 4 bases. However, the last three guanines (Gua-10–Gua-12) show base-stacking interactions (Fig. 1B). The bases of Gua-4 and Gua-10 are in the syn orientation, whereas all other ordered bases are in the anti orientation. 2'-endo sugar puckering is observed throughout.

**Protein–DNA interactions**

Detailed UP1–ssDNA interactions are shown in Figures 4 and 5A. Many of the contacts with ssDNA are similar for both RRMs, a feature consistent with the high degree of sequence homology between the two RRMs (Fig. 1A). Specifically, three consecutive nucleotides [T2, A3, and G4] in the first telomeric repeating unit superimpose extremely well with the corresponding nucleotides [T8, A9, and G10] in the second repeating unit (Fig. 5A). Two conserved phenylalanines [Phe-17 and Phe-59 in RRM1, and Phe-108 and Phe-150 in RRM2] located in the RNP-2 and RNP-1 submotifs of each RRM interact directly with AG dinucleotides [Ade-3–Gua-4, and Ade-9–Gua-10, respectively] by aromatic ring stacking (Fig. 5A). The same
four phenylalanines have been shown to be the major sites of covalent adduct formation when hnRNP A1 is UV cross-linked to oligo(dT) [Merrill et al. 1988]. A third phenylalanine, Phe-57 in RRM1 and Phe-148 in RRM2, does not contact the bases directly but, rather, interacts with the backbone of a guanine (Gua-3 and Gua-9) via van der Waals contacts. Another RNP-1 residue, Arg-55 in RRM1 and Arg-146 in RRM2, interacts with the backbone of a guanine (Gua-4 and Gua-10, respectively) via charge interaction with the phosphate and hydrogen bonding with O5'. A lysine (Lys-15 in RRM1, and Lys-106 in RRM2) immediately amino-terminal to the RNP-2 submotif forms a hydrogen bond with the N7 atom of this same guanine. This lysine contacts the next adjacent guanine via a water molecule in RRM1, but directly in RRM2. In addition, two charged residues (Glu-85 and Lys-87 in RRM1, Glu-176 and Arg-178 in RRM2) located in β4 interact with a thymine, Thy-2 in RRM1 and Thy-8 in RRM2, respectively. Arg-178 also interacts with Ade-9. Apart from the side-chain interactions, similar hydrogen bonds for main chain atoms of amino acids 88–90 and 179–181 are formed with AG dinucleotides, that is, Ade-3–Gua-4 and Ade-9–Gua-10, respectively.

One major difference between RRM1 and RRM2 interactions with DNA is seen in β2. In RRM1, the side chain of Asp-42 makes two hydrogen bonds with the N1 and N2 atoms of Gua-5, whereas the equivalent residue in RRM2, Val-133, does not contact DNA at all. Met-46, another residue located in β2 of RRM1, is within the range for making van der Waals contact with the base of Gua-5, whereas the RRM2 counterpart, Met-137, is fur-
group of Leu-181 hydrogen bond with the guanine rings of Gua-8 and Gua-9, respectively. The segment including residues 183–190 was disordered in the protein-only structure, but becomes ordered and forms an α-helix when bound to DNA. The amide group of Lys-183 makes a hydrogen bond with Gua-11, and its side chain interacts with the DNA backbone via a water-mediated inter-

Figure 3. Electron density maps showing the bound telomeric DNA. (A) Omit difference map \( (F_O - F_C, \phi_C) \) showing the path of the bound ssDNA. The map is contoured at 2.5σ. The DNA molecules are shown in a ball-and-stick model and the protein molecules in ribbon representation. The coloring scheme for the protein monomers is the same as in Fig. 1B. (B) Electron density surrounding the nucleic-acid-binding region in RRM1. The \( (2F_O - F_C, \phi_C) \) map was contoured at 1.5σ level, and the refined protein and DNA models are represented in a ball-and-stick model.

Figure 2. Conformational change of UP1 upon DNA binding. (A) Superposition of the Cα chains of UP1 from the UP1–TR2 complex (yellow) and from the protein-only structure (cyan) shows large domain movement attributable to DNA binding. The Cα atoms of RRM1 (residues 15–89) were used for least-squares alignment. (B) A close view of the salt bridges in both DNA-bound (yellow) and free (cyan) forms of UP1. The pairs of arginines and aspartates are shown in ball-and-stick representation. Green (C); blue (N); red (O).

ther away from the DNA bases. Instead, Arg-140 in RRM2 interacts electrostatically with the backbone phosphate of Gua-11, whereas its RRM1 counterpart, Pro-49, does not play a role in DNA binding.

The inter-RRM linker segment is defined to include amino acids 90–105. Three residues in this segment make direct contacts with DNA bases. Arg-92 makes contacts with three bases, Gua-4, Gua-5, and Thy-7, and the guanidino moiety is locked between the three bases [Figs. 4 and 5A]. Ser-95 makes a hydrogen bond with the N2 atom of Gua-4. The imidazole ring of His-101 stacks with the purine ring of Ade-3, which is sandwiched between His-101 and Phe-17.

We predicted that the amino-terminal 3₁₀ helix of UP1 would be involved in nucleic acid binding [Xu et al. 1997]. The present structure confirmed this prediction. O1 of Gln-12 forms a hydrogen bond with O6 of Gua-4 [Figs. 4 and 5A]. Glu-11 does not interact with DNA directly, but it stabilizes the conformation of Lys-15, which makes direct contact with DNA. At the carboxyl terminus, the main-chain amide group and carbonyl
action. Met-186 makes a van der Waals contact with Ade-9, such that the adenine ring is sandwiched between Met-186 and Phe-108.

Protein–protein interactions

In the present structure, DNA binding is achieved through a dimer of UP1, whereas in the absence of DNA, UP1 crystallized as a monomer (Shamoo et al. 1997; Xu et al. 1997). In the DNA complex there is an extensive interface between the symmetry-related protein monomers, which forms two contiguous DNA-binding clefts (Fig. 6A). A total surface area of 1574 Å² is shielded from the solvent by protein–protein interactions between the monomers. The dimer interface involves contacts that are distinct from the crystal lattice contacts in the protein-only structure. The intermolecular interaction is mediated mainly through six residues, of which four (Ile-164, Lys-166, Tyr-167, and His-173) are within RRM2 and two (Glu-11 and Asp-94) are outside of the two-RRM region (Fig. 1A). The intersubunit contacts include the following: hydrogen bonding between Glu-11 and His-173; a charge interaction between Asp-94 and Lys-166; hydrogen bonding between Tyr-167 and the carbonyl group of Ile-164 (Fig. 6B). Because of the twofold symmetry, each of these interactions occurs twice in identical fashion.

Discussion

Structural implications for interactions of hnRNP A1/UP1 with RNA

Biochemical studies have demonstrated that hnRNP A1 can bind specifically and with high affinity to single-stranded RNA or DNA sequences of similar sequence. Although physiological RNA targets remain to be better defined, some functionally significant RNA targets with sequences related to the high affinity motif have recently been found (Chabot et al. 1997; Li et al. 1997; del Gatto-Konczak et al. 1999; M. Caputi, A. Mayeda, A.R. Krainer, and A.M. Zahler, in prep.). The sequence specificity of hnRNP A1 appears to be similar for RNA and ssDNA, as no qualitative differences in binding were observed between these polynucleotides (Swanson and Dreyfuss 1988; Buvoli et al. 1990; Ishikawa et al. 1993; Abdul-Manan and Williams 1996). The binding similarity implies a similar underlying structural basis for sequence-specific interactions with RNA and ssDNA. We observed indistinguishable UP1 complexes by gel mobility shift with TR2 or with the RNA version of the same sequence (data not shown). These similarities enable us to infer the structural basis of RNA–hnRNP A1 interaction from the present structure of the UP1–ssDNA complex, at least in a qualitative sense. The presence of 2’ OH groups and the 3’-endo sugar puckering preferred by RNA should not interfere with the global features of the model, although small readjustments of the backbone conformation are expected. Moreover, the 5-methyl groups of the thymines are not involved in significant interactions with UP1.

In the cocrystral structure, RRM1 and RRM2 within the same protein monomer bind to two separate strands of ssDNA, which are antiparallel. Because the two RRMs within each monomer are also antiparallel, the 5’ → 3’ polarity of ssDNA with respect to the RRM orientation is the same for each RRM. Confirming our earlier conjecture (Xu et al. 1997), this nucleic acid directionality with respect to the RRM orientation is the same as that observed in the structures of an RNA–hairpin bound to the single amino-terminal RRM of U1A protein and also in the recently solved U2B–U2A–RNA ternary complex (Oubridge et al. 1994; Allain et al. 1996; Price et al. 1998). Therefore, it appears that RRMs bind RNA or ssDNA molecules in a preferred 5’ → 3’ direction.

Figure 4. Summary of contacts between UP1 and TR2. Residues enclosed in single-line boxes indicate side-chain contacts with DNA; residues enclosed in double-line boxes contact DNA with main chain atoms. (Dotted lines) hydrogen bonds; (wavy lines) charge interactions; (arrows) base-stacking or van der Waals interactions. For the DNA molecule, a thick line represents either carbonyl groups at the base or double-bonded phosphate oxygens. RRM1 residues from one monomer are shown in roman type, and RRM2 residues from the other monomer are shown in italics.

Figure 5. Structure of UP1 telomeric DNA complex
Direct interaction with 4 nucleotides (TAGG) is observed in RRM1 and with 5 nucleotides (TTAGG) in RRM2 (Fig. 4). These sequences are not only present in the high-affinity hnRNP A1-binding sequences selected from a random-sequence RNA pool by an in vitro iterative procedure (SELEX), but they can also be identified in the consensus sequences selected with hnRNP A1 lacking either RRM (Burd and Dreyfuss 1994). A 4-nucleotide sequence in an extended conformation is required to traverse the four β-strands in an RRM, following the nucleic acid path observed in the present structure and in the structures of U1A and U2Bβ and, therefore, this appears to be the minimal length required for recognition by an individual RRM. Interactions with the TAG trinucleotide [nucleotides 2–4 for RRM1 and nucleotides 8–10 for RRM2] are virtually identical and in register in both RRMs (Fig. 5B). This trinucleotide sequence is part of the consensus vertebrate 3′ splice site. In fact, binding of hnRNP A1 to 3′ splice-site sequences has been reported (Swanson and Dreyfuss 1988; Buvoli et al. 1990; Ishikawa et al. 1993). Interactions with the other nucleotides differ between the two RRMs. Asp-42 in RRM1 makes two hydrogen bonds with Gua-5 in RRM1, whereas the corresponding amino acid in RRM2, Val-133, does not interact with ssDNA at all. According to the present structure, replacing Gua-5 with an ad-
enzyme would maintain one of the two hydrogen bonds, but replacing it with a pyrimidine would prevent all the hydrogen bonds with Asp-42 because of distance constraints. Thy-7 is within the range of van der Waals interaction with Gly-111 and Ile-112 in RRM2, but the corresponding Thy-1 is not ordered in the structure.

The inter-RRM linker is highly conserved in length and sequence and has been implicated in nucleic acid binding and alternative splicing function [Burd and Dreyfuss 1994; Mayeda et al. 1998]. The present structure demonstrates the direct involvement of the linker segment in both protein–protein and protein–ssDNA interactions. Several residues located within this region contact ssDNA directly [Fig. 4]. Most interestingly, Arg-92 makes contacts with three nucleotides, Gua-4, Gua-5, and Thy-7. The two guanines are bound by RRM1, whereas the thymine is the first nucleotide of the TTAGG sequence bound by RRM2. The remaining di-

Figure 6. Protein–protein interactions in the UP1–TR2 dimer. (A) Molecular surfaces showing extensive contacts between the two protein monomers bound to the same two strands of DNA. The DNA fits into contiguous clefts in the two monomers. The molecular surfaces were generated with the program GRASP [Nicholls et al. 1991] with a 1.4 Å probe radius. The same color-code convention as in previous figures was used for the proteins, and the DNA molecules are shown in a CPK model colored in magenta. (B) Stereo figure showing amino acids involved in the protein–protein interface. (Magenta dotted lines) Hydrogen bonds; (red) oxygen atoms; (blue) nitrogen atoms; (brown) carbon atoms.
rect DNA contacts made by the linker are located in the RRM1 region. However, the presence of both RRMs and their spatial positioning are important for the linker to function in nucleic acid binding, because they constrain its spatial location and range of motion. The spatial arrangement of the two RRMs of hnRNP A1, which is largely determined by the two salt bridges, is such that the linker is exposed to the RNA-binding surface and is readily accessible for interaction with RNA or DNA. Conversely, the linker segment influences the spatial positioning of the two RRMs. In the cocrystal structure, the relative movement of the two RRMs appears to be primarily dictated by the covalent joining of the two RRMs by the linker. Thus, interaction with DNA on the RRM1 side pulls the linker toward RRM1, and this movement in turn pulls RRM2 closer to RRM1. This cooperative phenomenon is likely to be the origin of nucleic acid binding and functional synergy between the two RRMs (Shamoo et al. 1995, Mayeda et al. 1998 and references therein).

Additional contributions to nucleic acid binding from both the amino-terminal and carboxy-terminal regions outside of the RRMs of UP1 appear to be localized, that is, they contribute to nucleic acid binding by RRM1 and RRM2, respectively. Likewise, structural studies of the amino-terminal U1A RRM (Oubridge et al. 1994; Allain et al. 1996) and the RRM of hnRNP C (Gorlach et al. 1992), have shown that regions immediately adjacent to the classical RRM are often important in RNA binding. Interestingly, the RNA-binding role of the U1A RRM carboxy-terminal helix, which undergoes large conformational changes on RNA binding and makes contacts with bound RNA (Oubridge et al. 1994; Allain et al. 1996), appears to be analogous to that of e0, the amino-terminal 310-helix of UP1. When RRM2 of UP1 is superimposed with the U1A amino-terminal RRM, αC points in the opposite direction compared with the carboxy-terminal helix of the U1A RRM. This difference may reflect the fact that the U1A RRM binds to an RNA stem–loop, whereas hnRNP A1 binds to single-stranded nucleic acids.

**Significance of UP1 dimerization**

The structure reveals an interesting mode of nucleic acid binding by UP1, in which one single-stranded telomeric DNA binds to the amino-terminal RRM of one protein monomer and the carboxy-terminal RRM of another (Fig. 1B). This mode of binding may also contribute to the above-mentioned binding and functional synergy between the RRMs. The closely interacting UP1 monomers are related by a twofold crystallographic symmetry, raising the possibility that dimerization is induced by packing forces in the crystal lattice, and therefore is not physiological. The nucleic acid-free form of UP1 crystallized in a monomeric form (Shamoo et al. 1997, Xu et al. 1997), and the contact sites in the crystal lattice differ from those observed in the crystal of the UP1–TR2 complex. However, discounting the potential physiological relevance of the observed protein–protein interaction on the basis of these in vitro observations would be premature. Several lines of evidence suggest that this mode of dimerization may be important. (1) The extensive area of interface of 1574 Å² is indicative of a specific protein–protein interaction. For example, a survey of protease-inhibitor or antibody–antigen complexes revealed that the interfaces bury a surface area of 1500 ± 250 Å² (Janin and Chothia 1990; Janin and Rodier 1995), whereas a typical crystal-lattice contact buries <1200 Å²; (2) the residues that are critical for the dimer interface are all highly conserved in the hnRNP A/B family of proteins (not shown; see also Mayeda et al. 1998); (3) Gbp1p, a putative telomere-binding protein from C. reinhardtii that also contains two RRMs, can interact with single-stranded telomeric oligonucleotides as a monomer or as a dimer (Johnston et al. 1999). Dimeric Gbp1p shows strong preference for binding ssDNA; and (4) the conformational change, that is, the relative rotation of RRM2 with respect to RRM1 caused by nucleic acid binding is important for optimal dimer formation, because modeling the similar dimeric state with the protein-only UP1 structure gave an unfavorable protein–protein interaction (not shown). For the nucleic acids.

It is possible that UP1 dimerization is efficiently promoted by the particular oligonucleotide sequence used, which contains two tandem telomeric repeats. Nucleic acid-induced UP1 or hnRNP A1 dimerization may account for the unusually high binding affinity of hnRNP A1 for the SELEX winner sequence, which also contains two hexamer repeats separated by two nucleotides (Burd and Dreyfuss 1994; Abdul-Manan and Williams 1996; Abdul-Manan et al. 1996). On the other hand, purified hnRNP A1 can bind to oligoribonucleotides containing only one copy of UAGGGU/A [Burd and Dreyfuss 1994; Mayeda et al. 1998]. In such cases, it is not known whether the same protein dimerization and binding stoichiometry applies.

We have used a variety of physical methods to test whether UP1 can dimerize in solution in the presence of nucleic acid, but the results have been ambiguous, that is, we could neither demonstrate nor rule out that dimers can form. However, binding of UP1 to TR2 as-sayed by gel mobility shift required both of the telomeric repeat sequences and the wild-type forms of both RRMs, confirming the synergistic behavior of the RRMs evident in the structure (data not shown). It is possible that stable dimers cannot form under physiological conditions, and that the crystal structure and interface residue conservation reflect interactions of a more transient nature. Our recent hnRNP A1 domain-swap and domain-duplication data showed that although RNA binding is not severely affected in these variants, efficient alternative splice-site switching activity requires the presence of one copy of RRM2 preceding the carboxy-terminal glycine-rich domain, whereas the amino-terminal RRM can be either RRM1 or a copy of RRM2 (Mayeda et al. 1998). The alternative splicing activity of the different hnRNP A1 variants correlates well with the ability to model the
dimerization interface, which involves residues in RRM2 but not in RRM1. This observation suggests that protein dimer formation may be required for alternative splicing activity, and hence, that the observed dimer interface in the UP1–TR2 structure may have important physiological implications. It should be possible to test this hypothesis by mutational and functional analyses.

**hnRNP A1/UP1 and telomere length regulation**

As hnRNP A1, the UP1 fragment, and other very closely related proteins have been found to associate with the single-stranded overhangs of vertebrate telomeric repeats (McKay and Cooke 1992; Ishikawa et al. 1993; Erllitzki and Fry 1997), and more importantly, a recent study demonstrated a functional role for hnRNP A1 and/or UP1 in telomere length regulation in mouse cells (LaBranche et al. 1998), the present structure has important implications for the structure and function of vertebrate telomeres. Vertebrate chromosomes have single-stranded overhangs of vertebrate telomeric repeats (McKay and Cooke 1992; Ishikawa et al. 1993; Erllitzki and Fry 1997), and more importantly, a recent study demonstrated a functional role for hnRNP A1 and/or UP1 in telomere length regulation in mouse cells (LaBranche et al. 1998), the present structure has important implications for the structure and function of vertebrate telomeres. Vertebrate chromosomes have single-stranded overhangs of vertebrate telomeric repeats (McKay and Cooke 1992; Ishikawa et al. 1993; Erllitzki and Fry 1997), and more importantly, a recent study demonstrated a functional role for hnRNP A1 and/or UP1 in telomere length regulation in mouse cells (LaBranche et al. 1998), the present structure has important implications for the structure and function of vertebrate telomeres. Vertebrate chromosomes have single-stranded overhangs...

**Implications for alternative splicing regulation**

hnRNP A1 is an abundant protein associated with most nascent transcripts in the cell (for review, see McAfee et al. 1997). It has global concentration-dependent effects on alternative pre-mRNA splicing that require RNA...
binding via both of its RRMs [Mayeda et al. 1994]. The hnRNP A1 SELEX consensus hexamer sequence UAG-GGA/U coincides with the telomeric repeat TTAGGG and also bears some resemblance to portions of the vertebrate consensus 5’ and 3’ splice sites (C/AAG:GUAGU; YNYAG:G) [Burd and Dreyfuss 1994]. It is not clear at present whether binding to high-affinity sites, or to one or both splice sites, is necessary for the global effects of hnRNP A1 in alternative splicing. However, binding of hnRNP A1 to the sequence UAGAGU, which resembles the above consensus, within an intron of the hnRNP A1 pre-mRNA, has been implicated in autoregulation at the level of alternative splicing [Chabot et al. 1997]. Likewise, specific hnRNP A1 binding to other similar sequences, which in all cases include one or two copies of the UAG trinucleotide, is involved in splicing silencing of fibroblast growth factor receptor 2 and HIV pre-mRNAs, and in subgenomic mRNA transcription of mouse hepatitis virus RNA [Li et al. 1997; del Gatto-Konczak et al. 1999; M. Caputi, A. Mayeda, A.R. Kraimer, and A.M. Zahler, in prep.]. In view of its participation in many different cellular processes, it is likely that hnRNP A1 can function both as a sequence-specific and general nucleic acid-binding protein, depending on the process.

The structural basis for the sequence specificity of UP1 is predicted to be essentially identical for RNA and for ssDNA. With the short oligonucleotides used for cocrystallization in the present study, the structure shows that each RRM within a UP1 monomer binds to a different molecule of ssDNA. However, the crystal structure is consistent with the involvement of either one or both of the hnRNP A1 RRMs interacting with a single, long nucleic acid molecule [Shamoo et al. 1997]. For example, when there is only one high-affinity binding site, such as a splice site, it is possible that only one of the RRMs from a single hnRNP A1 molecule is used. When multiple binding sites are present in a single RNA molecule, each site may be bound by separate hnRNP A1 molecules and the protein molecules may associate with each other to bring distant binding sites closer together. It is also possible that these protein molecules do not interact with each other, that is, the sites are bound independently. Another scenario would be that the two binding sites interact with separate RRMs within the same protein molecule.

One or more of these scenarios may be relevant to the cellular functions of hnRNP A1. For example, the hnRNP A1-binding sites could be pre-mRNA splice sites. HnRNP A1 may then bring distant splice sites into close proximity and facilitate the proper folding of pre-mRNA for efficient splicing. In the case of alternative 5’ splice sites, which may be quite far apart on the same pre-mRNA molecule, binding to each of the RRMs within an hnRNP A1 monomer, or across a UP1 dimer, would place the two 5’ splice sites in an antiparallel orientation and within 25 Å of each other (Fig. 7D). This would present the two splice sites to the splicing machinery in a context in which they are easily distinguishable, thus explaining in part how hnRNP A1 can promote the selection of distal alternative 5’ splice sites [Mayeda and Krainer 1992]. The next step would be the base pairing of the 5’ terminus of U1 snRNA to the appropriate 5’ splice site, which may be facilitated by the RNA annealing activity of the carboxy-terminal domain of hnRNP A1 [Pontius 1993]. Each RRM may also bind to separate nucleic acid molecules to bring them together for efficient duplex formation [Shamoo et al. 1997]. Conversely, hnRNP A1 may unwind base-paired RNA or DNA by binding to transiently single-stranded regions and thus promoting the opening up of the duplex. Whether any of the above scenarios is realized in nature clearly warrants further structure-function studies.

Materials and methods

Crystallization and data collection

Recombinant human UP1 was expressed and purified as described [Mayeda et al. 1994; Jokhan et al. 1997]. Oligonucleotide TR2 was synthesized on an Applied Biosystems machine by standard phosphoramidite chemistry. The oligonucleotide was precipitated twice with ethanol from 20 mM MgCl₂ solutions and analyzed for purity by urea–PAGE and UV shadowing. The UP1–TR2 complex was formed by mixing UP1 protein and TR2 oligonucleotide at a 1:1 molar ratio and incubating on ice for 1 hr. The final protein concentration was ~12 mg/ml. The crystals were grown by the hanging-drop vapor diffusion method. The reservoir contained 0.1 M Tris (pH 8.5), 15% glycerol, and 2.0 M (NH₄)₂HPO₄. The crystals reached a maximal size of 0.2 x 0.2 x 0.4 mm after 3 days. The space group for these crystals is P₄₁₂₃, with cell dimensions of a = b = 51.20 Å, c = 171.09 Å. A native data set, Native-1, was collected with a one-cell Brandeis CCD detector at beam-line X12C, and all other data sets, except HgCl₂-1, were collected at beam-line X26C. Both beam-lines are at the National Synchrotron Light Source, Brookhaven National Laboratory. The derivative data set HgCl₂-1 was collected on a Rigaku X-ray generator [focused Cu Kα] with an Raxis-II imaging plate detector. All data were collected at 100°K. Derivatives were prepared by soaking the crystals with either 2.5 mM p-chloromercuribenzenesulfonate (PCMBs) or 2.5 mM mercuric chloride (HgCl₂) for two days. A native data set, Native-2, was collected from a crystal soaked for >2 months with 2.5 mM lead acetate, but no lead atom sites were detected. X-ray wavelengths used for synchrotron data sets were 1.15 Å for Native-1, 1.127 Å for PCMBs, and 1.115 Å for Native-2 and HgCl₂-2. All data reduction was carried out by the HKL program suite [Otwinowski 1993].

Phasing and refinement

One mercury site was identified for each of the derivatives by isomorphous and anomalous difference Patterson maps. Interestingly, the Native-2 data set, which was obtained from crystals presoaked with lead acetate, showed a higher degree of isomorphism with the mercury derivatives, and was therefore used for phasing. Refinement of heavy-atom parameters and phase calculations were done by the PHASES suite of programs [Furey and Swaminathan 1996]. The initial 3 Å MIRAS phases were improved by solvent flattening [Wang 1985]. The resulting solvent-flattened electron density map clearly shows the protein and DNA molecules. The protein model was placed into the electron density by molecular replacement with the coordinates of RRM1 and RRM2 from the protein-only UP1 structure [pdb

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code 1up1] with the AmoRe program (Navaza 1994). The molecular replacement solution has an R-factor of 42.9% and a correlation coefficient of 41.1%, by use of the data in the resolution range of 10.0–4.0 Å. In addition to the protein model, 11 (of 12) nucleotides can be built into the solvent-flattened MRAS electron density map. Modeling of the DNA molecule and protein model rebuilding were performed with the graphics program O (Jones et al. 1991). Model refinements were carried out with XPLOR and CNS (Brünger et al. 1998). Initially, data in the resolution range of 8.0–3.0 Å were used, and the starting crystallographic R-factor was 40.5%. Both higher and lower resolution data were then included with bulk-solvent correction. The crystallographic R-factor was 38.0%. Both higher and lower resolution range of 8.0–3.0 Å were used, and the starting model was refined with the PROCHECK program (Laskowski et al. 1993).

The atomic coordinates of the UP1–TR2 complex have been deposited with the Brookhaven Protein Data Bank [pdb code 2up1]. The coordinates can also be obtained from R.-M. Xu at xur@cshl.org.

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Crystal structure of the two-RRM domain of hnRNP A1 (UP1) complexed with single-stranded telomeric DNA

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