

Human tra2-beta1 autoregulates its protein concentration by influencing alternative splicing of its pre-mRNA

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HTRA2-BETA1 is an SR-like protein that regulates alternative splice site selection in a concentration-dependent manner. Its proper concentration is important as several pathological states are associated with its change. We investigated the mechanism that controls the cellular HTRA2-BETA1 concentration and found it utilizes a negative feedback loop to regulate the splicing of its exon 2. TRA2-BETA1 binds to four enhancers present in exon 2, which activates its inclusion. Inclusion of exon 2 generates mRNAs that are not translated into proteins. Mutations of exon 2 enhancers demonstrate that TRA2-BETA1 binds a degenerate sequence GHVVGANR, which is found more frequently in exons than in introns. Hyperphosphorylation of TRA2-BETA1 strongly reduces its binding to RNA. Presence of the CLK2 kinase prevents the usage of exons 2 and 3, generating the htra2-beta3 mRNA. The resulting HTRA2-BETA3 protein lacks the first RS domain of HTRA2-BETA1, is expressed in several tissues and has no influence on tra2-beta splice site selection. HTRA2-BETA1 interacting proteins promote exon 2 skipping by sequestering it, which upregulates the HTRA2-BETA1 protein synthesis. We propose that the regulation of the tra2-beta pre-mRNA alternative splicing provides a robust and sensitive molecular sensor that measures the ratio between HTRA2-BETA1 and its interacting proteins.

INTRODUCTION

The initial draft of the human genome has shown the number of genes in humans to be between 25 000 and 40 000, much smaller than previously expected (1,2). Higher eukaryotes use several mechanisms to generate protein diversity from this limited number of genes. As a result, the human proteome is estimated to be between 90 000 and one million (3,4). One of these protein diversifying mechanisms is alternative splicing, a process by which exons can be either included or excluded from a single pre-mRNA species resulting in multiple RNA isoforms (5,6). Studies on chromosome 21 (1) and EST database comparison (7) showed that about 47–59% of all human genes are alternatively spliced. Exons are defined by three major *cis*-elements, the 5' and 3' splice site and the branchpoint (8). Since these elements are short and degenerate (9), additional elements known as exonic or intronic enhancers/silencers regulate exon recognition (10,11). These additional elements are recognized by *trans*-acting factors,

such as hnRNPs and SR-proteins (12). Therefore, exons are recognized by the interaction of different *trans*-acting factors with multiple *cis*-elements on the pre-mRNA (13). This process occurs *in vivo* with high fidelity and can be modified according to cellular needs (14). Its complexity is further emphasized by the failure of current computer models to accurately predict exons in genomic DNA (15).

SR-proteins are essential splicing factors highly conserved in metazoa. They are composed of one or two N-terminal RNA recognition motifs and a C-terminal RS-domain rich in arginine and serine residues. They can change splice site selection in a concentration-dependent manner (16,17). SR-like proteins have domains similar to authentic SR proteins. Like SR proteins, they can direct splice site usage in a concentration dependent manner (18,19) and antagonize other SR proteins (20), but are not essential for the splicing reaction. Transformer-2 is an SR-like protein that was first described in drosophila as a sex-determining factor. Together with transformer it regulates

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doublesex exon 4 (21,22), which results in female drosophila DSX and development (23). Transformer-2 has two mammalian homologues tra2-alpha (24) and tra2-beta (25). Owing to alternative splicing both the drosophila and the human genes produce multiple mRNAs that encode either the full-length transformer protein consisting of two RS domains flanking a central RRM (dTRA2²²⁶, hTRA2-BETA1) or isoforms lacking the first RS domain (dTRA2¹⁷⁹/hTRA2-BETA3). Whereas dTRA2¹⁷⁹ is specific for the male germline, TRA2-BETA3 is predominantly expressed in brain, testis and liver (26). Its expression is regulated by developmental stages and by neuronal activity (26,27).

An increasing number of diseases are shown to be caused by missplicing (28–30) and several pathophysiological conditions demonstrate that the proper concentration of TRA2 is important for normal cellular function. Absence of dTRA2 in drosophila chromosomal females results in male specific splicing of both the doublesex and fruitless genes (31,32) and the transformation towards the male phenotype, as a large number of downstream sex-specific genes are regulated by this pathway (23). An excessive level of dTRA2²²⁶ in the germline results in male sterility (33). In mammals, a change of TRA2-BETA1 concentration is concomitant with hypoxia (34), silicosis (35) and arteriosclerosis (36). An increase of the TRA2-BETA1 concentration will affect splice site selection. For example, it causes exon inclusion of the SMN2 gene, which could be a therapeutic approach for spinal muscular atrophy (37). Cells precisely control the TRA2 concentration to avoid missplicing events. In drosophila, this is achieved by an autoregulatory loop. dTRA2²²⁶ inhibits removal of the M1 intron in this loop, which results in generation of the inactive variant dTRA2¹⁷⁹. Several genes encoding RNA binding proteins with roles in splice site selection autoregulate their expression, among them hnRNP1 (38), SRp20 (39), SC35 (40), 9G8 (41), drosophila and mammalian SWAP (19,42). Since the tra2-beta and dtra2 gene structures are not conserved, we investigated how human cells regulate their TRA2-BETA1 concentration. We showed that TRA2-BETA1 binds to enhancers that are highly degenerate and can contain significant number of pyrimidines. We found that TRA2-BETA1 causes inclusion of its own exon 2 by binding to four enhancers, which generates a mRNA that is not translated into protein. This autoregulatory loop keeps the protein concentration constant, even when the gene dosage is increased several-fold. When TRA2-BETA1 is sequestered by interacting proteins, exon 2 is no longer recognized, resulting in the up-regulation of TRA2-BETA1. As a result, the TRA2-BETA1 autoregulation provides a molecular sensor that measures and integrates the concentration of several splicing regulatory proteins.

RESULTS

TRA2-BETA1 regulates the splicing of tra2-beta gene exon 2 and APP gene exon 8

Through alternative splicing, alternative polyadenylation and alternative promoter usage the human tra2-beta gene (Fig. 1A) generates five RNA isoforms. These mRNAs contain three

different ORFs. The start codon in exon 1 precedes the longest open reading frame encoding TRA2-BETA1, as well as a short ORF present in tra2-beta2 that is not translated into protein (27). In the beta3 and beta4 isoforms, this start codon is followed by in frame stop codons and a start codon in exon 4 precedes an ORF encoding TRA2-BETA3. TRA2-BETA1 and TRA2-BETA3 differ in the presence of the first RS domain (Fig. 1B). To study how the alternative splicing of the tra2-beta pre-mRNA is regulated, we cloned a minigene (MGTra minigene) that spans the first four exons of the gene (Fig. 1A). Several factors regulating alternative splicing such as dTRA2, SWAP and SRp20 regulate splice site selection of their own pre-mRNA. We therefore tested the influence of TRA2-BETA1 on the splicing of its pre-mRNA in cotransfection experiments where we employed the minigene with increasing amounts of EGFP tagged splicing regulatory proteins. In all assays (Fig. 2A), the amount of DNA was kept constant by the addition of parental vector expressing EGFP (43). Identical results were obtained when using Flag-tagged constructs. The RNA was analyzed by semiquantitative RT-PCR or by quantitative real-time PCR (Fig. 8D). Both methods gave similar results. As shown in Figure 2B, transfection of 0.5 µg of the tra2-beta1 construct resulted in a moderate increase of EGFP-TRA2-BETA1 relative to the endogenous protein. This increase in the TRA2-BETA1 is sufficient to drastically switch the splicing of the MGTra minigene from tra2-beta1 to tra2-beta4. Thus, TRA2-BETA1 is promoting inclusion of exon 2. In contrast, transfection of the TRA2-BETA3 expression construct did not alter tra2-beta splicing, although comparable amounts of transfected proteins were expressed (Fig. 2C). This indicates a specific action of TRA2-BETA1 on its pre-mRNA.

In addition to tra2-beta exon 2, SMN2 exon 7 (37), CLB exon EN (44), tau exon 10 (45) and APP exon 8 (46) (our unpublished data) have been identified as exons influenced by tra2-beta1 concentration. The motifs identified in SMN2 and tau exon 10 have been shown to be TRA2-beta1 dependent ESEs (37,45). We used a Gibbs algorithm (47) to find locally conserved regions that could represent possible binding motifs for TRA2-BETA1. This approach identified a common purine-rich sequence in the alternative exons influenced by TRA2-BETA1 (Fig. 2D). Similar motifs were also detected in the constitutive exons flanking the alternative exon EN of CLB and exon 8 in the APP gene. The common motif GAARGARR is reminiscent of the purine-rich elements that have been identified *in vitro* using SELEX and gel shift experiments (48) as possible TRA2-BETA1 target sequences. It is present three times in exon 2, at positions 10–20, 182–189 and 205–218 of exon 2 (26). A related motif 2 GGARGARR is present at position 105–112 of exon 2 (Fig. 2D). This suggests that tra2-beta exon 2 contains four motifs that could be regulated by TRA2-BETA1.

Four sites in exon 2 are recognized by tra2-beta1

To find out whether the sequences we identified function in tra2-beta regulation *in vivo*, we mutated them individually. In each site, purines were substituted with either the other purine (Fig. 3A) or with pyrimidines (data not shown). Mutant minigenes were tested in cotransfection experiments with increasing

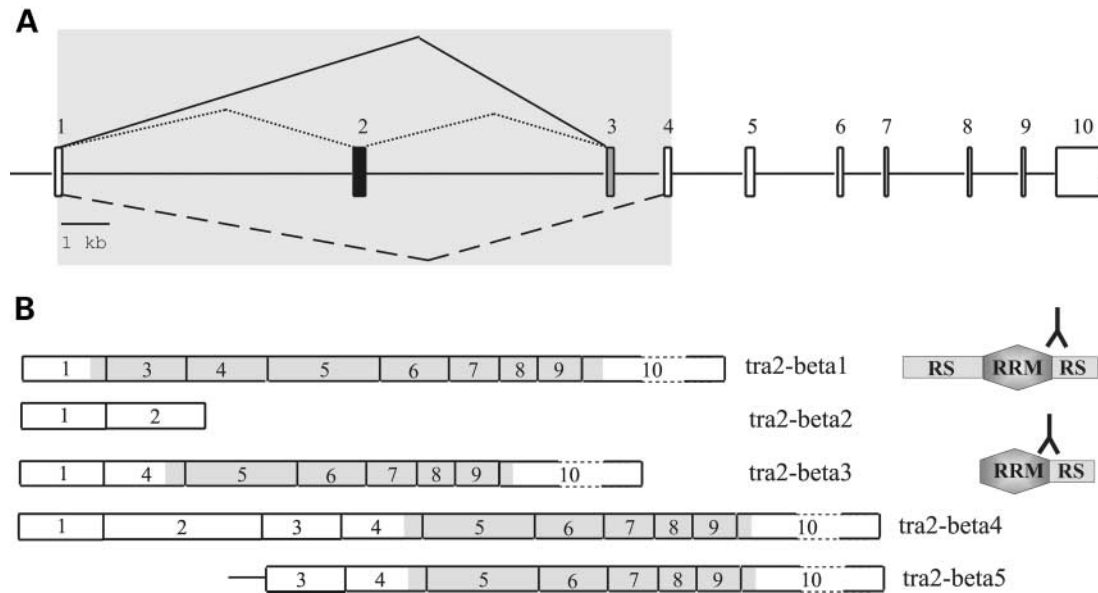


Figure 1. Genomic organization and isoforms generated by the human tra2-beta gene. (A) Genomic organization: structure of the htra2-beta gene. Exons are shown as boxes, introns as lines. The shaded region was PCR amplified and used to clone the MGTr mini-gene. Owing to alternative splicing and alternative promoter usage, several mRNA isoforms can be generated that are shown in (B). Connecting lines indicate the various splice choices. The drawing is to scale. (B) Structure of TRA2-BETA variants. Skipping of exon 2 (solid line in A) generates the tra2-beta1 mRNA that is translated into the TRA2-BETA1, whose protein structure is shown on the right. The triangle indicates the localization of the peptide used to generate the pan-tra2-beta antiserum. Inclusion of exon 2 and use of its polyadenylation site creates tra2-beta2, which is not translated into protein (27). Skipping of both exons 2 and 3 (dashed line in A) generates the tra2-beta3 mRNA, which is translated into TRA2-BETA3 that lacks the first RS repeat. The inclusion of both exons 2 and 3 generates the tra2-beta4 mRNA carrying an ORF coding for TRA2-BETA3. However, as shown in this paper, this ORF is not translated. Usage of an alternative promoter in the second intron generates the tra2-beta5 variant. The longest ORFs encoding TRA2-BETA1 and -beta3 are indicated in grey.

concentrations of TRA2-BETA1. These mutations decreased the TRA2-BETA1 dependent inclusion of exon 2. In addition to reducing exon 2 inclusion, the purine exchange in motif 4 generated a novel splice site leading to a shorter RNA isoform (Fig. 3A). Changing the purines into pyrimidines reduced exon 2 usage in a similar way (Fig. 3A). In contrast, changing exon 2 sequences outside these four motifs had no effect on TRA2-BETA1 dependent exon 2 inclusion. This suggests that four purine-rich motifs in the alternative exon 2 mediate the regulation by TRA2-BETA1.

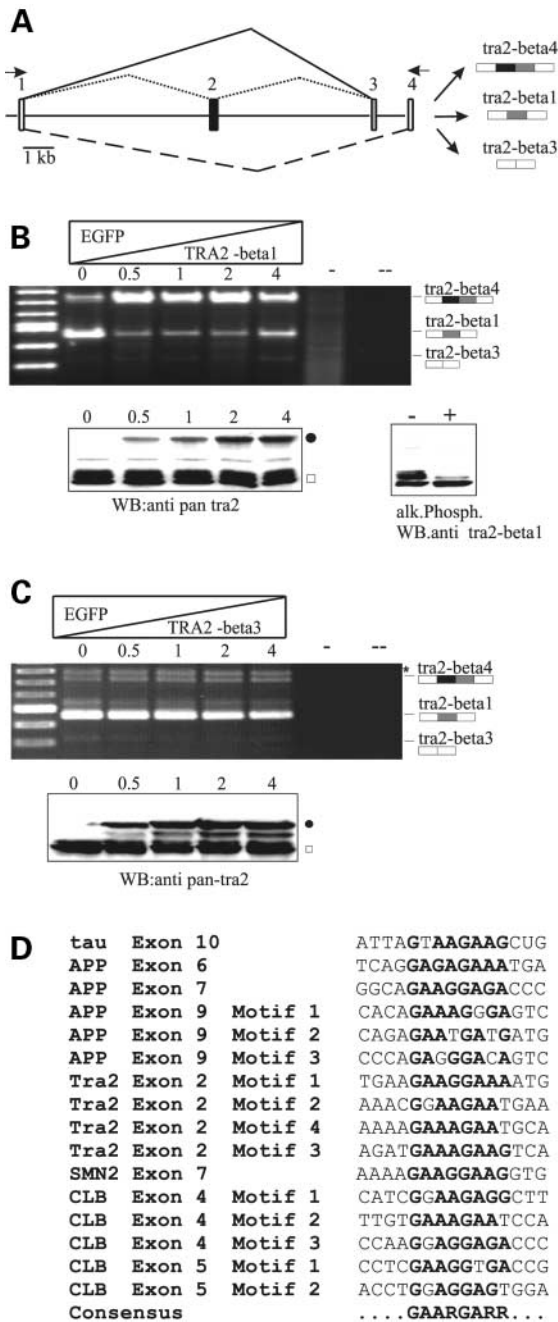
Mutations of the splicing motifs *in vivo* reveals TRA2-BETA1 acts through a degenerate ESE

Mutation of motif 3 had the strongest effect (Fig. 3A) and we investigated its composition in more detail by scanning nucleotide mutagenesis. At each position, we replaced the purine with either the other purine, cytosine or thymidine (Fig. 3B). Without coexpressed TRA2-BETA1, the inclusion of exon 2 was weak in the wild-type (Fig. 2B and C, lane 0) and absent in several mutants. Therefore, the effect of each mutation on the TRA2-BETA1-dependent exon 2 inclusion was compared with the wild-type in cotransfection experiments using a small amount of TRA2-BETA1-expressing cDNA. This allowed us to assay the effect of TRA2-BETA1 on the various mutants. A mutant giving a higher exon 2 inclusion than the wild-type was considered the preferred base at this position. As a result, we were able to derive a functional consensus for TRA2-BETA1 dependent ESEs: GHVVGANR (H=A/C/T; V=A/C/G; R=A/G; Fig. 3C). Thus, to our surprise, the mutants giving the strongest TRA2-

BETA1-mediated effect had cytosine instead of adenine in positions 2 and 4. The double mutant carrying cytosines instead of adenines in these positions showed exon 2 inclusion comparable to the wild-type and no cumulative effect was observed (data not shown). These data suggest that TRA2-beta1 can act through a highly degenerate ESE containing pyrimidins.

TRA2-BETA1 binds efficiently to the motif GHVVGANR

To determine whether TRA2-BETA variants interact directly with the motifs identified, we performed *in vitro* binding experiments. A biotinylated RNA oligonucleotide (motif3) that contained motif 3 was used to pull down TRA2-BETA1 from nuclear extract to streptavidin-coated metacrylate beads. Binding to this, RNA was competed with either non-biotinylated competitor with same sequence or an unrelated RNA. The protein that remained bound to the RNA was analyzed by western blot (Fig. 4A). In nuclear extract, TRA2-BETA1 is present in a hyper- and hypophosphorylated form (27) (Fig. 2B). Interestingly, preferentially the hypophosphorylated form binds to RNA. A 6-fold molar excess of RNA competitor containing either one of the purine-rich binding motifs 3 or 1 reduced the amount of bound TRA2-BETA1 to 40%. The same molar excess of RNA oligonucleotides RNA1 and RNA2 lacking purine-rich stretches reduced binding only to 75% (Fig. 4A). To test whether TRA2-BETA1 binds directly to the purine-rich motifs, we repeated the experiment with purified *in vitro* translated TRA2-BETA1. A 6-fold molar excess of purine-rich competitor RNA reduced



bound TRA2-BETA1 to 10%, whereas RNA1 had no effect and RNA2 reduced binding to 50% (Fig. 4B). To rule out tra2-beta1 binding to the RNA being mediated by factors present in the reticulate lysate, we repeated the experiment with tra2-beta Δ SR1 + SR2, a protein corresponding to the RRM of tra2-beta1. It was previously shown that the deletion of the two RS-domains in tra2-beta Δ SR1+SR2 almost completely abolished binding to other SR-proteins in immunoprecipitations (26). Again, we found that tra2-beta Δ SR1+SR2 binds significantly more strongly to purine rich RNAs than to unrelated controls (Fig. 4C). In all binding studies, we observed that the

Figure 2. TRA2-BETA1 influences the splicing of tra2-beta. (A) Structure of the TRA2-BETA (MG Tra) minigene. Constitutive exons are shown in white, alternative exons in grey and black, respectively. Arrows indicate the positions of the primers used for the reverse transcription and PCR. The structure of the isoforms are schematically indicated on the right. (B) TRA2-BETA1 promotes formation of the tra2-beta4 isoform. An increasing amount of the EGFP-TRA2-BETA1 expressing construct was cotransfected with 1 μ g of the MGtra Minigene. The top panel shows an ethidium bromide stained agarose gel of the RT-PCR products. Numbers indicate micro grams of expression plasmid used in transfection. The structure of the products are shown on the right. An equal amount of parental EGFP vector was added to ensure that the same amount of DNA was transfected. '-' and '- -' indicate reactions without reverse transcription and RNA, respectively. Below the ethidium bromide-stained gel is a western blot from parallel transfections that shows TRA2-BETA1 and EGFP-TRA2-BETA1 expression levels. Numbers indicate micrograms of EGFP-tra2-beta1 expression clone transfected. The pan-tra2-beta antiserum was employed for detection. The dot indicates the TRA2-BETA1 EGFP fusion and the open box indicates the endogenous protein. The multiple bands for the endogenous protein are due to phosphorylation and disappear after phosphatase treatment (small blot on the right). Numbers indicate micrograms of transfected expression plasmid. In these experiments TRA2-BETA1 was fused to an N-terminal EGFP tag. Identical results were obtained with a C-terminal EGFP-tag or Flag-tag. The quantifications of these data are shown in the doctoral thesis of Petar Stoilov, which is available on under www.stamms-lab.net/pdfs/thesis-Peter.pdf. (C) TRA2-BETA3 has no effect on the splicing of tra2-beta. An experiment similar to that in panel B is shown, but EGFP-tra2-beta3 is used for transfection. The asterisk on the right indicates a heterodimer that is visible when low amounts of tra2-beta4 are present. The western blot below shows protein expression levels detected by an antiserum against TRA2-BETA1. Dot and open box indicate the transfected and endogenous protein, respectively. (D) Alignment of the common motifs found in pre-mRNAs influenced by TRA2-BETA1. Sequences of the human genes for tau, APP, tra2-beta1 (Tra2), survival of motoneuron 2 (SMN2) and clathrin light chain B (LCB) are aligned. The consensus is shown in the bottom line. Matches to it are shown in bold in the individual sequences.

unrelated RNA sequence RNA2 could weakly compete with a purine-rich sequence for TRA2-BETA1 binding (Fig. 4).

To verify that the results from the single nucleotide mutagenesis experiment (Fig. 3B) reflect the binding affinity of TRA2-BETA1 to the third binding site in exon 2, we performed similar pull-down experiments (Fig. 4D). We incubated *in vitro* translated TRA2-BETA1 with an oligonucleotide (BS3-wt) containing the wild-type binding site 3. We then determined the ability of oligonucleotides containing mutagenized tra2-beta1 binding sites to compete for TRA2-BETA1 binding. The binding was competed with three RNA oligonucleotides containing sequences selected *in vivo*. In BS3(2A \rightarrow C), an adenine is substituted at position 2 with cytosine, in BS3(5G \rightarrow U), a guanosine is substituted with uracil, and in BS3(cons), two adenosines are substituted by cytidines at positions 2 and 4. These oligonucleotides corresponded to mutations that give exon inclusion higher [BS3(2A \rightarrow C)], or lower [BS3(5G \rightarrow U)] than the wild-type. The oligonucleotide BS3(cons) represents the derived consensus sequence (Fig. 3C). BS3(2A \rightarrow C) and BS3(cons) competed for binding similar to wild-type, whereas BS3(5G \rightarrow U) could not compete as efficiently for binding (Fig. 4D, top). Again, we observed that TRA2 lacking both RS domains has binding properties similar to TRA2-BETA1 (Fig. 4D, bottom), demonstrating that the RNA binding specificity is due to the RRM.

Together, these experiments demonstrate that hypophosphorylated TRA2-BETA1 binds to degenerate ESEs in a sequence-dependent manner. Furthermore, mutants responding poorly to a change in TRA2-BETA1 concentration *in vivo* also bind poorly to TRA2-BETA1. In contrast to previous reports,

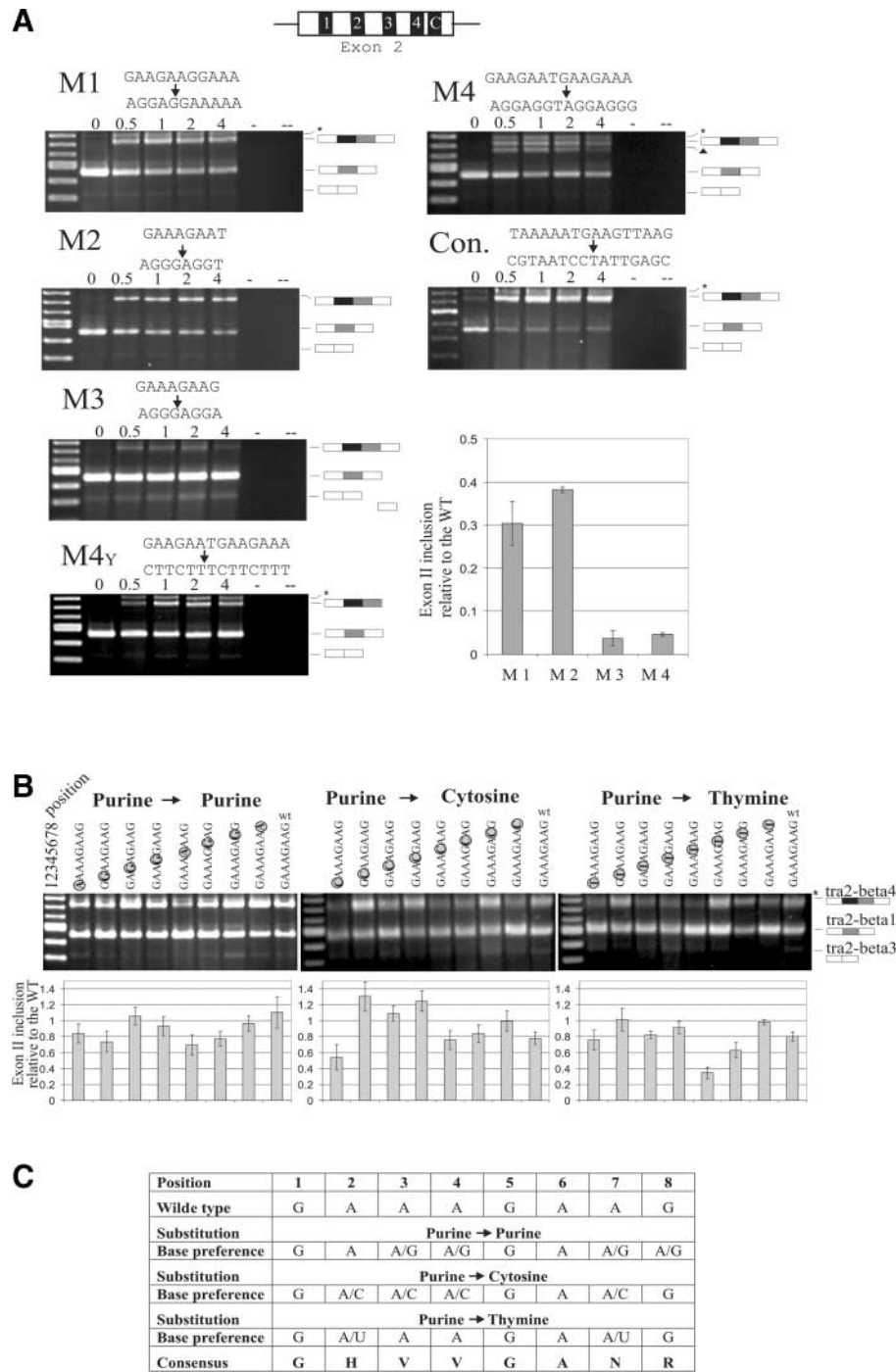


Figure 3. Tra2-beta1 acts through degenerate ESEs in exon 2. (A) Mutagenesis of the motifs identified by sequence comparison. The location of the four motifs identified in Figure 2D in exon 2 are schematically indicated. Each of these motifs (M1–M4) were mutated, as indicated above each gel. In motif M1 to M4 the purine was substituted with the other purine. M4y shows an exchange of pyrimidine into purine for motif 4. Constructs were transfected in HEK293 cells and analyzed by RT–PCR. Numbers indicate the micrograms of EGFP-Tra2-beta1 expression construct transfected. A decreasing amount of parental vector was added to ensure equal amounts of transfected DNA. The substitution of purines with pyrimidines gave similar effects for M1–M3 (data not shown). A control substitution of purine rich sequence with an unrelated purine rich sequence from the serotonin 2C receptor (73) had no effect on exon 2 splicing (con.). Heterodimers formed are indicated with an asterisk. The mutation in motif 4 creates a novel 5' splice site that gives rise to an additional band indicated with a triangle. The quantification of the data relative to the wild-type shown in Figure 2B is shown in the bar diagram. Error bars indicate standard deviations from at least three independent experiments. (B) Scanning mutagenesis of motif 3. Each nucleotide of motif 3 was mutated into either the other purine (left), cytosine (middle) or thymidine (right). The changed nucleotide is indicated with a circle and its position shown on the left. The minigenes were cotransfected with a constant amount (0.5 µg) of EGFP-TRA2-BETA1 expression plasmid and compared with the wild-type. The analysis of four independent experiments is shown under representative ethidium bromide-stained gels. Error bars indicate the standard deviation. (C) Summary of the scanning nucleotide mutagenesis experiments. A base is assumed to be preferred at a position if the inclusion of exon 2 was equal or greater compared with the wild-type minigene. The consensus sequence is the consensus of the preferred bases. H = A/T/C, V = G/A/C, R = A/G.

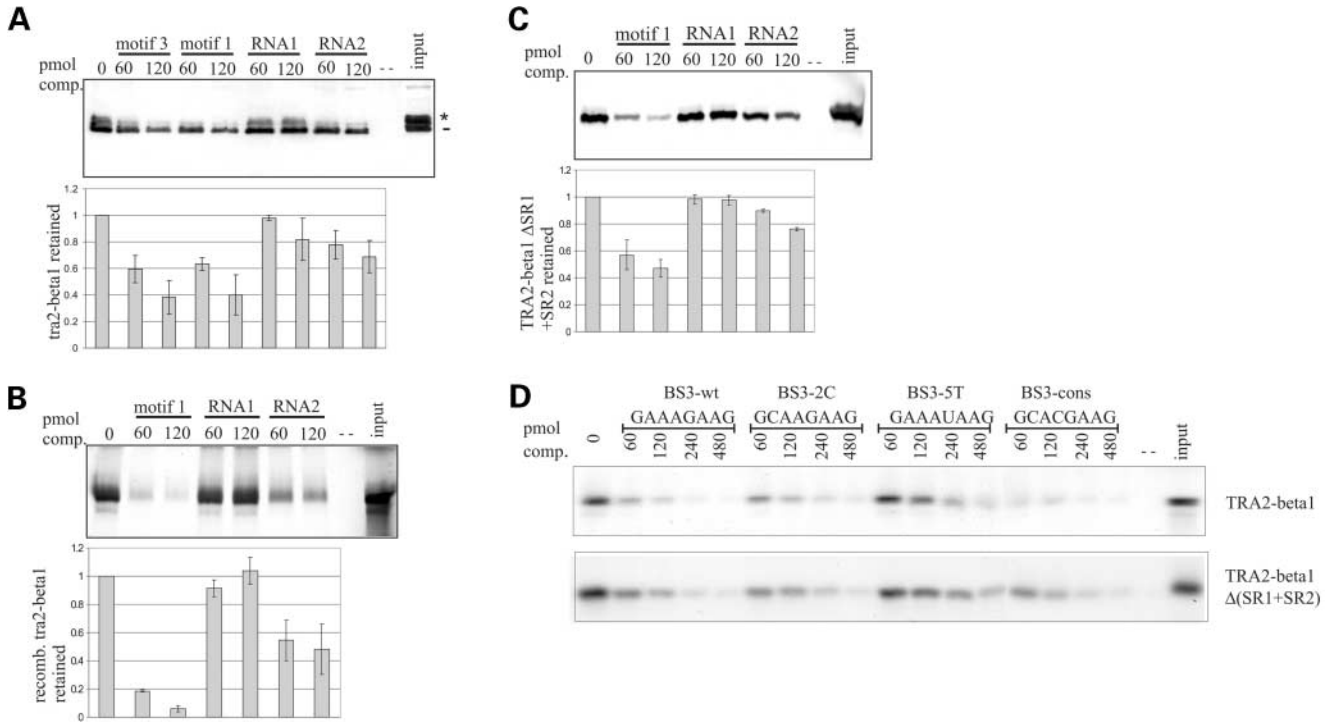


Figure 4. TRA2-BETA1 binds to purine-rich sequences and the motif GHVVGANR. (A) Pull-down of TRA2-BETA1 from nuclear extract with purine-rich RNA-oligonucleotides. In each reaction, 10 μ g nuclear extract was incubated with 20 pmol biotinylated RNA oligonucleotides containing motif 3 (GAAAGAAG). The bound protein was resolved using gel electrophoresis and detected by anti-TRA2-BETA1 antiserum. The circle and the square indicate the hyperphosphorylated and the hypophosphorylated forms, respectively. The unbiotinylated competitor RNA used is indicated on the top, the amount is given in pmol. '-' indicates an experiment lacking the biotinylated oligonucleotide. Motif 1, AAUGUUGAAGAAGGAAAAUGCGGAAGUCgT; RNA1, ACAAAGCGUUCUACCAGCAGCCAGAUGCUG; RNA2, ACAAAGCGAAGAUGGUGCAGCCAGAUGCUG. The statistical evaluation below shows the fraction of TRA2-BETA1 bound. Binding without competitor RNA was set to 100%. Error bars are the standard deviation from at least four experiments. (B) Pull-down of *in vitro* translated TRA2-BETA1. A similar experiment was performed using *in vitro* translated TRA2-BETA1. (C) Pull-down of the *in vitro* translated RRM of TRA2-BETA1. A TRA2-BETA1 deletion variant containing only the RRM [TRA2-BETA1 Δ (SR1 + SR2)] (26) was translated *in vitro* and tested for RNA binding. Similar to TRA2-BETA1 (PI B), binding was effectively competed by the RNA carrying motif 1 (GAAGAAGG), but not by the control oligonucleotides. (D) Binding of TRA2-BETA1 to degenerate sequences. *In vitro* translated TRA2-BETA1 (top) and the TRA2-BETA1 variant containing only the RRM [TRA2-BETA1 Δ (SR1 + SR2); Bottom] was pulled down using the RNA oligonucleotide containing motif 3 (motif 3). The binding was competed with either the wild-type motif 3 (BS3-wt), or three variants containing either single or double nucleotide substitutions (BS3-2c, -5T, -cons). The sequence of the motif in each oligonucleotide and the amount used for the competition in pmol is indicated.

our data show that TRA2-BETA1 binding sites can contain substantial numbers of pyrimidines.

Clk2 kinase inhibits exon 2 inclusion

The binding studies (Fig. 4) showed that preferentially hypophosphorylated TRA2-BETA1 binds to the regulatory sequences of exon 2, suggesting that phosphorylation of TRA2-BETA1 would interfere with exon 2 regulation. We therefore tested whether CLK2, a SR-protein kinase (49) would bind to TRA2-BETA1. EGFP-TRA2-BETA1 and versions lacking one or both RS domains were cotransfected with a Clk2 expression construct. The immunoprecipitation with a clk2 antiserum shows that TRA2-BETA variants containing at least one RS domain bind efficiently to CLK2 (Fig. 5A). To determine whether CLK2 influences tra2-beta1 splicing, we tested its influence on the MG Tra minigene in cotransfection experiments. We observed that active CLK2 reduces exon 2 inclusion. When larger amounts of the clk2 expression construct were used, it repressed the inclusion of both exon 2 and 3, promoting the formation of

the beta3 isoform (Fig. 5B). In contrast, the catalytically inactive form CLK2KR had no effect (Fig. 5C), demonstrating that the effect is phosphorylation-dependent. To determine whether CLK2 functionally interacts with TRA2-BETA1, we cotransfected a constant amount of a clk2 expression clone with an increasing amount of the EGFP-TRA2-BETA1 clone. We observed that CLK2 antagonizes the TRA2-BETA1-induced formation of the beta4 isoform (Fig. 5D, compare with Fig. 2B). These data suggest that CLK2-mediated hyperphosphorylation of tra2-beta1 promotes skipping of exon 2, most likely by reducing TRA2-BETA1 binding to RNA (Fig. 4A).

The tra2-beta locus generates two proteins *in vivo*

In vivo, tra2 pre-mRNA predominantly generates the tra2-beta1 mRNA (26,27). Our results show that minigenes comprising the first four exons recapitulate this behavior (Fig. 2B). In response to an increase in TRA2-BETA1, the beta4 isoform is produced whereas in response to CLK2, the beta3 isoform is formed. Neuronal activity regulates the ratio of these isoforms

in rat brain (27). Both beta3 and beta4 mRNAs contain the same open reading frame, which codes for a protein lacking the first RS-domain (Fig. 1B). We thus determined whether the regulation of alternative pre-mRNA splicing relates to the protein expression from the tra2-beta1 locus.

To detect protein generated by the mRNA isoforms, we constructed minigenes where the regulated first four exons of tra2-beta are fused to EGFP (Fig. 6A). In the TRA-EGFP minigene, exon IV was cloned in frame with the ORF of EGFP. A control minigene, TRA-FS-EGFP contained a frameshift in the exon 4/EGFP border. The ATG start codon of EGFP was converted to ATC to ensure that translation is initiated only from start codons present in tra2-beta. The TRA-EGFP minigene can create chimeric proteins carrying the N-terminal parts of TRA2-BETA1 and beta3, whereas the TRA-FS-EGFP minigene does not form EGFP containing proteins (Fig. 6A). These constructs were cotransfected with TRA2-BETA1 and Clk2 expression clones. Protein and RNA expression were analysed by western blot and RT-PCR, respectively. As shown in Figure 6B, in the absence of other factors, the TRA-EGFP minigene expressed a TRA2-EGFP fusion protein of the expected size. The TRA-FS-EGFP minigene did not express any EGFP-tagged protein. Coexpression of TRA2-BETA1 completely abolished TRA2-EGFP fusion protein expression, whereas Clk2 coexpression induced formation of TRA2-EGFP fusion protein corresponding to the beta1 and beta3 isoform. As expected, no protein was generated by the TRA-FS-EGFP minigene in the presence of TRA2-BETA1 and CLK2. RT-PCR analysis revealed that the constructs generated the expected RNA isoforms. Interestingly, in the presence of TRA2-BETA1, the TRA-EGFP minigene generated the tra2-beta4 isoform, but failed to express the corresponding protein (Fig. 6B), indicating that this mRNA is not translated into protein.

To investigate whether TRA2-BETA3 is formed under physiological conditions, we created a pan-tra2-beta antiserum able to recognize the resulting isoforms. Since tra2-beta3 was found predominately in brain and testis (26), we analysed protein lysates from these tissues for expression of TRA2-BETA3. As shown in Figure 6C, the antiserum detected bands of the sizes predicted for both TRA2-BETA3 and TRA2-BETA1. The pan-tra2-antiserum shows lower expression of TRA2-BETA3 compared with TRA2-BETA1, which approximately reflects the RNA expression detected by RT-PCR (26).

Together these experiments show that two proteins, TRA2-BETA1 and TRA2-BETA3, are generated from the tra2-beta locus. The tra2-beta4 mRNA is not translated although it contains the same open reading frame as the tra2-beta3 mRNA, which yields the truncated TRA2-BETA3 protein.

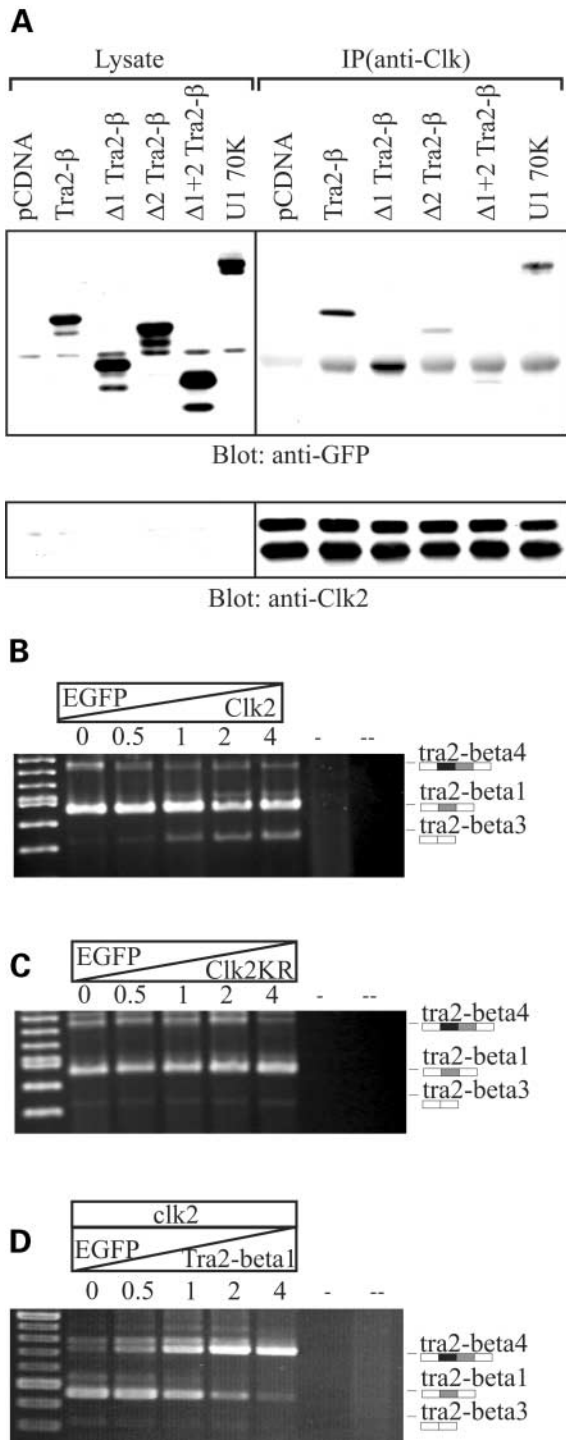
TRA2-BETA1 autoregulates its concentration

Since the presence of TRA2-BETA1 stimulates the formation of tra2-beta4 that is not translated into protein, we assumed that the regulation of tra2-beta exon 2 splicing would provide a mechanism to regulate the cellular TRA2-BETA1 concentration. To test this hypothesis, we created a TRA-prot minigene containing the first four exons of the genomic part of tra2-beta. Exon 4 was cloned in frame with the remaining flag-tagged ORF of TRA2-BETA1. A control minigene, TRA-FS-prot, contained a frameshift in the exon 4/TRA2-BETA border (Fig. 7A) and was not able to express TRA2-BETA protein.

The TRA-Prot minigene can express the three tra2-beta mRNA isoforms encoding functional TRA2-BETA1 and TRA2-BETA3. The minigenes were transfected into HEK293 cells and analysed by real time RT-PCR (Fig. 7B). Transfection of the TRA-FS-prot minigene showed that it expressed high levels of the beta1 mRNA and lower levels of the beta4 mRNA, similar to the original MG Tra minigene (Fig. 7B, 4/0). In contrast, the TRA-Prot minigene produces predominantly the beta4 mRNA that is not translated into protein (Fig. 7B, 0/4). This finding can be explained by the ability of TRA-prot to express the TRA2-BETA1 protein. Since TRA-prot expresses TRA2-BETA1, this minigene could alter the splicing pattern of the TRA-FS-Prot minigene when cotransfected (Fig. 7B, 3/1, 2/2, 1/3). Real-time PCR analysis shows that an increasing ratio of the TRA-prot minigene results in an increase of the tra2-beta4 and a decrease of the tra2-beta1 isoform. We monitored TRA2-BETA1 protein levels in these cotransfection experiments (Fig. 7C) by western blot and were unable to detect any increase of the total TRA2-BETA1 protein, even when significant amounts of TRA-Prot minigene were transfected. The ability of the TRA-Prot minigene to express TRA-beta1 protein was confirmed by probing for the C-terminal Flag-tag carried by the minigene-derived protein (Fig. 7C). This result demonstrates that TRA2-BETA1 protein tightly controls its levels by regulating the alternative splicing of its own exon 2.

Tra2-beta1 interacting proteins influence the tra2-beta splicing pattern

Our data show that TRA2-BETA1 tightly regulates its cellular concentration. Different tissues and cell types have, however, been shown to express both TRA2-BETA1 (26) and its interacting regulatory proteins (50) at different levels. We investigated how TRA2-BETA1 interacting proteins would influence tra2-beta pre-mRNA splicing. TRA2-BETA1 was shown to bind directly to several SR-proteins and hnRNPs, among them SF2/ASF, SRp30c, SAF-B and hnRNP G (25,51–53). We asked whether overexpression of these factors would change tra2-beta splicing patterns. As shown in Figure 8A, increasing the amount of those factors promoted skipping of exon 2 when cotransfected with the MG Tra minigene. To test whether these proteins bind to tra2-beta pre-mRNA directly to regulate exon 2 splicing, we used mutants lacking the RRM (SRp30c Δ RRM, hnRNP-G Δ RRM, SAF-B Δ RRM) or having a non-functional RRM (SF2/ASF FF-DD) in *in vivo* splicing assays (Fig. 8B). We found that all mutants promoted the formation of the tra2-beta1 isoform independently of their RNA binding ability, except SRp30c, which showed partial dependence on RNA binding. The influence of proteins with non-functional RRM on tra2-beta splicing could best be explained with a sequestration of TRA2-BETA1 through these proteins. To test this hypothesis, we employed the MG Tra minigene (Fig. 2A) and induced the formation of tra2-beta4 by cotransfecting TRA2-BETA1. SF2/ASF, SRP30c and SAF-B variants unable to bind RNA were then cotransfected to determine whether they can reverse the TRA2-BETA1 mediated effect. As shown in Figure 8C, SF2/ASF FF-DD and SAF-B Δ RRM can completely reverse the TRA2-BETA1 mediated exon inclusion, whereas SRp30c Δ RRM can partially reverse the effect.



Finally, we asked whether TRA2-BETA1 interacting protein could influence splicing of the TRA-prot minigene (Fig. 7A) that can produce functional TRA2-BETA1. Cotransfection and real-time PCR revealed an ~2-fold induction of the beta1 and a 3-fold induction of beta3 isoform (Fig. 8D, right), which is in agreement with the results observed with the MG tra minigene (Fig. 8A–C).

Figure 5. Clk2 kinase influences alternative splicing of TRA2-BETA. (A) CLK2 binds to TRA2-BETA isoforms containing at least one RS domain. A CLK2 expression construct was cotransfected with EGFP tagged expression clone of TRA2-BETA1 and the EGFP tagged variants TRA2-BETA1ΔSR1, TRA2-BETA1ΔSR2 and TRA2-BETA1Δ(SR1 + SR2) lacking the first, second or both RS domains. Lysates were immunoprecipitated with an anti-clk antiserum and interacting proteins detected using an anti-EGFP antibody. Below is a reblot probed with anti-clk2 indicating equal immunoprecipitation of CLK2. (B) CLK2 kinase inhibits exon 2 inclusion in *in vivo* splicing assays. Increasing amounts of an EGFP-Clk2 expression construct were cotransfected with the MGtra minigene. Numbers indicate the amount transfected in micrograms. A decreasing amount of parental vector was added to ensure equal amounts of transfected DNA. The RNA was analysed by RT-PCR. The structure of the products is indicated on the left and its generation is shown in Figure 2A. CLK2 expression inhibited the inclusion of both exons 2 and 3 in a concentration-dependent manner. (C) The catalytically inactive kinase CLK2KR has no effect. Similar to (B), a catalytically inactive variant, CLK2 KR, was cotransfected with the MGtra minigene. (D) Cotransfection of CLK2 inhibits the ability of TRA2-BETA1 to induce exon 2 inclusion. An increasing amount (0.5–4 μg) of EGFP-Tra2-beta1 expression construct was transfected in the presence of a constant amount (1 μg) of a CLK2 expression construct.

We conclude that tra2-beta1 interacting proteins antagonize the TRA2-BETA1 induced exon 2 inclusion, most likely through RNA independent interaction with TRA2-BETA1.

DISCUSSION

Alternative splice site selection is regulated by the cooperative action of multiple splicing factors (13,54). The recognition of an exon is often based on a finely tuned balance between antagonistic splicing factors (55–57). This balance implies that the active concentrations of the splicing factors are tightly controlled within very narrow concentration ranges. Several splicing factors have been shown to control the splicing of their own pre-mRNAs by autoregulatory loops (19,38,39,42,58). However, in most cases little is known about the physiological importance and the molecular mechanism of the autoregulation. In this study, we showed that a negative feedback autoregulatory loop regulating tra2-beta exon 2 usage is utilized to control the TRA2-BETA1 protein levels.

The principles, but not the details of autoregulation are conserved between drosophila and mammals

TRA2-BETA1 shows 38% sequence identity to drosophila TRA2 (25) and the highly related TRA2-alpha gene can functionally complement drosophila TRA2 (24). Although the structures of the tra2 genes are not conserved between drosophila and humans in both systems TRA2-BETA1 and the corresponding dTRA2²²⁶ proteins autoregulate the formation of their mRNA isoforms. In drosophila, retention of an intron is controlled; in humans, inclusion of an exon. Both the alternative intron and exon are flanked by suboptimal splice sites. In the drosophila male germ line, dTRA2²²⁶ causes intron M1 retention. As a result, translation starts from a downstream start codon and the dTRA2¹⁷⁹ protein is formed (33,58–60). Similar to the human TRA2-BETA3 isoform dTRA2¹⁷⁹ lacks the N-terminal RS repeat and is unable to complement the function of the full length protein *in vivo*. In the human autoregulatory loop,

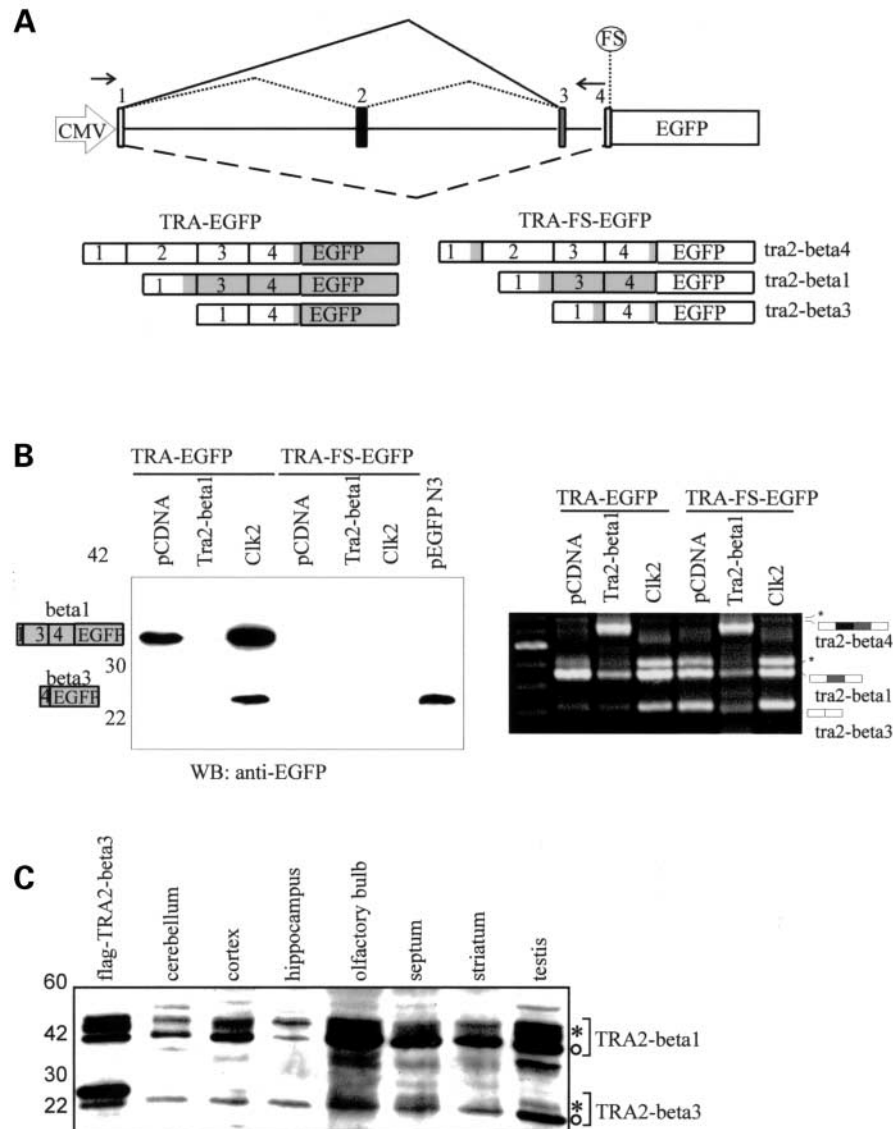


Figure 6. The tra2-beta4 mRNA is not translated into protein. (A) Structure of the TRA-EGFP and TRA-FS-EGFP minigenes. Both minigenes differ in a single nucleotide causing a frameshift at the exon 4/EGFP border (FS). The structure of the mRNAs generated from each of the minigenes is shown below. Arrows indicate localization of the primers used for RT-PCR. The gray shading indicates open reading frames. (B) Protein and RNAs generated from the TRA-EGFP and TRA-FS-EGFP minigenes. An antibody against EGFP was used to detect the chimeric TRA-EGFP proteins in western blot after cotransfecting 0.5 μ g TRA-EGFP and TRA-FS-EGFP minigenes with 1 μ g control vector (pcDNA) or 1 μ g tra2-beta1 and clk2 expression constructs. pEGFPN3 (Clontech) is a positive control for EGFP expression. A single band corresponding in size to the tra2-beta1-EGFP ORF of the TRA-EGFP minigene is detected in the pcDNA control lane. TRA2-beta1 expression induced exon 2 inclusion and completely blocks the protein synthesis from the minigene. Clk2 expression induced skipping of both exons 2 and 3 and results in a synthesis of a TRA2-BETA3-EGFP protein. No EGFP expression is detected from the TRA-FS-EGFP minigene where EGFP is out of frame with the tra2-beta start codons. The structure of the proteins is schematically shown on the left of the blot. The ethidium bromide stained gel on the right shows the RT-PCR analysis of these transfections. The band indicated with an asterisk indicates a heterodimer. RNA isoforms are schematically shown on the right. (C) Expression of TRA2-BETA3 in rat tissue. Ten micrograms of protein in lysates from different brain regions and testis were analysed in western blot with the pan-Tra2 antibody. The dot and the square indicate the hyper- and hypophosphorylated forms of the TRA2-beta proteins, respectively.

a mRNA isoform, tra2-beta4 that is not translated into protein is formed and fulfills a role like dTRA2¹⁷⁹. The tra2-beta3 mRNA is used in the dynamic regulation of the TRA2-BETA1 activity in response to the external stimuli. Its formation is controlled by protein phosphorylation and is regulated in neuronal stimulation models (27). Although the detailed molecular mechanisms of the human and drosophila negative feedback loops are different, they both serve the same purpose:

to ensure a constant concentration of the full-length TRA2 protein.

TRA2-BETA1 binds to highly degenerate pyrimidine-containing sequences *in vivo*

Previously, *in vitro* SELEX experiments were performed suggesting that TRA2-BETA1 binds to poly(A) and

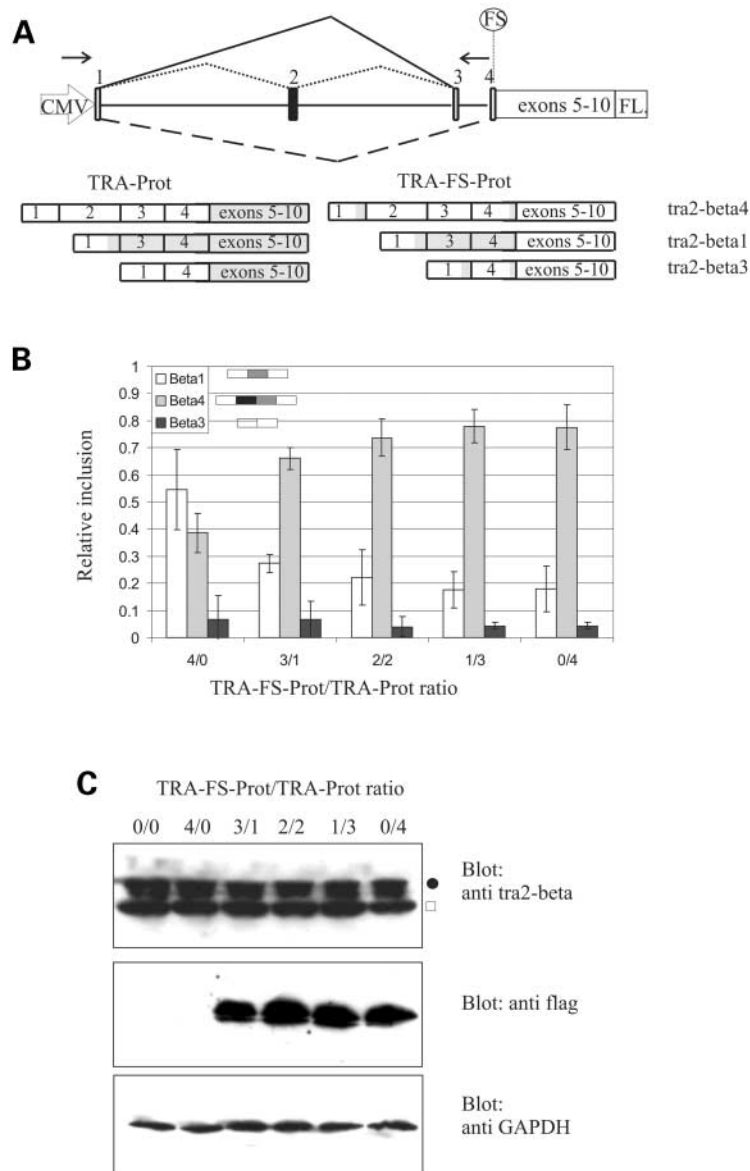


Figure 7. The TRA2-BETA1 protein is held constant by an autoregulatory loop. (A) Structure of the TRA-prot and TRA-FS-prot minigenes. The remaining tra2-beta exons 5–9 from cDNA are fused to exon 4 from genomic tra2-beta DNA. A four nucleotide insertion at the exon 4/5 border introduces a frameshift (FS) in the TRA-FS-prot minigene. The constructs contain a C-terminal flag-tag (FL). The various RNA isoforms generated by the minigenes are shown below and their open reading frames are indicated by shading. (B) Real time PCR analysis of TRA-prot and TRA-FS-prot cotransfection experiments. Varying ratios of the two minigenes were transfected in HEK293 cells. Different RNA isoforms were determined by real-time RT-PCR. Their structure is indicated on the top left. The TRA-FS-prot minigene expresses predominantly tra2-beta1 mRNA, but is unable to express TRA2-BETA1 protein. Transfection of the TRA-prot minigene resulted in high levels of the exon 2 containing tra2-beta4 mRNA. Owing to its ability to produce functional TRA2-beta1, it can change the splicing pattern of the TRA-FS-prot minigene when the TRA-FS-prot and TRA-prot minigene ratios are altered. (C) Constant TRA2-BETA1 expression in TRA-prot and TRA-FS-prot cotransfection. The ratio of the two minigenes was varied as indicated above. '0/0' indicates protein from mock transfected cells. The TRA2-BETA1 protein expression was determined using western blot (top). A dot and a square indicate the hyper- and hypophosphorylated form, respectively. The blot in the middle shows the detection of the transfected protein using an anti-flag antibody that detects only the transfected protein. GAPDH served as a loading control and is shown in the bottom blot.

oligo(GAA) sequences (48) present in splicing enhancers (61). Our sequence comparison of exons regulated by TRA2-BETA1 *in vivo* also identifies common purine-rich motifs. However, database analysis demonstrates that purine-rich enhancers are not highly abundant in naturally occurring alternative exons (62). We characterized the consensus binding site for TRA2-BETA1 by mutagenesis in a functional assay and found that it is highly degenerate and can contain substantial number of

pyrimidines, extending the previous reports (48). The sequence we obtained is found in all exons regulated by TRA2-BETA1 *in vivo* (Fig. 2D) indicating that it can work in different sequence contexts. Other studies that relied on functional assays to determine enhancer sequences resulted in degenerate sequences as well (63–65). This degeneracy seems to be typical for the binding sites of most splicing factors and most likely prevents interference with the coding capacity of the exons.

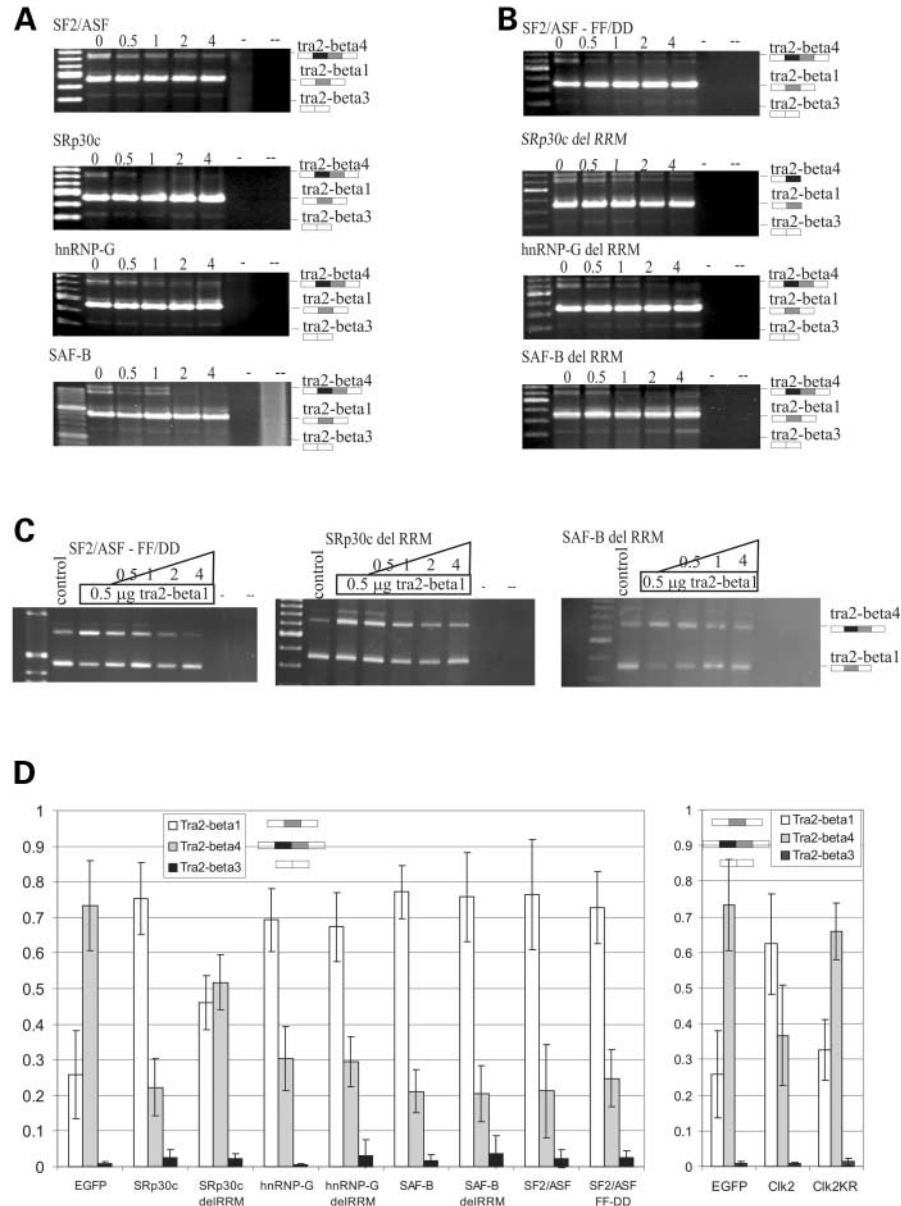


Figure 8. TRA2-BETA1 interacting proteins promote TRA2-BETA1 formation. **(A)** Cotransfection of TRA2-BETA1 interacting proteins with the MGTra minigene. Increasing amounts (0.5–4 µg) of EGFP tagged SF2/ASF, SRp30c, hnRNP-G and SAF-B expression clones were cotransfected with 1 µg MGTra minigene and the RNA analysed by RT-PCR. A decreasing amount of parental vector was added to ensure equal amounts of transfected DNA. **(B)** Variants of TRA2-BETA1 interacting proteins unable to bind RNA influence tra2-beta splicing. Interacting proteins that are deficient in RNA binding were cotransfected in an assay similar to A and are still able to suppress exon 2 inclusion. **(C)** TRA2-BETA1 interacting proteins can reverse the effect of TRA2-BETA1 on tra2-beta splice site selection. One microgram of the MGTra Minigene was cotransfected with 0.5 µg of EGFP-TRA2-BETA1 expression clone and increasing amounts (0.5–4 µg) of SF2/ASF, SRp30c and SAF-B expression clones unable to bind RNA. Parental vector was added to ensure constant amounts of DNA in the transfections. Control: MGTra minigene with parental vector. **(D)** Real-time PCR analysis of the influence of TRA2-BETA1 interacting proteins on the TRA-prot minigene. One microgram of the expression constructs in (A) were cotransfected with the TRA-prot minigene and their effect was quantified by real time RT-PCR. All factors suppressed exon 2 inclusion independent of their RNA binding ability except SRp30c, which shows partial dependence on its RRM. Similar to Figure 4A, CLK2 inhibits exon 2 inclusion, whereas the inactive KR variant (clkKR) has no effect.

ESEs show an uneven distribution between the exons and the introns (63,66). Indeed, screening the annotated exons and introns in the rough draft of the human genome with the consensus sequence for the TRA2-beta1 binding site showed a strong bias for exons, suggesting that TRA2-BETA1 could bind to and potentially regulate, numerous exons (Fig. 9A).

An interesting consequence of the TRA2-BETA1 binding site degeneracy is its partial overlap with the functional consensus binding sites of SF2/ASF (Fig. 7B). This could create splicing regulatory elements that can be recognized either only by SF2/ASF and TRA2-BETA1 or by both proteins. It explains why TRA2-BETA1 and SF2/ASF can regulate splicing through the

same ESEs, such as the A3 enhancer (48) while having opposite effects on the TRA2-BETA1 exon 2 splicing. In a broader perspective the degeneracy of the SR and SR like protein binding sites and their partial overlap could be the underlying reason for the both unique and redundant functionality of those proteins in *C. elegans* (67,68).

The TRA2-BETA1 concentration is determined by an auto-regulatory loop

Four mRNAs are generated from the human tra2-beta locus through the alternative processing of the exons 2 and 3 of the pre-mRNA. Exon 2 alternative splicing plays a major role in the regulation of the TRA2-BETA1 protein expression. Its inclusion in the mRNA is dependent on the TRA2-BETA1 protein, which binds to four ESEs in exon 2. Exon 2 carries several stop codons that interrupt the open reading frame and the resulting tra2-beta4 mRNA is not translated. The tra2-beta4 isoform is readily detectable by RT-PCR. The first in frame stop-codon is located 198 nt upstream of the junction of exon 2 and 3, which would destine this isoform to NMD. It remains to be determined why this isoform is abundant and whether it can escape NMD. Finally, sequestration of TRA2-BETA1 by interacting proteins results in exon 2 skipping and TRA2-BETA1 synthesis (Fig. 7).

The autoregulatory loop controlling tra2-beta pre-mRNA splicing and protein synthesis is strikingly sensitive and robust. Cotransfection experiments with TRA2-BETA1 expressing constructs show that a very small increase of the TRA2-BETA1 protein level is able to induce dramatic increase of exon 2 inclusion (Fig. 2). On the other hand, the negative feedback loop was able to completely compensate for the elevated tra2-beta gene dosage and keep the TRA2-BETA1 protein levels constant (Fig. 5).

We showed that binding of TRA2-BETA1 to RNA, and consequently exon 2 usage, is dependent on the phosphorylation status of the protein. The hyperphosphorylated TRA2-BETA1 binds poorly to RNA. Consistent with this observation, phosphorylation of TRA2-BETA1 by CLK2 kinase *in vivo* prevents the formation of the tra2-beta4 mRNA.

An intriguing feature of exon 2 alternative splicing is the role played by splicing factors binding to TRA2-BETA1. Exon 2 usage is suppressed by several TRA2-BETA1 interacting proteins, independently of their RNA binding ability. This implies that the effect is based solely on protein-protein interactions. We suggest that these factors exert their effect on exon 2 splicing by sequestering the TRA2-BETA1 protein or by saturating its protein interaction capacity. Although the precise mechanism requires further investigation, the data presented here clearly shows that splicing factors can control alternative exon usage through mechanisms that do not necessarily involve RNA binding. Physiologically, this system could determine and maintain constant the ratio between TRA2-BETA1 and several of its interactors, e.g. SF2/ASF, SRp30c, hnRNP-G and SAF-B. This allows TRA2-BETA1 protein levels to be regulated in respect to concentrations of other splicing factors that differ between the cell types and the physiological condition of each cell.

MATERIALS AND METHODS

Generation of the minigene

The part of the human tra2-beta gene that includes exons one to four was amplified from a genomic clone (26) using primers MGTra-F-Bam (GGGGATCCGACCGGCGCGTCTGCGG-GGCT and MGTra-R-Xho GGGCTCGAGTACCCGATTC-CCAACATGACG). The PCR product was cloned in pCR XL TOPO vector (Invitrogen) as described (43). The minigene was then excised using BamHI and XhoI restriction sites that were introduced by the primers and cloned in pCR3.1 (Invitrogen).

Mutagenesis

Site specific mutagenesis was performed as described (69). The primers used for the mutagenesis are at www.stamms-lab.net/publications/tra.

Generation of a pan tra2-beta antiserum

A rabbit polyclonal antiserum (pan tra2-beta) was raised against a tra2-beta1 peptide (GCSITKRPHPTPGIYMGRPTY) fused to keyhole-limpet hemocyanin as previously described (27). The peptide is located between the RRM and the second RS domain (Fig. 1B). The antiserum specificity was determined by western blot after pre-binding to the immunogenic peptide.

Western blot

Western blot using tra2-antibodies (1:3000 dilution) was performed as previously described. Briefly, proteins were separated by SDS-PAGE, transferred onto nitrocellulose and detected by ECL (27). The antibody dilutions used were as follows: GAPDH (RDI) 1:100 000, FlagM2 (Sigma) 1:5000, GFP (Roche) 1:10 000. HRP conjugated secondary antibodies were obtained from AP-Biotech and from Santa Cruz Biotechnology and used in 1:10 000 dilution.

Cotransfection experiments and RT-PCR

Cotransfection experiments were performed as described (43) using HEK293 cells and the calcium chloride method. For reverse transcription, a vector specific primer pCR-RT-reverse (GCCCTCTAGACTCGAGCTCGA) was used to avoid amplification of endogenous transcripts. For the following PCR amplification primers, MGTra-F-Bam and MGTra-R-Xho were used that annealed to both the endogenous and transfected cDNA. PCR products were then separated on agarose gels and quantified using Image Quant (AP Biotech). Expression constructs for SF2/ASF, SRp30c, hnRNP G, YT521-B, SAF-B and mClk2 driven by the CMV promoter and tagged by EGFP were all cloned in pEGFP-C2 (Clontech) and previously described (70). The SF2/ASF FF-DD expression construct was a generous gift from Javier Caceres (71).

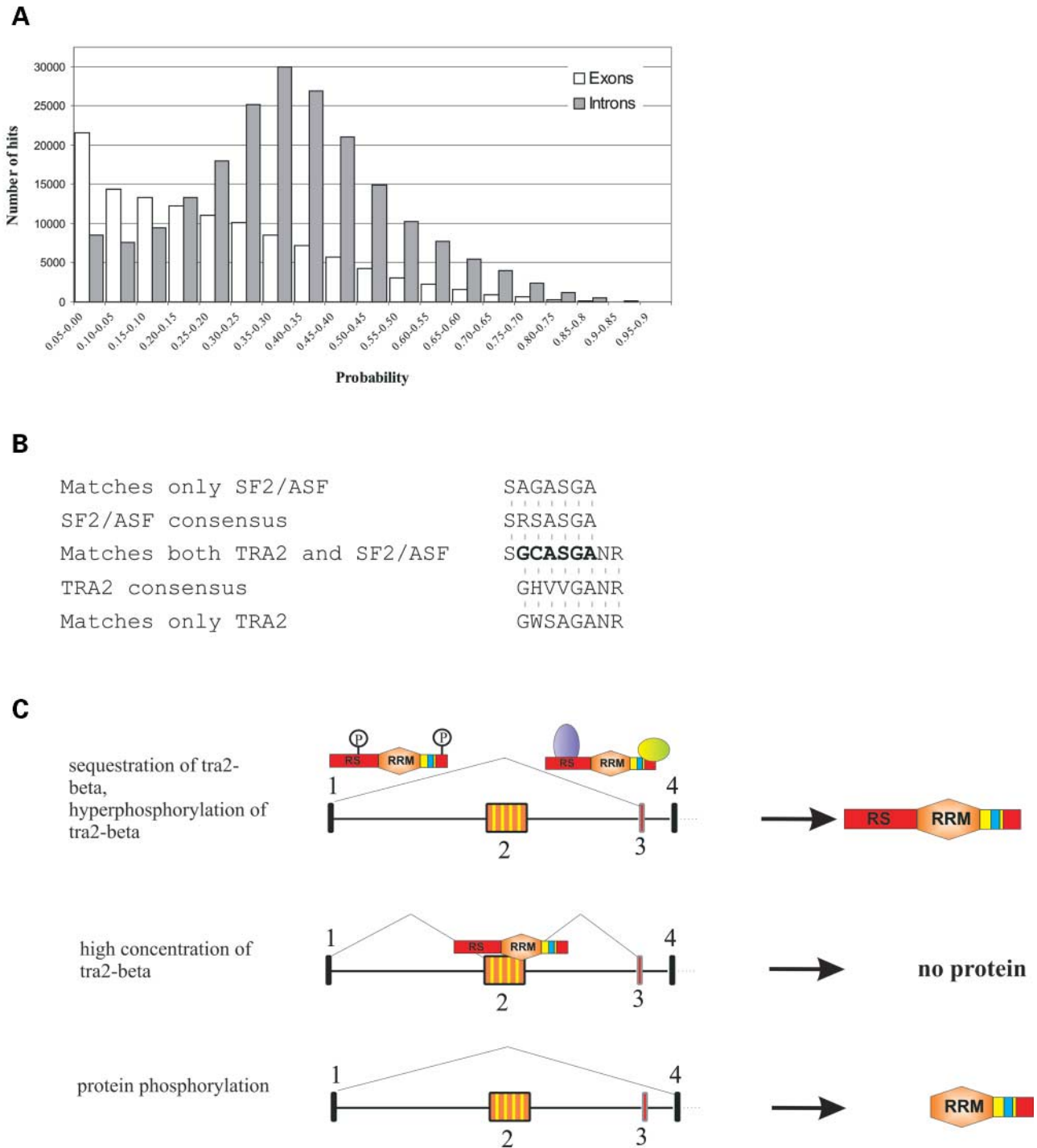


Figure 9. Model of TRA2-BETA1 regulation. (A) The TRA2-BETA1 binding motif is more abundant in exons than in introns. The human genome draft sequence was analyzed. The dataset consisted of 252 288 introns with total length of 1.18 Gbp and 280 064 exons totaling 42 Mbp. The probability distribution of the sequences containing matches to the sequence GHVVGANR is shown. The intron sequences (grey bars) peak at the probability for a random match of 0.35–0.30. This corresponds to the number of matches actually found equaling the number of matches expected to occur by chance. In contrast, the distribution of the exon sequences (white bars) is biased towards low probabilities of hits occurring by chance. Probability: probability that the number of hits in the dataset occur by chance. (B) Partial overlap of SF2/ASF and TRA2-BETA1 binding sites. The functional SF2/ASF and TRA2-BETA1 consensus site are shown in the second and fourth lines. Less degenerate sequences that would bind only to SF2/ASF, TRA2-BETA1 or both proteins are shown in the first, third and last line. S = G/C, H = A/C/T, V = G/A/C, R = A/G, W = A/T. (C) Model of tra2-beta pre-mRNA regulation. TRA2-BETA1 does not bind to exon 2 if its concentration is low, if it is sequestered or phosphorylated. As a result, exon 2 is skipped and TRA2-BETA1 formed (top). This will raise the TRA2-beta1 levels to a point where its interacting proteins will be unable to sequester it completely. The excess of TRA2-beta1 binds to exon 2 and activates its inclusion in the mRNA. The resulting tra2-beta4 mRNA cannot be translated and therefore the synthesis of TRA2-beta1 protein is effectively switched off (middle). Protein phosphorylation by CLK2 kinase can suppress the inclusion of both exon 2 and 3 in the mature transcript, generating the TRA2-beta3 mRNA. This mRNA is translated into a truncated protein that lacks the N-terminal SR repeat (bottom).

Pull-down assays

Twenty picomoles of biotinylated RNA oligonucleotide were incubated for 1 h with either nuclear extract (10 µg of protein) or 2 µl of ³⁵S-labeled *in vitro* translation mix and competitor RNA oligonucleotides in a total of 20 µl RNA binding buffer (10 mM HEPES pH 7.5, 100 mM KCl, 5 mg/µl Heparin, 1.5 mg/ml tRNA, 0.67 mg/ml BSA, 0.1% Triton-X100, 20U RNase inhibitor) at 4°C. Streptavidin coated metacrylate beads (Boeringer) equilibrated in the binding buffer were then added to the reaction. This was further incubated for 1 h at 4°C. The beads were washed three times with RNA binding buffer and resuspended in SDS gel loading buffer. The bound protein was detected either using western blot with TRA2-BETA specific polyclonal sera or by autoradiography. The sequences of the RNA oligonucleotides were: motif1, AAUGUUGAAGAAGGAAAUGCGGAAGUCGdT; motif3, GUGGGGAAGAUGAAAAGAAGUCAGAAUUUGdA; RNA1, ACAAAGCGUUCUACCAGCAGCCAGAUGCUG; RNA2, ACAAAGCGAAGAUGGUGCAGCCAGAUGCUG; motif3-C2, GUGGGGAAGAUGCAAGAAGUCAGAAUUUGdA; motif3-U5, GUGGGGAAGAUGAAAUAAGUCAGAAUUUGdA; motif3-cons, GUGGGGAAGAUGCACGAAGUCAGAAUUUGdA.

Computer analysis

The rough draft sequence of the human genome was obtained from ftp://ftp.ncbi.nlm.nih.gov/genome. Scripts written in Perl were used to extract the exons and introns from the genomic sequences according to the annotation data and to perform pattern searches of the datasets. The Perl scripts are available for download from http://stamms-lab.net/papers/tra. The probability of the matches in a given exon or intron to occur by chance was determined using the formula: $P = e^{-(L \cdot m/l)}$. In this formula, l is the length of the exon or intron, m the number of matches and L the length of the sequence with the same base composition as the inspected exon or intron, where one hit is expected to occur by chance (72).

Real-time PCR

Real time PCR quantification was performed on ABI prism 9700 system using QuantiTect SYBR Green PCRkit (Qiagen) following the manufacturer's protocol. Standard curves for the amplification of each isoform were prepared using serial dilution of linearized cloned template. All experiments were done in triplicate and the amount of each isoform was determined by comparison to the standard curve and used to calculate the isoform ratio. The specificity of the amplification was controlled using agarose gel electrophoresis. The tra2-beta mRNA isoforms were detected using isoform specific primer pairs as follows: tra2-beta1 was amplified using tra1/3forw (TACGGCGAGCGGGAA) and tra4rev1 (GTATAATGCCTTCGGGAGCTTC) primers; tra1-beta3 was amplified using tra1/4forw (TACGGCGAGCGGGAAT) and tra4rev; tra2-beta4 was amplified using tra2forw (TTGAGCAATTTGTGTGAA-GTGG) and tra2/3rev (AACGGGATTCTTAGCGTAGTGC).

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