Structural determinants for alternative splicing regulation of the MAPT pre-mRNA

Jolanta Lisowiec, Dorota Magner, Elzbieta Kierzek, Elzbieta Lenartowicz, and Ryszard Kierzek*

Institute of Bioorganic Chemistry; Polish Academy of Sciences; Noskowskiego, Poland

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Abbreviations: AD, Alzheimer disease; DMS, dimethyl sulfide; ESE, exonic splicing enhancer; ESS, exonic splicing silencer; FTD, frontotemporal dementia; FTDP-17, frontotemporal dementia and Parkinsonism linked to chromosome 17; ISM, intronic splicing modulator; ISS, intronic splicing silencer; MAPT, microtubule-associated protein tau; NMIA, N-methylisotoic anhydride; NMR, nuclear magnetic resonance; PPE, polypurine enhancer; pre-mRNA, pre-messenger RNA; RT-PCR, reverse transcription polymerase chain reaction; SHAPE, selective 2'-hydroxyl acylation analyzed by primer extension; SMA, spinal muscular atrophy; SRE, splicing regulatory element; U1 snRNP, U1 small nuclear ribonucleoprotein; WT, wild type

Alternative splicing at the MAPT gene exon 10 yields similar levels of the 3R and 4R tau protein isoforms.¹ The presence of mutations, particularly in exon 10 and intron 10–11, changes the quantity of tau isoforms. Domination each of the isoform yields tau protein aggregation and frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17). Here, we report for the first time the secondary structure of the 194/195 nucleotide region for the wild type (WT) and 10 mutants of the MAPT gene pre-mRNA determined using both chemical and microarray mapping. Thermodynamic analyses indicate that single nucleotide mutations in the splicing regulatory element (SRE) that form a hairpin affect its stability by up to 4 and 7 kcal/mol. Moreover, binding the regulatory hairpin of small molecule ligands (neomycin, kanamycin, tobramycin and mitoxantrone) enhance its stability depending on the nature of the ligands and the RNA mutations. Experiments using the cos-7 cell line indicate that the presence of ligands and modified antisense oligonucleotides affect the quantity of 3R and 4R isoforms. This finding correlates with the thermodynamic stability of the regulatory hairpin. An alternative splicing regulation mechanism for exon 10 is postulated based on our experimental data and on published data.

Introduction

The nervous system includes the highest number of tissue-specific proteins and isoforms. Such protein diversity is due to and mainly regulated through an alternative splicing process, and deep sequencing data show that 92–97% of human genes are alternatively spliced.²

The product of the MAPT gene (i.e., the tau protein) belongs to the microtubule-associated protein family. Alternative splicing of the MAPT gene transcript yields 6 tau isoforms.³ The tau protein is primarily observed in the adult human brain in the axonal portion of a neuron, and its main functions are polymerization and microtubule stabilization. Consequently, it is involved in morphogenesis, axonal extension and protein transport in the axon. In a pathological state, the tau protein aggregates and is present throughout the neuron. Moreover, it can form insoluble paired helical filaments and neurofibrillary tangles, which yield diseases that are referred to as tauopathies. Tau protein aggregates are found in Alzheimer disease (AD) and frontotemporal dementia

(FTD).⁴ Recently, tauopathies connected with dementia linked to chromosome 17 were named frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17). In 1998, the first mutation in the MAPT gene was discovered, and, currently, over 40 have been found.⁵ MAPT gene mutations can be divided into 2 types. The first type causes changes in biochemical properties of the tau protein. The second type disrupts alternative splicing of exon 10. The MAPT gene consists of 16 exons; furthermore, alternative splicing of exons 2, 3 and 10 yields 6 protein isoforms. Alternative splicing of exon 10 produces 2 tau protein isoforms, 3R (without exon 10) and 4R (with exon 10), and a healthy brain includes similar levels of 3R and 4R.⁶ One study reported that the MAPT gene region that includes the 3'-end of exon 10 and 5'-end of intron 10–11 is self-complementary and forms a hairpin (regulatory hairpin).⁷ Changes in the thermodynamic stability of this hairpin are induced by mutations and, consequently, cause the 3R or 4R isoform to dominate, which changes the 3R/4R tau isoforms ratio. A change in the quantity of 3R and 4R isoforms causes tau protein aggregation and FTDP-17 disease.^{8–10}

*Correspondence to: Ryszard Kierzek; Email: rkierzek@ibch.poznan.pl

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The alternative splicing process is controlled by trans-acting factors and cis-acting elements. Studies show that exon 10 splicing is regulated by a weak 5' splice site and 7 cis-acting elements: SC35-like enhancer, polypurine enhancer (PPE), A/C rich enhancer, exonic splicing silencer (ESS), exonic splicing enhancer (ESE), intronic splicing silencer (ISS) and intronic splicing modulator (ISM).^{11,12} However, it was reported that a regulatory hairpin (ISS with 5' splice site) is the main factor responsible for regulating exon 10 splicing.^{11,12} Researchers have suggested that changes in the stability of the regulatory hairpin affect the interaction of this fragment with the U1 snRNP or indirectly affect trans-acting element binding (e.g., the PSF protein and helicase p68).^{13,14}

The main goal of this study was to determine the secondary structure of the 194/195 nucleotide fragment (195 nucleotide fragment for certain mutations) of the MAPT gene pre-mRNA. Studies of the mutations in regulatory hairpin indicated changes in thermodynamic stability. In the context of longer RNA molecule those mutations may possibly affect the modulation of the secondary structure.^{8–10} Structural studies were performed using 11 RNA molecules.^{8–10,15} The RNAs (194/195 nucleotide molecules) consist of 39 nucleotides from intron 9–10, 93 nucleotides from exon 10 and 62/63 nucleotides from intron 10–11. To determine the secondary structure of the pre-mRNA MAPT gene, chemical mapping (DMS and SHAPE) and isoenergetic RNA microarray approaches were used. The data from these experiments were used to predict the secondary structure using the vsfold5 and Shapeknots programs. The 25/26 nucleotide-long pre-mRNA MAPT gene fragments were used to determine thermodynamic parameters of 11 RNAs with the same mutations as in structural studies. Finally, in vitro experiments using cos-7 cells showed that the 194/195 fragments studied are active in the MAPT gene exon 10 alternative splicing process, and this process can be controlled by antibiotics or antisense oligonucleotides. Combining the information on secondary structure, data for cell line experiments described here and results by other investigators on the influence of certain regions on MAPT pre-mRNA alternative splicing regulation, allowed hypothesized about this process.^{11,12}

Results

The secondary structure of the MAPT pre-mRNA 194/195 nucleotide fragments

The main goal of these investigations was to determine the secondary structure of the pre-mRNA MAPT gene fragment that contains the hairpin involved in regulating alternative splicing at exon 10. Previous studies focused only on regulatory hairpin structure.^{7,16,17} Herein, for the first time, the secondary structures of 194/195 nucleotides

long tau pre-mRNAs were investigated. It was suggested that in SMA (spinal muscular atrophy) disease mutation of single nucleotide change the pre-mRNA secondary structure could disrupts splicing.¹⁸ Based on the RNAstructure program analysis, the shortest fragment was selected in which the hairpin studied and its adjacent fragments did not change the predicted secondary structure.^{19,20} As the smallest MAPT pre-mRNA model, the RNA composed of 39 nucleotides from intron 9–10, 93 nucleotides from exon 10 and 63/64 nucleotides from intron 10–11 was selected. To obtain the 194/195 nucleotide MAPT pre-mRNA fragment, including the hairpin with the following mutations: 11C, 12U, 13G, DD-PAC, 16U, 19G, DD-10C, DDI-17T, WT-10C, and WTI-17T, 11 DNA templates were prepared and used for in vitro transcription (Fig. 1). To determine these secondary RNA structures, microarray and chemical mapping (both DMS and SHAPE methods) were used.^{21–26} Native gel electrophoresis at 4°C was performed to verify that the model RNAs fold into one structure. The experiment confirmed that WT RNA from in vitro transcription forms a single structure irrespective of the buffer and folding conditions (Supplementary Fig. S1).

Microarray mapping

Microarray mapping is based on complementary binding pentanucleotide probes to single stranded regions of the target RNA only.²⁷ Application of the LNA modified probes results in their isoenergetic binding to RNA. The nucleotide complementary to the middle nucleotide of a strongly binding probe was constrained in RNA folding programs. Hybridizations using an isoenergetic microarray demonstrate that all 11 194/195 nucleotide-long RNAs form almost the same secondary structure (Supplementary

Figure 1. The structures of the studied RNA. (A) Naturally occurring mutations in FTDP-17^{8–10}, and (B) mutations proposed by Donahue et al.¹⁵ The symbols 3R and 4R next to the indicated mutation denote formation of a preferred tau protein isoform due to the mutation introduced

Data, Table S1). The data presented show that mutations in the regulatory hairpin do not affect the secondary structure of the 194/195 MAPT pre-mRNA nucleotide fragments. Analyses of the microarray mapping demonstrate that certain probes may have alternative binding sites. Among the 182 probes used to prepare the isoenergetic microarray, 48 yielded strong and medium binding signals, only 20 signals of which can be considered as such because the probe did not have alternative binding sites.

Hybridization of target RNA molecules on the microarray was used to define the single-stranded RNA fragments. Within the target RNA, the following regions bind the microarray oligonucleotide probes: 7–12 (probes 71, 530), 15–28 (probes 569, 164, 41, S-1, 129), 37–41 (probe 96), 53–61 (probes S-6, S-7, 638), 67–71 (probe 451), 105–109 (probe 909), 118–125 (probes 347, 855, 470, 374), 141–145 (probe 899), 174–179 (probe S-13) and 185–189 (probe 328) (Supplementary Data, Table S1). To predict the secondary structure of the target RNAs, only unambiguous binding sites from microarray probes were used.

Chemical mapping (SHAPE and DMS approaches)

The SHAPE approach

SHAPE is chemical method used to map RNA secondary structure, in which NMIA reacts with the 2'-hydroxyl of a flexible ribose, and the most reactive regions are single stranded regions.^{22,28} The SHAPE mapping data show small differences in modifications between RNA mutants (Supplementary Data, Table S2). Medium 2'-hydroxyl SHAPE reactivity ranges from 0.3 to 0.7, and strong reactivity is above 0.7. The nucleotides in the following regions of the target 194/195 MAPT pre-mRNA nucleotide fragment indicate strong and medium reactivity upon SHAPE mapping: 24–25, 39, 49–55, 58, 85–87, 95, 105–109, 123–124, 131, 139–142, 154–156, 161, and 165–166. The following nucleotides showed differences in reactivity: 49, 54, 55, 86, 95, 109, and 156. These differences likely do not indicate the secondary structure rearrangement but rather small changes in flexibility.

DMS approach

In the second chemical mapping method, nucleotides were modified with dimethyl sulfate (DMS), which introduces a methyl on the N1 of adenine and N3 of cytosine.²³ The DMS mapping results correlate with microarray mapping and the SHAPE approach. The model RNA hairpins exhibit a similar mapping pattern (except nucleotides 85 and 109) (Supplementary Data, Table S3). Medium DMS reactivity ranges between 0.3 to 0.7, and strong reactivity is above 0.7. The nucleotides in the following regions of the target 194/195 MAPT pre-mRNA nucleotide fragments indicate strong and medium reactivity in DMS mapping: 37, 55, 56, 58, 85, 86, 95, 104, 106, 108, 109, 123, 130, 139, 140, 141, 152, and 153.

Owing to the lack of significant differences between the microarray and chemical mapping, the average mapping for all type of mutant was used to predict the secondary structure.

Pre-mRNA MAPT gene fragment secondary structure determination

The strong microarray and chemical (SHAPE and DMS approaches) mapping results were used to predict the secondary structure of the target 194/195 MAPT pre-mRNA nucleotide fragment using RNAstructure5.6.¹⁹ The same RNA molecule was also folded using vsfold5, which does not allow to apply experimental mapping constraints.^{29,30} In this case, the microarray and chemical (SHAPE and DMS) mapping data were indicated on the lowest free energy RNA structure predicted by vsfold5. Both programs proposed different folding at certain target RNA regions because RNAstructure does not predict pseudoknot formation (Fig. 2). As shown in Figure 2A, the chemical and microarray mapping results do not match well with the predicted secondary structure. Thus, vsfold5 was also used to determine the fold for the same RNA molecule.

As shown in Figure 2, both structures have 2 common hairpins (H1 and H6). Hairpin H6 is formed by the MAPT pre-mRNA fragment ISS involved in alternative splicing regulation, whereas H1 is composed of 16 nucleotides at the 5'-end. In the structure predicted using vsfold5 (Fig. 2B), hairpins H2, H6, H7 and H8 were formed, and formation of pseudoknots PK1 and PK2 is postulated. The data show that certain MAPT pre-mRNA fragments contribute to the alternative splicing process; they are indicated with various colors in Figure 2.^{11,12} The strong and medium hits from the microarrays, SHAPE and DMS mapping support structure B in Figure 2. Moreover, in this structure, the functional regions that regulate alternative splicing are located in single structural motifs, which is the opposite of structure A. Experimentally, it was shown that several regions that are indicated by colors act as silencer or enhancer and modulate alternative splicing (Fig. 2).^{11,12} Structural studies were performed on 11 pre-mRNA MAPT gene mutants. The tested mutations were placed in the hairpin H6, and the predicted RNA folding for each mutant is practically identical. These structural similarities in the mutated RNA molecules and, simultaneously, the differences in levels of 3R and 4R tau protein isoforms obtained during the splicing process suggest a more complex mechanism for alternative splicing regulation. Thermodynamic verification of the pseudoknot PK1 (Fig. 2B) was performed because certain mapping results (particularly hits at 53–59) do not support pseudoknot formation (Supplementary Data, Table S4). Based on the reported results, the thermodynamic stability of RNA pseudoknots are approximately the sum of the composing hairpin stabilities.^{21,31} The pseudoknot PK1 stabilities in the model PK1 and the component hairpins H3 and H4 in 1 M sodium chloride were -1.24 , -1.01 and -1.28 kcal/mol, respectively. The thermodynamic stabilities of these RNA models in the presence of 10 mM MgCl₂ were -1.92 , -0.90 and -2.47 kcal/mol, respectively. The thermodynamic stabilities of PK1 in NaCl and MgCl₂ are similar, which suggests that the model PK1 forms the hairpin H4, and the remaining oligonucleotide PK1 fragment (H3) is unfolded. However, inside of the cell, in a crowded environment, folding preferences may be different than in the NaCl and MgCl₂ solutions.³² Moreover, binding the RNA processed

Figure 2. Secondary structure of the pre-mRNA MAPT gene fragment predicted using RNAstructure5.6 (A) and vsfold5 (B) based on microarray mapping as well as SHAPE and DMS data. The red and green squares represent strong and medium binding sites for microarray probes, respectively. The black and gray circle represent strong and medium SHAPE reactivity sites, respectively. The black and gray squares represent strong and medium DMS reactivity, respectively. The mutations studied and cis-acting elements are indicated on proposed secondary structure B.

by proteins that contribute to splicing may also influence pre-mRNA folding.

Postulated secondary structure of the 194 nucleotide MAPT RNA

To predict folding model of pre-mRNA MAPT the Shapeknots program was also used.³³ This program allows on prediction of RNA folding, including pseudoknots, based on SHAPE mapping results and it postulates formation of the pseudoknot PK3 including base pairing regions 87–92 and 109–114. The secondary structure of fragment of pre-mRNA MAPT predicted by vsfold5 but also including PK3 predicted by Shapeknots is shown on Figure 3.

The RNA secondary structure (Fig. 3) is composed of 7 hairpins (only H2 and H6 contain single nucleotide bulges in the stem), pseudoknot PK3 and 3 single-stranded regions that are postulated as primarily on the 5'-side of the RNA molecule. In particular, the structure is compact in the pre-mRNA fragment surrounding the 3'-splice site. More information on the secondary structure of the pre-mRNA fragment is below, where the postulated alternative splicing regulation is presented. The secondary structure of the MAPT pre-mRNA fragment is supported by the

following results: (1) chemical and microarray mapping. The eleven mutants of the model RNAs were mapped; the structure is shown in Figure 3 and is supported by mapping results for all of the mutants; (2) the regions important for alternative splicing regulation (see Figs. 2 and 3) are in single structural motifs. Thus, observations reported by others that describe their influence on alternative splicing regulation are consistent.

Pre-mRNA MAPT gene fragment tertiary structure modeling

The tertiary structure of the pre-mRNA MAPT gene generated by the RNAComposer program is shown in Figure 4. The total calculated free energy (ca. −3500 kcal/mol) of this structure is optimal for such a long RNA molecule.³⁴ The tertiary structure generated by the RNAComposer program, based on homologous structures of RNA present in the database, shows that the proposed secondary structure of the 194 nucleotide fragment of the pre-mRNA MAPT gene does not have secondary structural motifs, that prevent optimal tertiary structure. The proposed model of the tertiary structure of tau pre-mRNA allows, for the first time, to see potential tertiary interactions, essential for alternative splicing regulation. For example, the fragments 5'GGG

Figure 3. Secondary structure of the model MAPT gene pre-mRNA predicted by vsfold5 with a pseudoknot supported by Shapeknots.

(region 120–122 in hairpin loop H5 within the ESE) and 5'CCC (region 151–153 within the ISM) could interact with each other. The microarray mapping revealed binding of oligonucleotide probes to the region of 120–122, and chemical mapping gave the signal for the C151 and C152. However, interactions other than

canonical GC pairing or contribution of other functional groups and of divalent metal cations could be also involved in interactions within those regions. This could indicate that single strand characteristic of ISM is structurally protected, at the stages preceding the alternative splicing.

Figure 4. The tertiary structure 194 nucleotide fragment of the pre-mRNA MAPT gene modeled by the program RNAComposer based on experimentally defined secondary structure (**A** ribbon model and **B** surface model). Color indicated cis – acting regulatory elements: pink - SC35-like enhancer, blue - polypurine enhancer (PPE), yellow - A/C rich enhancer, green - exonic splicing silencer (ESS), salmon - exonic splicing enhancer (ESE), gray - intronic splicing silencer (ISS), brown - intronic splicing modulator (ISM).

Thermodynamic stabilities of the model RNA hairpins and their small molecule complexes

Eleven 25/26 nucleotide RNAs related to tau exon 10 alternative splicing regulation were UV-melted to obtain the thermodynamic parameters; they contained a fragment from exon 10 and intron 10–11 of the pre-mRNA MAPT gene. Studies have reported that they form hairpin structure and are a major factor involved in alternative splicing regulation of the MAPT gene.^{7,12,35} WT, 6 RNA molecules with FTDP-17 disease mutations and 4 RNA molecules with artificial mutations proposed by Donahue et al were examined.¹⁵

As expected, the experiments confirmed that mutations in the non-coding region of intron 10–11 significantly change the RNA hairpin stability (Fig. 5). In buffer A, compared with WT RNA, the presence of occurring

Figure 5. The influence of mutations (11C, 12U, 13G, DD-PAC, 16U, 19G, DD-10C, DDI-17T, WT-10C, WTI-17T), neomycin (neo), kanamycin (kan), tobramycin (tob) and mitoxantrone (mtz) on thermodynamic stabilities of the MAPT pre-mRNA model hairpins in buffer A. Data are reported as the average $\pm$ standard deviation ($n = 9$). * $P < 0.05$ as determined by a Student t-test.

mutations decreased the thermodynamic stability of RNA hairpins by 2.16, 3.85, 2.12, 3.29, 2.82, 0.46, 2.95 and 0.88 kcal/mol for the 11C, 12U, 13G, DD-PAC, 16U, 19G, DD-10C and DDI-17T RNA hairpins, respectively. For the WT-10C and WTI-17T hairpins, the stability was enhanced by 0.45 and 2.13 kcal/mol, respectively. Because the 19G mutation cause 3R tau production to dominate, it was expected to form a thermodynamically more stable regulatory hairpin, but the experimental data show the opposite trend for stability.³⁶ The additive character of the double-mutated RNA hairpins and thermodynamic effects of a single mutation demonstrate that the stabilities of the single- and double-mutated RNA hairpins follow general RNA thermodynamic rules.³⁷

The MAPT pre-mRNA regulatory hairpin binds various small molecule ligands.^{16,17,38} For the first time, the effect of 4 antibiotics on thermodynamic stability alternative splicing regulatory hairpin was investigated. To quantitatively evaluate the influence of ligands on thermodynamic stability, 3 aminoglycoside antibiotics (neomycin, kanamycin and tobramycin) and mitoxantrone were used. The results show that, except in one instance (tobramycin), the presence of the ligands enhanced the thermodynamic stability (Fig. 5). The stabilization effect depends on the nature of the ligand and RNA hairpin. A significant excess of each antibiotic was applied to force formation of at least 90% complex antibiotic/RNA hairpin.³⁸ For mitoxantrone, equimolar quantities of the RNA and ligand were used. Higher concentrations significantly disrupted monitoring of the UV melting process at 260 nm (Supplementary Data, Table S5).

Regulation of MAPT pre-mRNA gene exon 10 alternative splicing through small molecule ligands

Exon-trapping analyses were used to visualize the changes in the 3R and 4R tau proteins isoforms due to mutations in the regulatory RNA hairpin (Fig. 6A). Experiments using cos-7 cells showed that the 194/195 nucleotide pre-mRNA fragment folds in the structure were sufficient for tau exon 10 splicing.

The levels of 3R and 4R isoforms formed and the thermodynamic stability of the mutated regulatory hairpins were interesting when compared with the WT pre-mRNA (Fig. 6B). The lower thermodynamic stabilities of the regulatory hairpins decreased the levels of the tau protein 3R isoform presumably because the 5' splice site in exon 10 is more accessible to the U1 snRNP. The only exception is the 19G mutation, which does not affect the hairpin motif stability but increases the levels of the 3R tau isoform.

Moreover, studies have shown that the antibiotics neomycin, tobramycin and kanamycin as well as mitoxantrone bind the splicing regulatory hairpin.^{16,17,38} Small molecule ligands indicate the potential to regulate alternative splicing exon 10 protein tau for DD-PAC mutant.³⁹ For the first time, it has been shown that these antibiotics may influence the alternative splicing of the MAPT pre-mRNA in a cell line.

The nature of interactions between the WT regulatory hairpin and neomycin or mitoxantrone was studied using NMR spectroscopy.^{16,17} The cell media contained 50 μ M, 0.1 mM, 0.5 mM, 5 mM and 10 mM of the respective ligand. After 24 h, the total RNA was isolated and analyzed using RT-PCR. Mitoxantrone application yielded no RT-PCR products, it is possible that it strongly interacted with the lipofectamine blocking mini-gene pSPL3b transfection. MTT assay showed that even the concentration of 10 mM small molecule ligands does not affect the viability of cell lines cos-7.

Figure 6. (A) The influence of the mutations tested in MAPT gene exon 10 pre-mRNA alternative splicing. (B) The relationship between the ΔG°_{37} value and the quantity of tau protein isoforms.

Figure 7. The influence of various concentrations of neomycin, kanamycin and tobramycin on MAPT pre-mRNA exon 10 alternative splicing. Data are reported as the average $\pm$ standard deviation ($n \geq 2$). * $P < 0.05$ as determined by a Student t-test.

The presence of antibiotics in the medium decreased the levels of the 4R tau protein isoform (Supplementary Data, Table S6). The Figure 7 shows the influence of various concentrations of neomycin, kanamycin and tobramycin concentrations on the change in levels of 4R tau protein formed using the different mutants in the MAPT gene pre-mRNA relative to WT. The results support the following conclusions: (1) the thermodynamic stability of the regulatory hairpin (H6 on Fig. 3) influences the ratio of 3R and 4R isoforms, (2) the presence of neomycin, kanamycin and tobramycin in the cos-7 medium result in a concentration-dependent decrease in 4R isoform by up to 22%, 24% and 29%, respectively, for the 10 mM concentration, (3) the level of 4R isoform could be decreased to the WT range, which was observed for the mutant 13G with 10 mM neomycin, DD-PAC with 10 mM tobramycin and 16U with 10 mM neomycin as well as 5 mM tobramycin, (4) for most pre-mRNA mutants, much lower concentrations (in the range 0.1–5 mM) of antibiotics significantly reduced the levels of the 4R isoform to not much higher than for WT. The effect of tobramycin on exon 10 alternative splicing is interesting. In cell-based experiments, tobramycin caused the greatest change in levels of tau isoform; however, in the UV melting experiment, tobramycin exhibited the lowest impact on the RNA hairpin. This phenomenon requires further investigations, but it seems that it is due to the weak affinity binding of tobramycin to other RNA molecules in cos-7, in comparison with neomycin and kanamycin. Strong and specific binding of tobramycin to some structured RNA was demonstrated.⁴⁰

Regulation of MAPT gene exon 10 pre-mRNA alternative splicing through modified antisense oligonucleotides

The ligands used here were dedicated to binding the regulatory hairpin H6, however, for the first time the 20–21 nucleotide long modified antisense oligonucleotides were designed to bind to following regions of the pre-mRNA: AB1 (13–26 and 46–52 of pre-mRNA), AB2 (58–75 of pre-mRNA), AB3 (94–113 of pre-mRNA) and AB4 (152–171 of pre-mRNA) (Supplementary Data, Fig. S2). Cos-7 cells were transfected with minigene pSPL3b constructs containing WT and 11C, 12U, 13G, DD-PAC, and 16U inserts. The concentration of the antisense oligonucleotides was 40 nM.

Depending on the type of antisense oligonucleotide, 4R was changed in the range –5% and 19% as shown in Figure 8. The antisense oligonucleotides used here were designed to bind to the dedicated region of pre-mRNA involved in alternative splicing regulation (Fig. 3). Particularly, the antisense oligonucleotides AB3 and AB4 that bind the exon splicing silencer (ESS) and intron splicing modulator (ISM), respectively, intensely influenced alternative splicing. Presumably, both antisense oligonucleotides contribute to changes in pre-mRNA folding and binding of proteins involved in alternative splicing regulation.

The previous studies based on the use of modified oligonucleotides have shown no effect on the ratio of 3R and 4R tau isoforms formation.⁴¹ Using morpholino antisense oligonucleotides, the authors demonstrated that some of the oligonucleotides significantly reduce MAPT transcript and tau protein level, but do not alter the amount of isoforms.

As mentioned above, MAPT gene exon 10 alternative splicing yields comparable levels of the 3R and 4R tau isoforms.⁶ The mutations tested in cells (11C, 12U, 13G, DD-PAC and 16U) increased the quantity of 4R isoforms by 20.2%, 24.2%, 18.0%, 20.5% and 21.5%, respectively, compared with the WT form. Applying antibiotics and antisense oligonucleotides is important for decreasing the level of 4R isoforms to WT levels (Supplementary Data, Tables S6 and S7) because a decrease in the 4R isoform below this level causes FTDP-17.³⁶

Figure 8. Quantitative analysis of the 2'-O-methyl RNA antisense oligonucleotide contributions to alternative splicing regulation. The following ASO were used: AB1: 5'AAUUUAAAAAGGAUGAGU3', AB2: 5'UUGCUAAGAUCCAGCUUCUUA3', AB3: 5'UGUUUGAUUAUCCUUUGA3', AB4: 5'AGCCACAGCACGGCGCAUGG3'. Data are reported as the average $\pm$ standard deviation ($n \geq 2$). * $P < 0.05$ as determined by a Student t-test.

Discussion

Thus far, data published on the structure of the MAPT gene pre-mRNA are limited to the regulatory hairpin that contains the 5'-splice site (hairpin H6 on Fig. 3).^{7,16,17} Here, for the first time, the structure of the 194/195 pre-mRNA nucleotide fragment was determined. The structure contains many structural motifs, including pseudoknots. Combining the information on secondary structure described here and results by other investigators on the influence of certain regions on MAPT pre-mRNA alternative splicing regulation supports a hypothesis for this process.^{11,12}

The secondary structure of the 194 MAPT gene nucleotide fragment pre-mRNA shown in Figure 3 correlate with the chemical and microarray mapping results described here. The structure presented here concerns RNA that is not involved in interactions with other biomolecules (RNAs or/and proteins), and presumably, it is the RNA structure after transcription. In MAPT gene exon 10 pre-mRNA alternative splicing, the tau protein 3R and 4R isoforms are formed at similar levels, and exceptions to these results yield FTDP-17.^{6,8-10} Neurons require certain factors to regulate the equilibrium of both isoforms. In patients with FTDP-17, the tau isoforms are formed with a high dominance by one isoform. These observations suggest that, in neurons, the splicing intermediate equilibrium that produces 3R and 4R must be dynamic and thermodynamically or kinetically driven.

The splicing process and its regulation involve the spliceosome and various splicing regulating factors. Currently, ca. 20 proteins have been identified as involved in regulating MAPT exon 10 pre-mRNA alternative splicing.^{13,14,42-51} Thus far, in addition to the regulatory hairpin, the significant secondary structure of the pre-mRNA fragment is unknown; nevertheless, binding regions have been established for certain splicing regulatory factors.

Here, we postulate that the secondary structure of RNA that is shown in Figure 3 represent its folding before protein binding. The first step in alternative splicing regulation is binding hairpin H4.2 at the pseudoknot PK3 by the protein(s) SRp55, hnRNPG or/and SRp30c.⁴⁸ These proteins bind the exon splicing silencer (ESS) region (Fig. 9)⁴⁸ and should result in rearranging PK3 to form the pseudoknot PK2. This pseudoknot was originally postulated by vsfold5; however, mapping fragment 152–156 does not support formation of this pseudoknot by protein-free pre-mRNA. PK2 seems more thermodynamically stable than PK3. In addition to pairing the fragments 87–92 and 152–157, this helix can be further stabilized by 2–3 kcal/mol due to coaxial stacking with the H7 hairpin and, presumably, with hairpin H6 after bulging-out by C151.⁵²⁻⁵⁴ In the next step, U1 snRNP must bind the 5' splice site; therefore, the RNA hairpin must unwind. In the proposed model, it is postulated that helicase p68 binds the ISM, whereas the splicing regulatory factor PSF interacts with the ISS.^{13,14} The literature reports that PSF only binds the WT regulatory hairpin (not DD-PAC) and enhances its stability.¹⁴ When only helicase p68 binds the MAPT gene pre-mRNA, it unwinds the double-stranded region of the PK2, and consequently, the U1 snRNP can interact with the 5' splice site, and the 4R isoform is formed (Fig. 9B). By contrast, the

regulatory protein PSF may bind the regulatory hairpin and increase its stability. Despite the presence of helicase p68, U1 snRNP cannot bind the acceptor site in a more stable regulatory hairpin, and the 3R tau isoform is formed (Fig. 9C).

The arguments that support the regulatory model for MAPT gene exon 10 pre-mRNA here are based on presented herein results as well as results published by other research groups, as follows.

1. Hairpin H6 (Fig. 3) is the main regulatory element that determines alternative splicing for MAPT gene exon 10 pre-mRNA.^{11,12} Mutations in the isolated H6 hairpin of the 194/195 pre-mRNA nucleotide fragment do not influence RNA folding. However, the mutations change the thermodynamic stabilities of the H6 hairpin in the range 2.1 and –6.9 kcal/mol (depending on the buffer used). UV melting demonstrates that neomycin, kanamycin, tobramycin and mitoxantrone bind the WT and mutated hairpins and enhance their thermodynamic stabilities. Experiments using cos-7 cells indicate that the presence of ligands in the medium decreases the quantity of 4R tau isoform due to binding the H6 hairpin and enhancing its thermodynamic stability.
2. Currently, ca. 20 proteins have been identified as involved in regulating MAPT exon 10 pre-mRNA alternative splicing.^{13,14,42-51} Certain such proteins bind the exon splicing silencer (ESS), intron splicing silencer (ISS) and intron splicing modulator (ISM) regions. Other groups have shown that deleting fragments in the ESS, ISS and ISM influences splicing.^{11,12} Such deletions influence RNA folding (and the thermodynamic stability of hairpin H6 when the deletion includes this region) and the binding capacities of the proteins involved in splicing regulation. The quantity of data on the proteins that regulate pre-mRNA alternative splicing is limited, but SRp55, hnRNPG, SRp30c, PSF and helicase p68 are involved, and binding to ESS, ISS and ISM in WT and DD-PAC was demonstrated.^{13,14}
3. A pseudoknot rearrangement is postulated here as the first step of the alternative splicing regulation. The program vsfold predicted folding for the 195 nucleotide fragment as shown in Figure 3B with formation of the pseudoknots PK1 and PK2. However, mapping results do not support structural motif formation. Binding to the region 103–108 proteins SRp55, hnRNPG and/or SRp30c may break the weak binding within the fragments 87–92 and 109–114. The released single-stranded 87–92 region bound fragment 152–157 of ISM and formed the more favorable pseudoknot PK3 with the helix 87–92/152–157 further stabilized through coaxial stacking with H7 and, presumably, hairpin H6. In addition to the cos-7 medium antisense oligonucleotides AB3 (2'-O-methylated 5'UGUUUGAUUUAUCCUUUGA) that bind region 94–103 of ESS (the region with the binding site for proteins SRp55, hnRNPG and SRp30c) should also unwind PK3 and form the PK2 pseudoknot. A reduction in the 4R tau isoform upon adding AB3 antisense oligonucleotides was observed, and this effect is similar to binding by splicing regulatory proteins. D'Souza's studies demonstrated that various

Figure 9. Postulated structural rearrangement and alternative splicing regulation mechanism for MAPT exon 10 pre-mRNA. (A) MAPT gene pre-mRNA folding before the binding of splicing factors. (B) scheme of binding the pseudoknot PK3 by the protein(s) SRp55, hnRNPG or/and SRp30c, which results in binding helicase p68 to ISM and U1 snRNP to regulatory hairpin (4R isoform is formed), (C) scheme of binding PSF protein to regulatory hairpin which inhibits the binding of U1 snRNP to H6 (3R isoform is formed).

deletions and mutations in the ESS region resulted in more 4R isoforms.³⁵ Presumably, splicing regulatory proteins could not bind the altered ESS region, and PK3 cannot be rearranged into PK2. Consequently, the region ISM remains single stranded, which is the opposite of when ISM is part of the PK2 pseudoknot, and it changes splicing protein binding abilities.

4. The 19G mutation also supports the proposed mechanism for regulating alternative splicing. The 19G mutation induces domination of the 3R tau protein isoforms.³⁶ Here, it is postulated that substituting C151 with G adds G151-C93 as shown in Figure 10. This mutation extends and enhances the thermodynamic stability of the duplex formed by the ISM region (formally part of the PK2 pseudoknot); helicase p68 must unwind a more stable helix that includes PK2. Consequently, binding of U1 snRNP is less efficient, and the 3R tau protein isoform dominates.

Alternative splicing of the MAPT gene exon 10 pre-mRNA is a complex, multistep process regulated by the pre-mRNA structure and splicing regulatory factors. It is a dynamic process because formation of similar quantities of 3R and 4R tau protein suggests 2 simultaneous exon 10 splicing pathways. The thermodynamic stabilities of the regulatory hairpin (H6 on Fig. 3) and its ability to bind splicing regulatory factors is crucial for regulating alternative splicing. We postulate that binding between the splicing regulatory protein and the exon splicing silencer (ESS) rearranges the pseudoknot PK3 into PK2. The postulated alternative splicing regulatory mechanism supports the following: (1) the secondary structure of the 194 pre-mRNA gene MAPT nucleotide fragment, (2) the structural rearrangement capacity of the pre-mRNA fragment involved in splicing regulation, (3) the location of alternative splicing regulatory regions (such as ESS and ISM) within individual structural motifs in the proposed secondary structure of the pre-mRNA, (4) the binding capacities and specificities of certain splicing regulatory proteins in WT and mutated pre-mRNA gene MAPT exon 10 fragments.

Materials and Methods

Materials

The oligonucleotide library from the authors' laboratory was used to prepare a penta-/hexanucleotide isoenergetic RNA microarray. Furthermore, 16 additional oligonucleotide heptamers

Figure 10. Structural rearrangement of the MAPT gene pre-mRNA structure with the 19G substitution. (A) folding of WT MAPT pre-mRNA, (B) folding of 19G mutated MAPT pre-mRNA, resulting in formation of the C(93) – G (151) base pair.

(composed of 2'-O-methyl and LNA nucleotide residues) were synthesized with 3'-terminal pyrene pseudonucleotide.⁵⁵ Taq polymerase, T7 polynucleotide kinase and dNTPs were from Thermo Scientific. Reverse Transcriptase SuperscriptIII (used in primer extension), Lipofectamine 2000 and Trizol were from Invitrogen. T-7 Mega ShortScript Transcription Kit and DNaseI were from Ambion. ddNTPs were from Roche. The iScript Kit was from Bio-Rad. The N-methylisatoic anhydride (NMIA) was from Molecular Probes. Silanized slides and HybriSlip hybridization covers, dimethylsulfide, DMEM, Antibiotic Antimycotic Solution and MEM Vitamin Solution, tobramycin were from Sigma-Aldrich. Fetal bovine serum was from Gibco. Neomycin and kanamycin were from Bioshop. Mitoxantrone was from abcam.

Oligonucleotide synthesis and purification

The oligonucleotides were synthesized using Applied Biosystems DNA/RNA and MerMade12 (BioAutomation) synthesizers and β -cyanoethyl phosphoramidite chemistry.⁵⁶ Commercially available A, C, G and U phosphoramidites with 2'-O-tertbutyldimethylsilyl were used to synthesize RNA (ChemGenes, GenePharma). To synthesize DNA (GenePharma), 2'-O-methyl RNA (GenePharma) and LNA (made in house and Exiqon), their respective phosphoramidites were used. The details of oligoribonucleotide deprotection and purification were previously described.^{57,58} Thin-layer chromatography (TLC) purification of the oligonucleotides was performed using Merck 60 F₂₅₄ TLC plates with the mixture 1-propanol/aqueous ammonia/water = 55/35/10 (v/v/v).

UV melting experiments

The oligonucleotides were melted in buffer containing 100 mM NaCl, 20 mM sodium cacodylate, 0.5 mM Na₂EDTA, pH 7 (buffer A); 8 mM MgCl₂, 10 mM Tris-HCl and 0.5 mM Na₂EDTA, pH 7 (buffer B); and 5 mM MgCl₂, 150 mM KCl, 20 mM sodium cacodylate and 0.5 mM

Na₂EDTA, pH 7 (buffer C). The single-strand oligonucleotide concentrations were calculated based on absorbance at 80°C, and the single strand extinction coefficients were approximated using a nearest-neighbor model.^{59,60} The measurements were performed for 9 different concentrations of each RNA hairpin in the range 10⁻⁵–10⁻⁶ M. Absorbance vs. temperature melting curves were measured at 260 nm at the heating rate 1°C/min from 0 to 90°C using a Beckman DU 640 or JASCO V-650 spectrophotometer with a thermoprogrammer. The melting curves were analyzed, and the thermodynamic parameters were calculated using a 2-state model with the program MeltWin 3.5.⁶¹ For most sequences, the ΔH° derived from the TM^{-1} vs. $\ln(C_T/4)$ plots is within 15% of the ΔH° derived from averaging the fits to the individual melting curves, which is expected if the 2-state model is reasonable.

DNA template construction and RNA synthesis

The DNA templates used for RNA in vitro transcription (WT, 11C, 12U, 13G, DD-PAC, 16U, 19G, DD-10C, DDI-17T, WT-10C and WTI-17T) were constructed using several PCR reactions and chemically synthesized oligonucleotides. The DNA templates (17 nucleotides from the T7 promoter, 39 nucleotides from intron 9–10, 93 nucleotides from exon 10, and 62 or 63 nucleotides from intron 10–11) were cloned into pUC19 to determine the proper sequences (sequenced using the seq-pUC19 primer 5'TGCAAGGCGATTAAGTTGGGTA). The 194/195 RNA oligonucleotides using in vitro transcription and the T7 MegaShortScript Transcription Kit were synthesized. The eleven pre-mRNA MAPT gene fragments were recloned into the exon-trapping vector pSPL3b using EcoRI and PstI endonucleases. The minigene pSPL3b constructs included 39 nucleotides from intron 9–10, 93 nucleotides from exon 10, and 62 or 63 nucleotides from intron 10–11. The primer seq-pSPL3b (5'TCCTTGGAATGTTGATGATCTGTAG) was used to verify all construct sequences.

Native gel electrophoresis

A sample of 20,000 cpm-radiolabeled RNAs (obtained using in vitro transcription with phosphorous radioactive α ATP) were folded in 10 μ l of one of 4 buffers [1 M NaCl, 4 mM MgCl₂, 10 mM Tris-HCl pH 7.5 (I); 200 mM NaCl, 4 mM MgCl₂, 10 mM Tris-HCl pH 7.5 (II); 100 mM NaCl, 15 mM MgCl₂, 10 mM Tris-HCl pH 7.5 (III); and 100 mM NaCl, 0.5 mM Na₂EDTA (IV), and 10 mM Tris-HCl pH 7.5] and under one of 3 folding conditions [(A) without denaturation, (B) denaturation for 3 min at 75°C then cooling on ice and (C) denaturation for 3 min at 75°C and slowly cooling at RT]. Next, 1 μ l of 50% glycerol and a trace of tracking dye to the samples were added. The samples were analyzed through non-denaturing

polyacrylamide gel electrophoresis (PAGE) in TBM buffer (100 mM Tris-HCl pH 8.3, 100 mM boric acid and 10 mM MgCl₂) and TBM running buffer. The electrophoresis experiments were performed at 20W and 4°C. The dried gels were analyzed through exposure to a phosphorimager screen followed by MultiGauge program calculations.

Preparing isoenergetic microarrays

To prepare the isoenergetic microarrays, 166 probes (2'-O-methyl oligonucleotide pentamers and hexamers with incorporated LNA nucleotides and 2'-O-methyl and LNA 2,6-diaminopurineriboside derivatives) from our probes library were used. Additionally, 16 heptamers were prepared using a previously described method.²¹

Hybridization on the isoenergetic microarrays and microarray mapping

Radiolabeled RNA oligonucleotides were obtained through *in vitro* transcription with phosphorous radioactive α ATP and purified through denaturing PAGE. Three types of buffer [1 M NaCl, 4 mM MgCl₂ and 10 mM Tris pH 7.5 (A); 200 mM NaCl, 4 mM MgCl₂ and 10 mM Tris pH 7.5 (B); and 100 mM NaCl, 15 mM MgCl₂ and 10 mM Tris pH 7.5 (C)] were used for hybridization on the isoenergetic microarray. The RNA was incubated for 3 min at 75°C and renatured for 20 min at 25°C to obtain the native fold. For single hybridization on the isoenergetic microarray, 200 000 cpm-radiolabeled RNA oligonucleotides were used in 200 μ l buffer. The folded RNA molecules were placed on a microarray slide and covered with a HybriStrip. The microarray was placed in a chamber with 100% humidity and incubated for 18 h at 4°C, 22°C or 37°C. Thereafter, the HybriStrip was removed, and the microarray slide was washed in a buffer with the same concentrations as during hybridization for 3 min at 0°C. The slides were dried through centrifugation (2000 rpm, 2 min) and covered with plastic wrap. The hybridization results were visualized through exposure to a phosphorimaging screen and scanned. Quantitative analyses were performed using Array Gauge 2.1. The results were normalized, and the binding was defined as strong, medium or weak when the integrated spot intensity was $\geq 1/3$, $\geq 1/9$ or $1 \geq 27$ compared with the strongest integrated spot intensity, respectively. For each RNA molecule, hybridization was repeated 3 times, and the results were averaged. Only strong and medium binding signals were used to predict the secondary structure. The results with alternative binding sites were rejected based on the constraints.

SHAPE mapping

One picomole of each RNA molecule was dissolved in buffer H (300 mM NaCl, 50 mM HEPES and 5 mM MgCl₂, pH 7.5), incubated 5 min at 75°C then cooled in a metal block and 15 min at RT. The folded RNA molecules were modified for 3.5 h at RT using the NMIA reagent (the final NMIA concentration was 20 mM).²⁸ The mapping results were visualized through primer extension with the DNA/LNA primer 6-FAM, which was labeled at the 5'-end.⁶² Capillary electrophoresis was used to generate and identify the reactive nucleotides using the

Peak Scanner program and Excel. The results were normalized using the Weeks protocol.²² Three SHAPE analyses were performed for each RNA molecule, and the results were averaged.

DMS mapping

The DMS reactions proceeded under the same conditions as the SHAPE mapping.²³ For DMS mapping, an RNA molecule was treated with DMS at the final concentration 50 mM and incubated for 15 min at RT. The results were normalized using the Weeks protocol.²² Triplicate reactions using DMS were performed for each RNA molecule, and the results were averaged.

Folding of target RNAs

To predict the secondary structure of target RNAs, the RNAs-structure5.6 and vsfold5 programs were used with standard settings. In the case of vsfold5, the folding of RNA in presence of Mg⁺² was performed. For folding RNA with use of the Shape-knots program, results of the chemical mapping (SHAPE) were applied as constraints.

Experiments using cos-7 cells

Cos-7 cells were cultivated in DMEM with 10% FBS supplemented by vitamins and antibiotics. The passage of 100.000 cells in each of the 24 wells was performed. To evaluate influence on MAPT pre-mRNA alternative splicing regulation, cos-7 cells were transfected with the plasmid pSPL3b containing WT or 11C, 12U, 13G, DD-PAC, 16U, 19G, WT-10C, WTI-17T, DD-10C, DDI-17T inserts.⁶³ After 24 h, transfection was performed using Lipofectamine 2000 in accordance with the manufacturer's recommendations. The transfected cells were grown for 24 h at 37°C in a 5% CO₂ atmosphere. The tested antibiotics were added to the antibiotic free medium during transfection with the plasmid pSPL3b. ASOs (AB1: 5'AAUUAUAA AAAAGGAUGAGU3', AB2: 5'UUGCUAAGAUCAGCUU CUUA3', AB3: 5'UGUUUGAUUAUCCUUUGA3', AB4: 5'AGCCACAGCACGGCGCAUGG3') were co-transfected with minigene pSPL3b constructs. The RNA from the cultured cells using the Trizol reagent in accordance with the manufacturer's protocol was isolated, and the RNA was treated with DNase I. The quality of the isolated RNA was analyzed by agarose gel electrophoresis. Earlier prepared 500 ng RNA template was used for cDNA synthesis, using an oligo-T₁₈ primer and the iScript Kit. To visualize the splice effects, the primers SD6 (5'TCTGAGTCACCTGGACAAC) and SA2 (5'ATCTCAG TGGTATTGTGAG) were used in a standard PCR reaction. For the control purposes, the reactions with samples of transfected WT minigene pSPL3b and non-transfected samples (no products) were performed, also the β -actin gene levels were checked. The PCR products were analyzed by agarose gel electrophoresis, followed by MultiGauge program calculations.⁶⁴ Based on the intensity of the bands (3R and 4R), the program automatically computed the percentage ratio of the 2 isoforms. All experiments were repeated at least twice.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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