

## Review

# Nuclear functions of heterogeneous nuclear ribonucleoproteins A/B

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**Abstract.** The hnRNP A/B proteins are among the most abundant RNA-binding proteins, forming the core of the ribonucleoprotein complex that associates with nascent transcripts in eukaryotic cells. There are several paralogs in this subfamily, each of which is subject to alternative transcript splicing and post-translational modifications. The structural diversity of these proteins generates a multitude of functions that involve interactions with DNA or, more commonly, RNA. They also recruit regulatory proteins associated

with pathways related to DNA and RNA metabolism, and appear to accompany transcripts throughout the life of the mRNA. We have highlighted here recent progress in elucidation of molecular mechanisms underlying the roles of these hnRNPs in a wide range of nuclear processes, including DNA replication and repair, telomere maintenance, transcription, pre-mRNA splicing, and mRNA nucleo-cytoplasmic export.

**Keywords.** hnRNP A/B, RNA, RNA-protein interactions, telomere, DNA repair, pre-mRNA splicing, nucleo-cytoplasmic RNA export.

## Introduction

Heterogeneous nuclear ribonucleoproteins (hnRNPs) constitute a large family of proteins that associate with nascent pre-mRNAs, packaging them into hnRNP particles [1–3]. This family includes about 20 major polypeptides, hnRNPs A1 to U, which range in size from 34 to 120 kDa [2]. Many putative hnRNP genes that encode minor hnRNP proteins remain to be characterized [4].

Each hnRNP protein contains at least one RNA-binding motif such as an RNA recognition motif

(RRM), hnRNP K homology domain (KH) or arginine/glycine-rich (RGG) box [1, 5]. Many manifest a high affinity for specific nucleic acid motifs [6, 7]. Some hnRNPs contain auxiliary domains with unusual amino acid compositions [1, 5], which mediate protein-protein interactions [5, 8]. Correlated with these diverse structural features, a multitude of cellular functions has been ascribed to hnRNP proteins, including roles in DNA maintenance and recombination, transcription and processing of primary transcripts, and nuclear export, subcellular localization, translation and stability of mature mRNA [5, 9, 10].

The A/B subfamily of hnRNPs (hnRNPs A/B) [11] were originally described as two low-molecular-

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weight groups of hnRNP proteins isolated from the 40S “core” hnRNP particles of HeLa cells [12]. hnRNPs A0 [13] and A3 [14] were later included, as these proteins have modular structures that parallel their A1 and A2 paralogs (Fig. 1), with two tandem RRM domains near the amino-terminus and a glycine-rich domain (GRD) near the carboxyl-terminus [15]. These hnRNP A/Bs share a high level of amino acid sequence identity, especially in their structural motifs [13, 14, 16]. Human hnRNPs A1 and A2 exhibit ~80% and 58% identity in the RRM and glycine-rich regions, respectively [16, 17]. The amino acid sequence of the hnRNP A3 tandem RRM domains has high sequence identity with A1, though its GRD domain is more like that of A2 than A1 [14]. hnRNP A0 differs more; it has about 56% identity with human hnRNP A2 over the two RRM domains and GRD. The unrooted consensus neighbour-joining tree of tandem RRM domains encoded by 10 human genes obtained from a bootstrap analysis [18] supports the view that these hnRNP A/B proteins are evolutionary products that have arisen from a single, archetypal RNA-binding protein by gene duplication [14, 19–21]. The insertion of small peptides, resulting from alternative pre-mRNA splicing, generates some of the diversity among them [16].

Another two more distantly related hnRNP proteins, B2 and AB, have also been included in this subfamily [22, 23]. hnRNP B2 may be an alternatively spliced isoform of hnRNP A1 [24] or A2 [25]. hnRNP AB was previously classified as a type C hnRNP [26], but was later found to have two RRM domains and a GRD domain like the A/B type proteins [27]. This protein, however, shares limited sequence identity with hnRNP A/B subfamily proteins: it is more closely related to hnRNP D [18]. In this review we have focussed on hnRNPs A1, A2, A3, and A0, their alternatively spliced isoforms and UP1, which is a proteolytic product of hnRNP A1 generated by an unidentified trypsin-like protease [28].

hnRNPs A/B are among the smallest but most abundant hnRNP proteins [29], except for hnRNP A0, which is a minor hnRNP transcribed from a processed pseudogene [13] that has rarely been studied. hnRNPs A1 and A2 constitute 60% of the total protein mass of hnRNP particles, representing the most abundant nuclear proteins [12]. hnRNP A1 is present in  $7-10 \times 10^7$  copies per HeLa cell [30].

hnRNPs A/B localize predominantly in the nucleus but are excluded from nucleoli [31–33]. Most of these proteins also shuttle between the nucleus and cytoplasm [5, 9, 34–36]. hnRNPs A1, A2, B1, and B2, together with C1 and C2, form the 40S particle obtained by sucrose gradient sedimentation of sonicated nuclei digested with RNase A [1, 37]. hnRNPs

A2, B1 and B2 form (A2)<sub>3</sub>(B1) tetramers and (A2)<sub>3</sub>(B1)(B2) pentamers at the centre of core particles [38, 39], with hnRNPs A1, C1, and C2 positioned peripherally [37]. hnRNP A3 was not initially described as a component of the 40S particle, but recent mass fingerprinting has shown some of its minor isoforms to be present [40].

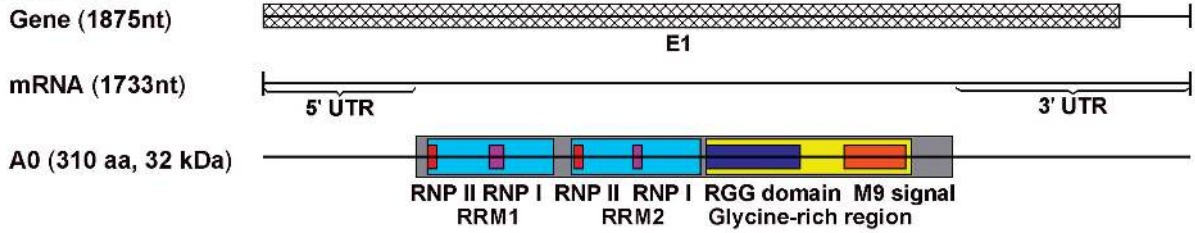
The tandem RRM-Gly structures of hnRNP A/B proteins enable them to bind other proteins and nucleic acids, hence their pivotal roles in packaging of nascent RNA and in many other aspects of nuclear and extra-nuclear mRNA processing. The major functions of these proteins include telomere biogenesis/maintenance [41–44], transcription [45, 46], alternative pre-mRNA splicing [24, 47–51], nuclear import [52] and export [53, 54], cytoplasmic trafficking of mRNA [14, 55–58], mRNA stability and turnover [59], and translation [60, 61]. This review focuses on the nuclear functions of these proteins. The interaction between hnRNP A/B proteins and polynucleotides or nucleic acids is reviewed first as it bears on the full repertoire of hnRNP A/B protein functions.

### Association of hnRNP A/B proteins with nucleic acids

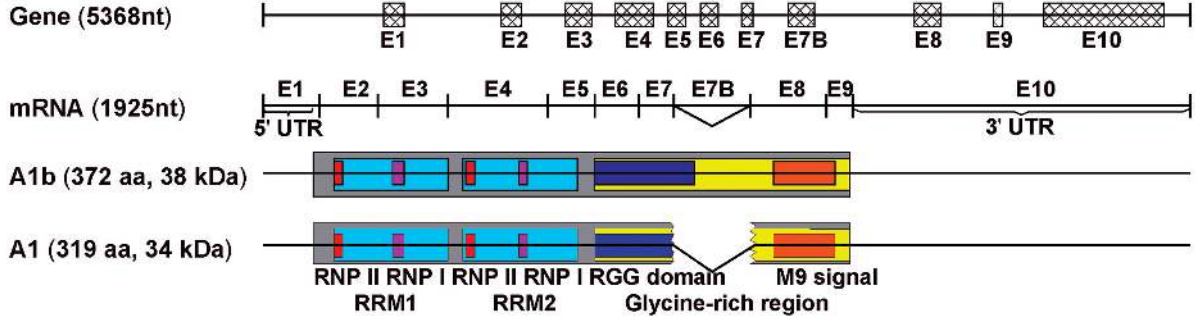
The interaction of hnRNP A/B proteins with polynucleotides was first observed for UP1, which passed through a column loaded with native dsDNA but was retained on a ssDNA-cellulose column in early attempts to identify eukaryotic DNA-binding proteins [62]. Subsequent studies have shown that hnRNP A1 and UP1 do associate with dsDNA [15, 63], suggesting that they may regulate gene expression. hnRNPs A1 and A2 interact *in vivo* with a number of elements in dsDNA, including hormone response elements [64] and other regulatory elements [46, 65]. These proteins also bind single-stranded DNA-agarose *in vitro* with low sequence specificity (Table 1, and references therein) [2].

Both the RRM and Gly-rich domains of these proteins are involved in binding DNA [66]. The hnRNP A1 tandem RRM domains are sufficient for the interaction with ssDNA, but they bind less tightly than the full-length protein. The C-terminal domain interacts with nucleic acids directly or indirectly through cooperative protein-protein interactions [66]. Post-translational *in vitro* methylation of HeLa hnRNP A1 arginine residues 193, 205, 217, and 224 [67] also affects its binding to ssDNA; compared with the unmethylated protein, the methylated A1 requires a lower concentration of NaCl to be released from a ssDNA-cellulose column [68]. As noted above, early *in vitro* data showed a preferential binding of UP1 and hnRNP A1 (cooperative for the latter) to ssDNA

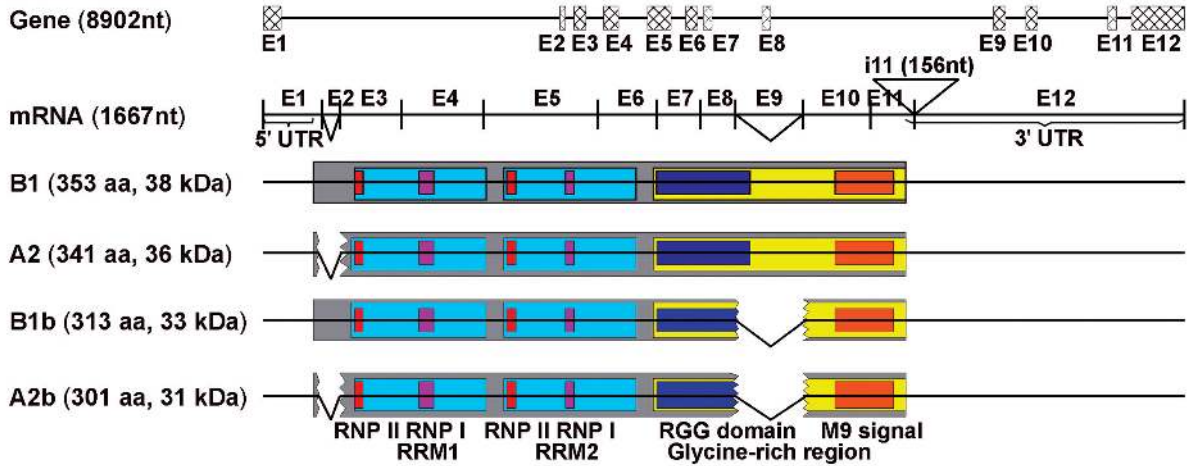
**hnRNP A0**



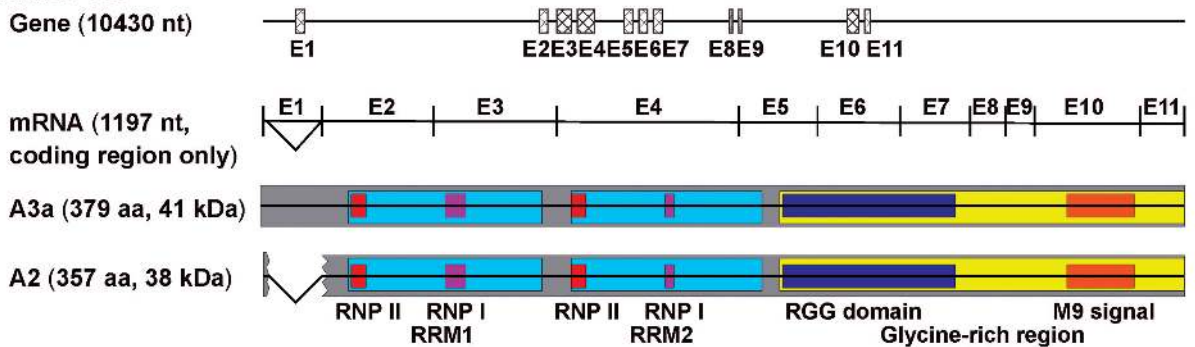
**hnRNP A1**



**hnRNP A2/B1**



**hnRNP A3**



**Figure 1.** Structure and characteristics of main hnRNP A/B proteins (E: exon; i: intron; nt: nucleotide; UTR: untranslated region)

**Table 1.** DNA-binding sequences/structures for hnRNP A/B proteins

| Binding sequence or structure | hnRNP           | Reference      |
|-------------------------------|-----------------|----------------|
| Native DNA-cellulose          | UP1             | [62]           |
| P(dT) <sub>8</sub>            | A1, UP1         | [196]          |
| Poly(dA-dT)                   | A1              | [63]           |
| ssDNA                         | A1              | [15, 63, 66]   |
| TGCTCTC                       | A1              | [46]           |
| Telomeric DNA repeats         | UP1, A1, A2, A3 | [44, 116, 117] |
| dsDNA                         | A1              | [15, 63]       |
| DNA triplexes                 | A2/B1           | [71]           |
| G-quartets                    | A1, A3          | [86]           |

rather than dsDNA [15, 63]. In accord with this, DNA duplex-destabilizing activity has been reported for A1 and UP1 [63], but under other conditions *in vitro* they can promote rapid renaturation of complementary strands of DNA and RNA [69]. The response of DNA to the presence of A1 is a complex function of temperature and A1 concentration. Whether A1 stabilises or destabilises dsDNA is dependent on the temperature relative to the melting temperature [70]. Above this temperature A1 destabilises dsDNA and the fraction of ssDNA is a function of the A1 concentration [70]. The interaction of A1 with ssRNA is also stronger than with ssDNA [15] and is attributed approximately equally to the tandem RRM domains of hnRNP A1 (or UP1) and the GRD [63]. Other data suggests that the GRD is needed for co-operative binding to nucleic acids [63, 69].

hnRNP A/B proteins bind structural motifs in DNA: hnRNP A2/B1 was pulled down by a DNA triplex probe together with hnRNPs K, L, E1, and I [71], and hnRNP A1 has been shown to interact with, and destabilize, G-quartets, the quadruplex structure of G-rich sequences [72]. This is believed to constitute one of the mechanisms by which these proteins trigger and coordinate their molecular functions [71].

Interaction of hnRNP A/B proteins with RNA has been well established, particularly for A1 and A2. hnRNP A1 preferentially associates with a so-called “winner” RNA sequence: UAUGAUAGGGA-CUUAGGGUG, in which the two closely-arranged UAGGGA(U) short sequences are critical [73]. Recombinant hnRNP A1 also binds to RNAs containing AUUUA-rich sequences *in vitro* [74]. For example, the granulocyte-macrophage colony-stimulating factor mRNA, which has an AUUUA-rich region in its 3'-UTR, can be immunoprecipitated using an antibody against hnRNP A1 [74]. With more extensive research on hnRNP A1 in the past two decades, additional binding sequences have been identified, as listed in Table 2.

One of the better-characterized hnRNP A2 binding sequences is the 21 nt hnRNP A2 response element (A2RE), or the derivative 11 nt oligonucleotide (A2RE11), which is essential for the cytoplasmic transport of several mRNAs in oligodendrocytes and neurons [55, 75, 76]. The A2RE sequence is evolutionarily conserved, and has been found in a number of transcripts, including *PRM2*, *MOBP81A*, *GABAR $\alpha$* , *GFAP*,  *$\alpha$ -CaMKII* and *ARC* [44, 75]. The RRM of hnRNP A2 are required to act in concert to ensure sequence-specific binding: single RRMs appear to be only capable of non-specific binding [56]. hnRNP A2 may also interact with the A2RE-like sequences, such as the A2RE-1 and A2RE-2 sequences found in a region of overlap between the *vpr* and *tat* genes of the HIV-1 virus *in vitro* [77].

The A2RE and A2RE-like sequences are not the only RNA structures that bind hnRNP A2. Early *in vitro* data suggested that hnRNP A2/B1 binds the UUAGGG sequence in addition to A1 [78]. Recently, in a microarray study to identify the downstream targets of hnRNP A2/B1 proteins, a group of transcripts was found which formed complexes with hnRNP A2/B1, but contained no A2RE or AU-rich elements (AuRE) [79], suggesting that hnRNP A2 may either associate directly with other unidentified RNA binding sequences or bind indirectly. The RNA-binding of hnRNPs A0 and A3 has been less studied, but *in vitro* evidence indicates that hnRNP A3 associates with A2RE and AuREs in the 3'-UTR of COX-2 mRNA [80].

In summary, the hnRNP A/B proteins are capable of binding a range of DNA and RNA sequences. Each of these proteins has high- and low-affinity nucleic acid binding sites [1, 56]. The eclectic binding of the hnRNP A/B proteins to DNA and RNA, specifically and non-specifically, and to consensus sequences and secondary or tertiary nucleic acid structures, can generate diverse regulatory roles.

**Table 2.** RNA-binding sequences for hnRNP A/B proteins

| Binding sequences                             | hnRNP     | Reference |
|---|-----------|-----------|
| hnRNA   | A1        | [15]      |
| Coliphage MS2 RNA                             | A1        | [15]      |
| Poly(rU), Poly(rC), Poly(rA), or Poly[r(A+U)] | A1, UP1   | [15, 63]  |
| Py-rich 3' splice site of introns             | A1        | [160]     |
| UUAGGG  | A1, A2/B1 | [44, 78]  |
| <u>UAGGGACUUAGGGU</u>                         | A1        | [73]      |
| AUUUA   | A1        | [74]      |
| d(GGCAG) <sub>n</sub>                         | A1        | [197]     |
| <u>UAGACUAGA</u>                              | A1        | [149]     |
| <u>UAGAGUAGG</u>                              | A1        | [149]     |
| <u>UAGAUUAGA</u>                              | A1        | [149]     |
| UAG binding site                              | A1        | [198]     |
| nYAGG <sub>n</sub>                            | A1        | [199]     |
| <u>UACCUUUAGAGUAGG</u>                        | A1        | [157]     |
| <u>AUAGAAGAAGAA</u>                           | A1        | [144]     |
| <u>UUAGA UUAGA</u>                            | A1        | [200]     |
| <u>UAGGGCAGGC</u>                             | A1        | [147]     |
| <u>UAUGAUAGGGACUUAGGGUG</u>                   | A1        | [201]     |
| UUAG  | A1        | [78]      |
| A2RE, A2RE-1, A2RE-2, A2RE11                  | A2, A3    | [55]      |
| pri-miR-18a                                   | A1        | [202]     |

### hnRNP A/B proteins in chromosome maintenance, DNA replication and repair

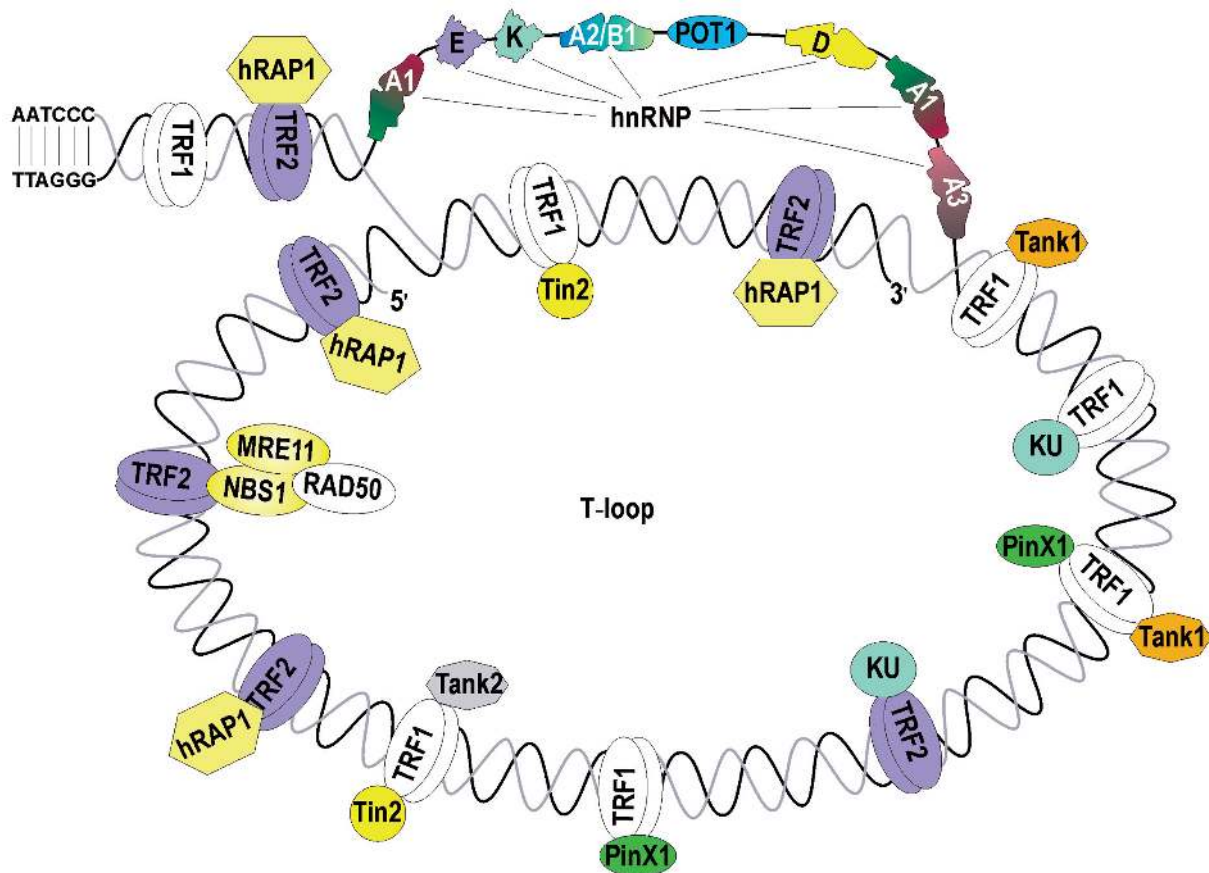
As discussed in the previous section, studies of helix-destabilizing activity for the full-length hnRNP A1 protein have not yielded consistent results [15, 26, 63]. It can be a potent regulator of DNA annealing within a single strand [81] and between two complementary strands [81, 82], but it can also destabilise dsDNA.

Targets for hnRNP A1/UP1-mediated destabilization include G-quartets, which are believed to be crucial for the regulation of DNA replication, transcription, and telomere maintenance [83–85]. hnRNPs A2/B1 and A3 also associate with G-quartets [86], but no studies of the destabilization of these structures have been reported. However, hnRNP A2/B1 is capable of destabilizing G<sub>2</sub> d(CG<sub>n</sub>), a tetraplex structure similar to the G-quartet but formed by two G-rich molecules. The conserved RNP1 and RNP2 motifs of the A/B hnRNPs mediate destabilization and stabilization, respectively, of the tetraplex structure [87].

The hnRNP A/B proteins play many roles in DNA replication. UP1 stimulates the activity of DNA polymerase  $\alpha$  [15, 88], an enzyme that synthesizes an RNA-DNA primer (the  $\alpha$ -segment) and initiates the formation of the Okazaki fragments during lagging strand DNA synthesis [89]. hnRNPA1 then stimulates

the activity of human flap endonuclease 1 (FEN-1), an enzyme that mediates processing of the  $\alpha$ -segment, and possibly the removal of the RNA primer, during the maturation of the Okazaki fragments [89]. hnRNP A2 binds the SET oncoprotein, a key regulator of DNA replication, chromatin remodelling, and gene transcription. Both proteins act as inhibitors of protein phosphatase 2A [90], an enzyme that regulates cell proliferation and differentiation. The unfolding of tetraplex structures, which appears to be widespread across the human genome [91], by UP1, hnRNPs A1 and A2/B1 may facilitate DNA replication [92]. Finally, hnRNP A1 interacts with nuclear DNA topoisomerase I (Top1) [93], which reversibly cleaves one strand of duplex DNA, relaxing DNA supercoiling, and thereby regulating DNA topology during replication, chromosome condensation, and transcription [94]. Top1 activity is inhibited by binding to G-quartets [95].

The roles of hnRNPA/B proteins in DNA metabolism also include the maintenance of telomeres, the protein-DNA complexes that cap the chromosome ends in some cells, preventing them from being illegitimately fused by the repair machinery for DNA double-stranded breaks [96]. The telomeres for vertebrates are comprised of a TTAGGG repeat [97, 98], with a G-rich, single-stranded 3' overhang



**Figure 2.** The putative telomere T-loop structure. The G-rich 3' overhang of the telomere loops back, invading the duplex repeat region of the same telomere and forming a T-loop structure by annealing with the C-rich strand. The displaced strand is associated with a number of proteins including POT1 and hnRNPs A1, A2/B1, A3, D, E, and K. Binding of telomeric repeat binding factors and their interacting proteins is also indicated (Adapted from a review by Neumann and Reddel [103]).

[99–101], which invades the double-stranded region of the telomeric DNA, forming a T-loop structure (Fig. 2) [102]. To stabilize their telomeres, cells synthesize new telomeric repeat DNA using telomerase [103], or, less frequently, lengthen the telomeres using a mechanism possibly involving recombination [104]. The replication capacity of cells that lack any means of maintaining their telomeres is limited by induction of cell cycle arrest, senescence and, in a subset of cells, apoptosis [105]. Failure to detect telomeres shortened beyond a critical length leads to chromosome instability and triggers malignant transformation [106, 107].

All of the A/B hnRNP paralogs, except A0, have been demonstrated to associate with the 3' single-stranded telomeric extension and protect it from nuclease attack. *In vivo* and *in vitro* studies have shown that hnRNPA1 and UP1 bind telomeres or single-stranded telomeric repeats [43, 108]. The crystal structure of UP1 complexed with a 12-nucleotide single-stranded telomeric DNA repeat revealed that a UP1 dimer binds to two strands of DNA, each strand interacting

with the RRM1 of one monomer and RRM2 of the other [109]. Murine hnRNP A2 associates with the single-stranded telomeric repeat (TTAGGG)<sub>n</sub>, as well as its RNA equivalent, UUAGGG [110]. hnRNP A2 protects the telomeric repeat sequence but not the complementary sequence [44]. A similar protective role has recently been reported for the binding of hnRNPA3 to the single-stranded telomeric repeat [42, 86] (S. Sara and R. Smith, unpublished observations). Telomeres are shorter in mouse erythroleukemic cells that do not express hnRNPA1, and are lengthened by the restoration of UP1 or hnRNPA1 expression [108], suggesting a positive role of this hnRNP in telomere elongation. Supporting this, a telomere repeat amplification protocol (TRAP) assay performed with a cell extract from HEK293, a human embryonic kidney cell line, showed that hnRNPs A1 [43] and the two isoforms of A2 that have the 12-residue N-terminal exon inclusion (B1 and B1b; Fig. 1) [111] stimulate telomerase activity. A *Caenorhabditis elegans* hnRNP A/B protein ortholog, HRP-1, also promotes telomere elongation *in vivo* [112]. At the molecular level,

hnRNP A/B proteins may serve as a bridge between the telomeric DNA template and the RNA component of telomerase. In a chromatography assay, the tandem RRM of hnRNP A1 were found to simultaneously bind a telomeric repeat DNA oligonucleotide and the RNA component of human telomerase, suggesting that hnRNP A1 may help recruit telomerase to the ends of chromosomes [113]. hnRNP A2 also binds the RNA template of telomerase (hTERT) [44]. However it is not known if it can bind to telomeric DNA and hTERT simultaneously. The unfolding of G-quartets by these proteins also suggests a positive role for hnRNPs A/B in telomere elongation. The telomeric repeat sequence is capable of forming a quadruplex structure [114] which inhibits telomerase activity [115]. Unwinding of the G-quartets may facilitate telomerase translocation and promote telomere extension [43].

The function of hnRNP A/B proteins in telomere elongation has been controversial since inhibitory effects have also been reported. Telomerase assays using a HeLa cell extract indicated that binding of hnRNP A1 to single-stranded telomeric repeat prevented extension by telomerase [116]. hnRNP A3 appears to have a similar inhibitory effect on telomerase activity [42, 117]. Most of these studies were performed *in vitro* and additional *in vivo* studies in mammalian models are needed to fully define the effects of hnRNP A/B proteins on telomerase activity. Recently, chromatin precipitation assays with antibodies to hnRNP A3 have shown an interaction with telomeric DNA repeats in rat brain extracts (S. Sara and R. Smith, unpublished observations).

hnRNP B1, which is over-expressed in the early stages of lung cancers, may play a role in DNA repair [118]. This protein associates with the DNA-dependent protein kinase (DNA-PK) complex, which mediates the repair of DNA double-strand breaks [119] and inhibits its activity, whereas hnRNPs A1 and A2 have no effect [118]. When the expression of hnRNP A2/B1 was suppressed by siRNA, DNA repair was faster in normal human bronchial epithelial (HBE) cells. It has been suggested that this causes inappropriate rejoining of double-strand breaks, triggering cell transformation.

### Functions of hnRNPs A/B in gene transcription

Although hnRNP A/B proteins preferentially bind RNA, rather than DNA [63], some have been shown to associate specifically with multiple promoter sequences and thus participate in regulation of transcription. hnRNP A1 binds the promoter regions of *c-myc* [120], *APOE* [121], thymidine kinase (*TK*) [122],

and the genes encoding  $\gamma$ -fibrinogen [46] and the vitamin D receptor [64]. It is a component of the transcription complex of an interferon-regulated gene, protein kinase regulated by RNA (PKR), which regulates virus multiplication and cell growth, differentiation, and apoptosis [123]. hnRNP A2/B1 shares some targets with A1, such as *c-myc* [120], *APOE* [121], and the vitamin D receptor gene [64], and additionally interacts with the promoter sequences of breast cancer 1 (*BRCA1*) [124] and gonadotropin-releasing-hormone 1 (*GnRH1*) [125]. hnRNP A3 also acts as a transcription factor, binding to the regulatory region of the *Hoxc8* gene [126].

Several different oligonucleotide motifs have been reported to mediate hnRNP A/B binding to transcriptional regulatory regions. They include the ATTT motif within the cell cycle regulatory unit of the human *TK* promoter [122], the TGCTCTC box in the  $\gamma$ -fibrinogen promoter [46], and the hormone-response elements of the vitamin D receptor [64]. It is not clear if the differences in these binding motifs determine the regulatory role of hnRNP A/B proteins in transcription. Some of these proteins, including A1 and A2, can act as either a transcriptional activator or a repressor. hnRNP A1 suppresses transcription from the *TK* [122] and  $\gamma$ -fibrinogen promoters [46], as well as both basal and induced expression from vitamin D-responsive promoters [64], but it activates the apolipoprotein E (*APOE*) promoter [121]. hnRNP A2 also represses expression of the vitamin D receptor, but it is more likely to be an activator for *BRCA1* transcription because suppression of this hnRNP led to a decrease of *BRCA1* at both the mRNA and protein levels [33].

How hnRNP A/B proteins contribute to transcriptional regulation is unknown. However, both direct and indirect mechanisms may be involved. Destabilization of G-quartets by hnRNP A/B proteins is likely to be a factor, considering the enrichment of putative G-quadruplex formation sites in the promoter regions [91, 127]. Transcription of *c-myc* is a good example: its regulation is associated with the formation of a G-quadruplex in the promoter region [127], where the interacting sites for hnRNPs A1, A2 and B1 are located [120]. hnRNP A/B proteins can indirectly participate in control of transcription through protein-protein interactions. hnRNP A2 interacts with the SET oncoprotein, which stimulates transcription by altering histone-DNA interactions [90]. Recent pull-down assays using a glutathione S-transferase (GST)-fused p53 transcriptional activation domain (residues 1–73) detected an hnRNP A2/B1 peptide [128], suggesting the possibility of A2/B1 forming a complex with p53, which is a multi-targeting transcription factor [129]. In addition, the association of hnRNPs

A1 and A2 with 7SK RNA (an snRNA) is critical for the release of P-TEFb, a transcription elongation factor required for transcription by RNA polymerase II, from the P-TEFb–HEXIM1–7SK RNA complex [130]. Simultaneous inhibition of hnRNP A1 and A2 expression reduced the transcription-dependent dissociation of P-TEFb–HEXIM1–7SK complexes.

Apart from their role in the initiation of transcription, hnRNP A/B proteins may contribute to termination of transcription, as evidenced by studies on two yeast hnRNP A/B proteins, Npl3 and Hrp1 [131]. Hrp1 forms the B component of the CFI polyadenylation factor and, when overexpressed, increases recognition of a weakened polyadenylation site and suppresses the defective transcription termination. Npl3 also functions in polyadenylation site recognition, by competing with the CFI polyadenylation factor for an RNA binding site. It will be of interest to see if mammalian hnRNP A/B proteins share a similar biological role in transcription termination.

### hnRNP A/B proteins as splicing repressors

Constitutive co-transcriptional splicing of nascent pre-mRNA results in intron removal and the fusion of exons to generate functional mRNA, a prerequisite for most eukaryotic genes [132]. Intron excision and exon ligation are directed by special sequences at the intron/exon junctions (splice sites) and catalyzed by the spliceosome, a large macromolecular complex assembled on the splice sites [133]. Alternative splicing results in excision of not only introns but also of specific exons. The hnRNP A/B proteins are essential components of the spliceosome and participate in both constitutive and alternative splicing (recently reviewed in [134]). *In vitro* dissociation of the spliceosome releases almost all hnRNP proteins, except A0 and AB, although some are only recruited to spliceosomes during certain stages of the splicing reaction. The major hnRNP A/B proteins, A1, A2, and A3 are among the few hnRNP proteins that are assembled into spliceosomes at all major splicing stages [135–142].

hnRNP A1 regulates pre-mRNA splicing by association with exonic splicing silencers (ESSs) and intronic splicing silencers (ISS), inhibiting the use of 3' splice sites or promoting the use of more distal 5' splice sites. This is supported by the established roles of hnRNP A1 and A2/B1 proteins as regulators of alternative splicing, which allows the cells to produce varied mRNA and protein isoforms from an identical gene by altering splice site choice, thus differentially including exons and introns, or portions of them [133]. These hnRNPs antagonise the action of serine/arginine-rich

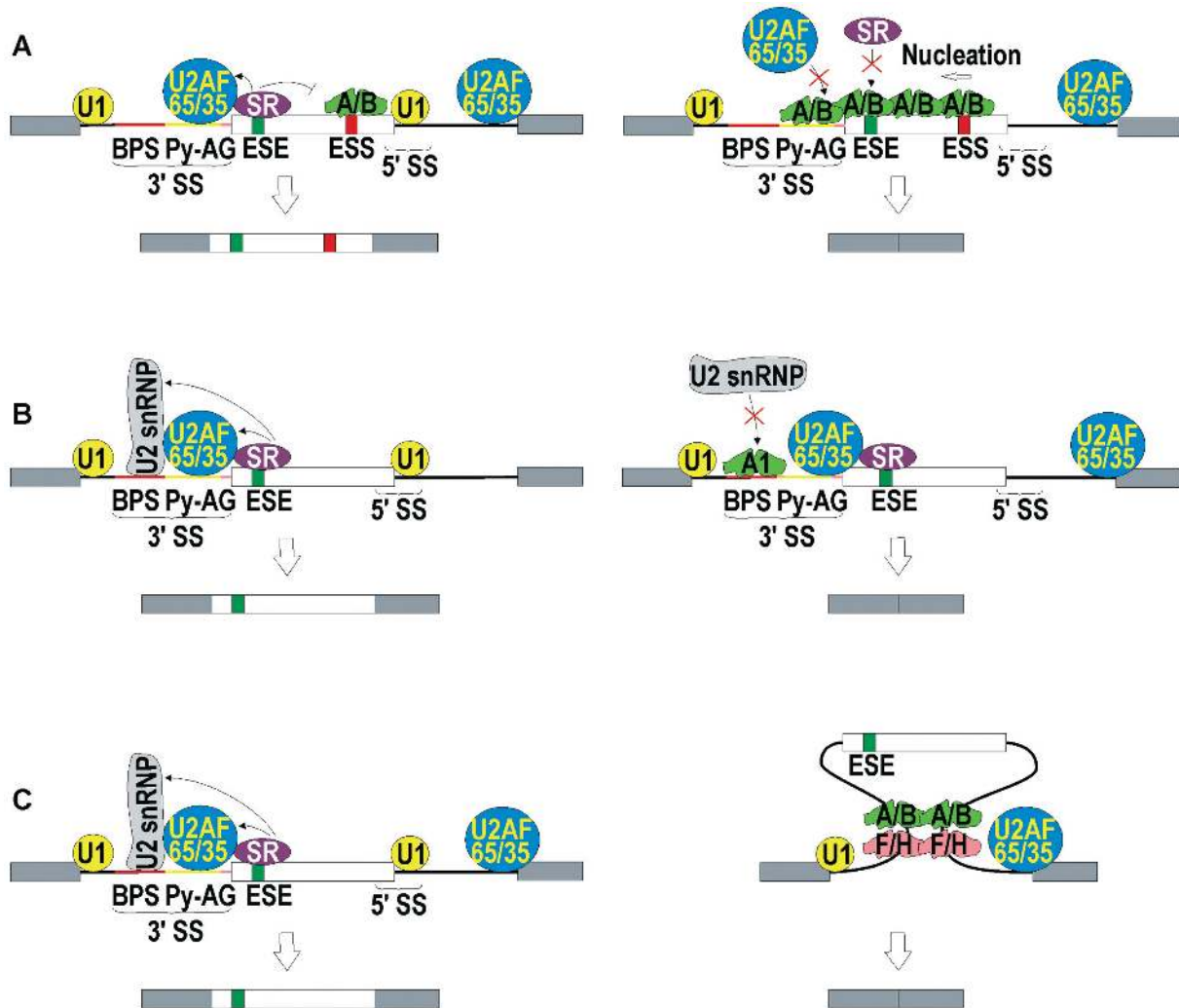
(SR) proteins, which bind to exonic and intronic splicing enhancers (ESE and ISE) and promote splicing [134].

As in most nuclear aspects of these proteins, the molecular mechanism of action of hnRNP A1 has been most intensively studied. However, hnRNP A2 appears to act similarly and it is more effective than A1 in splice-site switching. At this stage there is no direct evidence that hnRNP A3 mediates the splicing of any transcript, but it has been detected as a spliceosomal component by mass spectrometry and it is anticipated that it will function in a similar manner to A1 and A2.

The level of hnRNP A1 relative to the alternative splicing factor/splicing factor 2 (ASF/SF2) was first identified as a switch for splicing site selection using model and adenovirus E1A pre-mRNAs [47]. Later observations on bovine growth hormone (bGH) [143], HIV-1 tat and rev [144], c-Src [145], and INK4a pre-mRNA [146] correlated with this, suggesting hnRNP A1 as a *trans*-acting alternative splicing regulator *in vivo*. The two RRM, particularly the Phe residue in the RNP-1 submotif (Fig. 1), are essential for the specific hnRNP A1-pre-mRNA interaction and for modulating alternative splicing [48]. For some transcripts, such as human fibroblast growth factor receptor 2 (FGFR2), the repression of alternative splicing may be mediated by the GRD alone [147]. These domains are conserved across the hnRNP A/B subfamily, consistent with the observation that hnRNPs A2/B1 and A1<sup>B</sup> also favour distal splice-site selection [48].

Binding of hnRNP A/B proteins to ESS elements blocks proximal exon recognition [148]. Several ESS elements in different transcripts have been identified for hnRNP A1 [50, 147, 149, 150] and hnRNP A2/B1 [50, 149, 151]. Some alternatively spliced exons, such as bGH exon 5 [152] and HIV-1 tat exon 2 [153], have ESSs that overlap with an ESE element that specifically binds SR proteins. Thus, the outcome of competition between hnRNP A/B and SR proteins for common binding sites determines the splice site selection (Fig. 3A) [145, 150, 154]. When ESE and ESS elements are overlapping, the binding of one hnRNP A1 molecule may suffice to eliminate SR association with the ESE. When these sites do not overlap, the hnRNP A/B proteins may bind cooperatively along the exon, favouring splicing repression (Fig. 3A) [47, 134]. This co-operative binding results in exclusion of the proximal exon. However, a specific binding site is not essential for hnRNP A1 to antagonize SF2 in splicing control. Cooperative, indiscriminate, and low-affinity binding of A1 to the 5' splice site (5'SS) of  $\beta$ -globin mRNA inhibits U1 snRNP (small nuclear ribonucleoprotein) binding,



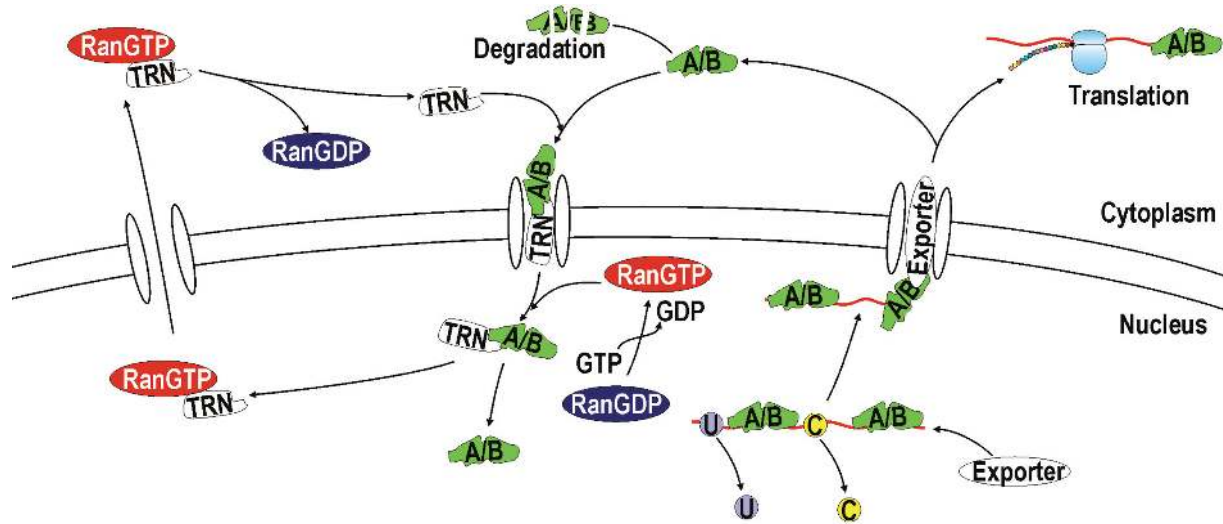


**Figure 3.** hnRNP A/B proteins in splicing. The intron-exon boundaries are defined by the U2 ribonucleoprotein particle (U2 snRNP), which binds a branchpoint sequence (BPS), and splicing factor U2AF, which recognizes a polypyrimidine tract (Py) at the 3' splicing site (SS), and U1 snRNP at the 5' SS. Binding of splicing factors, such as SR proteins, to an exonic splicing enhancer (ESE) promotes the exon definition process and suppresses the usage of exonic splicing silencers (ESS). (A) Binding of hnRNP A/B proteins to an ESS leads to assembly of additional hnRNP molecules along the intron-exon junction, and limits ESS accessibility to the spliceosome. (B) Splicing is repressed by hnRNP A/B proteins, which associate with the BPS and block entry of U2 snRNP. (C) The A/B- or F/H-type hnRNP proteins binding to the intronic splicing silencing sequences, which flank an exon, interact with each other and loop out the exon.

which is crucial for 5'SS recognition during spliceosome assembly, while ASF/SF2 enhances U1 snRNP binding at all 5'SSs [155].

Specific binding sites for hnRNP A/B proteins also exist in introns. For HIV-1 tat exon 3, an ISS for hnRNP A1 was found to overlap one of the branch points, a specific binding site for U2 snRNP that is required for efficient cleavage at the 3' splice site (3'SS). Binding of hnRNP A1 physically blocks the entry of U2 snRNP and inhibits spliceosome assembly (Figure 3B) [156]. hnRNP A1 pre-mRNA exon 7b is flanked by multiple ISSs for hnRNP A/B and F/H proteins [49, 51, 157–159]. The interaction between hnRNP molecules bound to the ISSs helps to loop out exon 7b (Fig. 3C) [49].

There are other mechanisms that elicit the participation of hnRNP A/B proteins in alternative splicing control. Association of any of these proteins with pre-mRNA may represent an early step in spliceosome formation. hnRNP A1 preferentially binds the 3' splice sites of introns in the presence of U1 and U2 snRNPs, two spliceosomal complexes, mediating the splicing of 5' and 3'-ends of introns, respectively [160–162]. hnRNP A1 also interacts with U2 and U4 snRNPs, and RNase H excision of U2 nucleotides 28–42 impacts on the U2 snRNP-pre-mRNA interaction by abolishing the A1-U2 snRNP interaction [163]. The RNA annealing capacity regulated by the GRD domain of hnRNP A1 [48] may be involved in the annealing of the RNA components of the snRNP



**Figure 4.** hnRNP A/B proteins participate in the nuclear export of mature mRNA. The exclusively nuclear-localized hnRNPs, such as hnRNPs C and U, dissociate from the mature transcripts, while the A/B type hnRNPs accompany the RNA through the nuclear pores. This may be mediated by an unidentified exporter. After directing the mRNA to its translation site, the hnRNP A/B proteins are released from the transcript, and destined for degradation or returned to the nucleus with the help of transportins (TRN). The hnRNP A/B proteins are then dissociated from transportins by the GTPase Ran (RanGTP) in the nucleus where they participate in their multiple nuclear roles.

particles and pre-mRNA [109]. Taken together, these studies support the concept that hnRNP A1 participates in the early stages of spliceosome assembly. Disruption of alternative splicing is associated with cancer, growth hormone deficiency, Frasier syndrome, Parkinson's disease, cystic fibrosis, retinitis pigmentosa, spinal muscular atrophy, and myotonic dystrophy [164]. The targets of hnRNP A/B-mediated splicing include two transcripts essential for the replication of HIV-1 virus [165]. In addition, these hnRNPs modulate the inclusion of alternatively spliced exons of several oncogenes, such as the *K-SAM* exons of human *FGFR2* [166], exon N1 of the *c-src* gene [145], and exon 1 $\alpha$  or 1 $\beta$  of *INK4a* [146]. The functions of hnRNP A/B proteins in the alternative splicing of these oncogenes or tumour-related genes may underlie the observation that hnRNP A/B proteins are frequently dysregulated in different types of cancer [167–171]. It is not known how closely the effects of hnRNP A/B proteins in alternative splicing are related to pathological conditions.

#### Shuttling of hnRNP A/B proteins and the nuclear export of mRNA

Mature transcripts are exported from the nucleus accompanied by an hnRNP complex [172]. The exclusively nuclear-localized hnRNPs, such as C and U dissociate from the complex and are retained in the nucleus whilst shuttling proteins, such as A1, E, and K, migrate into the cytoplasm together with mRNAs and

later return to the nucleus [172, 173]. hnRNP A1 is bound to poly(A)<sup>+</sup> RNA in both the nucleus and cytoplasm, suggesting it is exported together with the mRNA [34]. More convincingly, the hnRNP A1 ortholog in *Chironomus tentans*, *hrp36*, has been observed under the electron microscope to accompany mRNA through the nuclear pores to polysomes [174], suggesting association between hnRNP A1 shuttling and mRNA export.

The nucleocytoplasmic transport of hnRNP proteins requires import and export factors that target them to nucleoporin [175]. Two transport receptors of the karyopherin- $\beta$  family, transportin 1 (Trn1) and transportin 2 (Trn2), have been identified as regulators for the nuclear import of hnRNP A/B proteins [52, 176, 177]. Transportin is capable of binding nucleoporin and docks the hnRNP A1 at the nuclear pore complex during nuclear import (Fig. 4) [178]. Once in the nucleus, the transportin-hnRNP A/B complex is dissociated by the binding of transportin to the GTPase Ran (RanGTP) [177, 179]. The released hnRNP A/B proteins are available for their multiple nuclear functions, and the transportin returns to the cytoplasm where it is dissociated from RanGTP by the binding of the latter to the Ran binding protein (RanBP) and the GTPase activating protein (RanGAP) [173, 180].

In the GRD of hnRNPs A1 and A2/B1, a 38-residue M9 motif which bears no sequence similarity to the classical nuclear localization signal mediates their interaction with transportins [52, 176, 177]. Residues 3–21 of the M9 motif form the core signal peptide, of which residues 3–8 and 20–21 are particularly

important: a single mutation in one of the two sequences abolishes the binding of transportin and hence nuclear uptake [181]. However, the GST-tagged M9 signal exhibits different affinity and specificity for transportin compared to the full-length hnRNP A1, possibly because the M9 sequence is presented in different ways [177]. The potential conformational difference may affect the interaction of the M9 sequence with transportin, which is believed to recognize its cargo protein through secondary/tertiary structural features rather than primary sequence [179]. A 19-amino acid “F-peptide” adjacent to the M9 signal regulates the bidirectional transport of hnRNP A1. Its phosphorylation weakens the hnRNP A1-transportin interaction and decreases the nuclear import of hnRNP A1. Phosphorylation of the F-peptide may mediate the accessibility of the M9 signal by changing its tertiary structure [182].

Newly synthesized mRNA acts as an inducer for the nuclear import of hnRNP A/B proteins, although the signalling cascade is not understood. Inhibition of RNA polymerase II in HeLa cells by actinomycin D leads to hnRNP A1 retention in the cytoplasm [34, 183]. Similarly, in transcriptionally inactive mouse embryos, hnRNP A1 diffuses passively through the nuclear pores. When the actinomycin D is removed, hnRNP A1 starts to accumulate in the nucleus. Production of the new transcripts in the nucleus is necessary and sufficient to induce the nuclear accumulation of hnRNP A1, while the presence of newly synthesized RNAs in the cytoplasm has no such effect [183]. Blocking of transcription with actinomycin D or  $\alpha$ -amanitin also disrupts the localisation of A2/B1 hnRNPs within the nucleus [184].

The molecular mechanism by which hnRNP A/B proteins regulate nuclear export of mRNA is not well understood. Although the M9 sequence is believed to be a signal for nuclear import and export of these hnRNPs [53], it is uncertain whether transportins function as bidirectional factors [177, 185, 186]. hnRNP A1 associates with the mRNA export factor [172] which represents a family of proteins involved in TAP/Mex67p-mediated mRNA export [187]. Nuclear export of hnRNP A2/B1 can be induced by the activation of the chemokine receptor CXCR4, in the presence of cyclophilin A, which forms a complex with hnRNP A2/B1 [188]. It is uncertain whether CXCR4 functions as a nuclear export factor in the M9-mediated pathway.

Other factors might also influence hnRNP A1-mediated nuclear export. The nuclear export of the *Saccharomyces cerevisiae* hnRNP A1 homolog, Hrp1p, and another hnRNP A/B family protein, Npl3p, requires arginine methylation by Hmt1p. When Hmt1p is inhibited, the two hnRNP proteins

are retained in the nucleus [189]. Although methylation of human hnRNP A1 is not known to regulate the nucleocytoplasmic transport of the protein, methylation at four arginine residues (R193, R205, R217, and R224) within the RGG box affects its RNA-binding properties [67, 88].

Involvement of phosphorylation in the regulation of nuclear export and import of hnRNP A/B protein is suggested by the accumulation of hnRNP A1 in the cytoplasm when either protein kinase C  $\zeta$  (PKC  $\zeta$ ) or protein kinase A (PKA) is over-expressed [190, 191]. An hnRNP A1 peptide including Ser<sup>199</sup> has been identified as the substrate for the two kinases. In addition, phosphorylation may also affect the interaction between hnRNP A/B proteins and their cargo mRNA. The MAP kinase signal-integrating kinase (Mnk)-mediated phosphorylation of hnRNP A1 inhibits its binding to tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) mRNA *in vivo* [192].

hnRNP A2 is a *trans*-acting factor involved in the trafficking of mRNAs possessing an A2RE11-like *cis*-acting element. It has been proposed that tetramers of A2 bind A2RE11-containing mRNAs in the nucleus and orchestrate their export from the cell nucleus [180, 193]. After export, the complex binds hnRNP E1, which represses translation until the trafficking granule has moved along the microtubules and reached its destination in the periphery of the cell.

### The future and concluding remarks

The hnRNP A/B subfamily exhibits affinity for a spectrum of nucleic acid motifs, including the ssDNA telomeric repeat, the U-rich motif and A2RE. These proteins appear to possess two classes of nucleic acid binding sites, both of which largely map to the tandem RRM. One class associates with single-stranded nucleic acid without a strong preference for a particular nucleotide sequence: these sites bind less tightly and are disrupted by addition of polyanions, such as heparin, which are commonly added to suppress non-specific protein/nucleic acid interactions [56]. These interactions are typified by the use of native ssDNA in the purification of hnRNP A/B proteins.

The second class of sites binds more tightly, with dissociation constants typically in the 10–50 nM range, and shows preference for particular motifs, but not for a strictly defined consensus sequence. These motifs may include a few highly conserved nucleotides within a matrix of less conserved or non-conserved nucleotides (e.g. the A/B hnRNPs bind the telomeric repeat sequence, but this interaction is largely unaffected by the substitution of nucleotides in many positions [44]). A second example is the binding

to exonic and intronic splicing silencer motifs and the competitive interactions that lead to the antagonistic binding directed against ASF/SF2 during splice site selection.

The hnRNP A/B glycine-rich domain binds other proteins but it may also contribute to interactions between the hnRNPs A/B and nucleic acids. For example, the full-length hnRNP A2 binds the cytoplasmic trafficking motif A2RE more tightly than does the tandem RRM of this protein [56]. The possession of two or more binding sites on these hnRNPs may enable them to act as adaptors between DNA or RNA and other functionally specific factors. For example, hnRNP A1, A2 and A3 may help to recruit the telomerase to the telomeric RNA template [44, 113].

It will be of great interest to determine whether the individual mammalian hnRNP A/B proteins also associate with groups of functionally related transcripts. For example, the yeast homologs of two hnRNP A/B proteins, Npl3 and Nab4/Hrp1, manifest RNA binding profiles of functional significance [194]. Npl3 favours binding to mRNAs encoding ribosomal proteins and other highly expressed transcripts, whereas the transcripts for proteins involved in amino acid metabolism are enriched among the Hrp1-binding molecules [194].

Many, if not all, of the hnRNP proteins appear to be multifunctional, as noted above for the A/B hnRNPs. Some of these functions overlap for different proteins. For example, both hnRNPs A/B and K [195] have been implicated in transcription, RNA processing, translation and signal transduction processes and pathways. hnRNPs A1, A2 and A3 may manifest some similar functions, but our recent studies [184] show that all three are not expressed in the same location. In HeLa cells, A1 is concentrated close to the nuclear envelope whereas A2 and A3 are instead prominent in the perinucleolar region, suggesting that they have different intra-nuclear roles.

Further functional diversity is generated by post-transcriptional (including alternative splicing) and post-translational regulation of gene expression. Another field that has only been superficially addressed is the extent, species variation, and spatial and temporal distribution within tissues of protein molecules which have undergone different post-translational modifications, particularly methylation and phosphorylation.

Alternatively spliced isoforms may be directed to separate locations where they have different functions. One might expect, for example, to find different hnRNP A/B isoforms associated with components of the telomeres compared with the spliceosome. The clearest evidence we have to date for the hnRNPs is

the differences in localisation of hnRNP A2 isoforms. The exon 9-expressing hnRNP A2 and B1 isoforms are confined to the nucleus, whereas the A2b (and probably B1b) isoform is present at far lower concentrations in the nucleus and more abundantly in the processes of oligodendrocytes and dendrites of neurons (unpublished data). There are also strong temporal variations: in rodents, the levels of A2b and B1b are low at birth, rise sharply for a few days and then decline to being barely detectable in mature adult animals. Does this molecular distribution point to differences in function for these isoforms? It is tempting to speculate that the A2b and B1b isoforms are positioned to participate in two of the major processes with which hnRNP A2 has been associated: cytoplasmic mRNA trafficking and local regulation of translation.

Some of the roles of the hnRNPs A/B can be inferred from the molecules with which they interact and the location of these proteins. It is currently difficult to predict the binding partners for these hnRNPs, but the evolving genomic or proteomic technologies present powerful new tools for high-throughput identification of the interacting molecules. In recent experiments, shRNA-induced knockdown of hnRNP A2 was used with DNA microarrays to identify downstream targets of A2 [79]. A substantial number of transcripts with no known hnRNP A2-specific binding sequence were found to form complexes with this protein, possibly by indirect binding. The increasing use of siRNA techniques to knock down target genes and the generation of conditional knock-out mice offer considerable promise in exploration of the wide range of activities associated with individual hnRNP A/B proteins, their alternatively spliced isoforms and breakdown products.

Finally, complexes within cells may exist transiently or change their composition with time, but even transient interactions may fulfil important biological functions. Thus, while there is substantial evidence to support the involvement of the hnRNPs in alternative splicing of RNA, at some stages spliceosomes can be isolated that do not include the A/B hnRNPs. Yet this protein sub-family is one of the few classes of protein that is present at most stages of splicing. The major challenge is deciphering the reasons, at the molecular and cellular levels, for the genomic and proteomic complexity of the hnRNPs.

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