Published in final edited form as:

Biochem Soc Trans. 2012 August ; 40(4): 784–788. doi:10.1042/BST20120036.

How does Tra2 β protein regulate tissue-specific RNA splicing?

David J. Elliott*, Andrew Best, Caroline Dalglish, Ingrid Ehrmann, and Sushma Grellscheid

Institute of Genetic Medicine, Newcastle University, Central Parkway, Newcastle NE1 3BZ

Abstract

The splicing regulator protein Tra2 β is conserved between humans and insects and essential for mouse development. Recent identification of physiological RNA targets has started to uncover molecular targets and mechanisms of action of Tra2 β . At a transcriptome-wide level, Tra2 β protein binds a matrix of AGAA-rich sequences mapping frequently to exons. Particular tissue-specific alternatively spliced exons contain high concentrations of high scoring Tra2 β binding sites and bind Tra2 β strongly *in vitro*. These top exons were also activated for splicing inclusion *in cellulo* by co-expression of Tra2 β protein and significantly down-regulated after genetic depletion of Tra2 β . Tra2 β itself seems to be fairly evenly expressed across several different mouse tissues. Here we review the properties of Tra2 β and its regulated target exons, and mechanisms through which this fairly evenly expressed alternative splicing regulator might drive tissue-specific splicing patterns.

Keywords

RNA splicing; gene expression; RNA binding proteins; Tra2 β ; transcriptome; HITS-CLIP; development

Tra2 β protein is a splicing activator conserved between flies and mouse

Alternative splicing introduces new coding information into mRNAs, and so plays a pivotal role in expanding genome capacity to encode more proteins than just the ~23,000 that would be expected if each gene encoded a single protein [1]. Alternative splice events are controlled in part by a number of different RNA binding proteins attaching to pre-mRNAs, although links with transcription and epigenetic modification of the template chromatin are also important. A large group of splicing regulator proteins contain domains which are enriched in arginine and serine (so called RS domains, based on the one letter amino acid code) [2,3]. These include Tra2 proteins, which have a modular organisation comprising a single central RNA Recognition Motif (RRM) flanked either side by RS domains [4,5].

A single Tra2 protein is found in fruit flies, where Tra2 is one of the classical splicing regulators controlling sexual differentiation as well as being essential for spermatogenesis [6,7,8]. The fly gene is the source of the acronym Tra2, which is short for transformer2 since mutations in this gene transform sexual phenotype. The *Tra2* gene has duplicated in vertebrates, resulting in two mammalian Tra2 proteins with 63% amino acid identity (aligned at <http://www.ebi.ac.uk/Tools/emboss/align/>). These proteins are called Tra2 α (encoded by the *Tra2a* gene on mouse chromosome 6) and Tra2 β (encoded by the *Sfrs10* gene on mouse chromosome 16). Tra2 β binds to exons to regulate their alternative splicing inclusion. For example, Tra2 β binds to the testis-specific *T* exon in the *Homeodomain*

*Corresponding Author David Elliott, David.Elliott@ncl.ac.uk, Telephone 0191 241 8694, Fax 0191 241 8666 .

Interacting Protein Kinase 3 gene to regulate its splicing inclusion in the testis [9,10]. Recently the details of exactly how Tra2 β protein binds to both AGAA and CAA target RNA sequences have been revealed at atomic resolution, and involve protein-RNA interactions with both the RRM and flanking regions [11,12].

Transcriptome-wide identification of splicing targets for Tra2 β

Despite similar amino acid sequences and RNA binding specificities between Tra2 α and Tra2 β [13], genetic deletion of the *Sfrs10* gene still results in embryonic lethality even though the *Tra2a* gene remains intact [14]. *Sfrs10*^{-/-} mice die at around 12 days gestation. This indicates either non-redundancy with Tra2 α at this stage of development, or that expression levels achieved from both genes are needed for embryonic development [14]. Similarly, Tra2 β is essential in the embryonic brain [15].

Because of the known functions for Tra2 β protein in splicing, it is likely that defects in splicing regulation are a major contributing factor to the embryonic death of *Sfrs10*^{-/-} mice. Such defects would likely lead to downstream changes in mRNA and protein isoforms impacting on development. Previously there were just a handful of known splicing targets for Tra2 β which were not clearly mis-regulated in the absence of Tra2 β [10,16]. Recently Cross Linking Immunoprecipitation coupled to deep sequencing (HITS-CLIP) has been used to comprehensively identify endogenous RNA targets for Tra2 β during mouse germ cell development [15]. In this procedure, endogenous target RNAs are crosslinked by ultraviolet radiation, then short fragments bound to Tra2 β rigorously purified, amplified, deep sequenced and mapped onto the genome. As an illustration of the resolution of this technique in identifying binding sites in vivo, the CLIP tags mapping to one of these identified target exons (*Nasp-T*) is shown in Figure 1 [17].

Target exons which depend on Tra2 β for splicing inclusion should be mis-spliced in the absence of Tra2 β protein. Splicing analyses in the brains of mice which contain *Sfrs10*^{-/-} neurons indicated splicing inclusion of at least two of the identified target exons (within the *Nasp* and *Tra2a* genes) identified from the HITS-CLIP screen were indeed strongly down-regulated in the absence of Tra2 β protein, thus identifying these as physiologically regulated target exons [15].

The identification of these target exons reveal for the first time biological functions operating downstream of Tra2 β protein in mouse development. The *Nasp* gene itself is essential for mouse development [18] and encodes a protein which mediates histone import into the nucleus and assembly of chromatin after replication. The Tra2 β -regulated *Nasp-T* exon is very long at 975 nucleotides [19], making this single exon almost as long as the rest of the *Nasp* mRNA. This 975nt exon length is divisible by three, and inserts the open reading frame for a peptide cassette into the *Nasp* mRNA in those tissues where it is spliced. In adult mice the *Nasp-T* exon is most actively spliced in the testis (it is also spliced in the embryo). In the testis the longer encoded Nasp protein isoform is associated with meiotic chromosomes where it forms part of the complex which monitors the completion of double strand break repair [18,20,21,22]. Hence by regulating splicing of the *NaspT* exon, Tra2 β operates as one of the key upstream regulators for this crucial physiological process in mouse male germ cells.

Tra2 β also physiologically regulates splicing inclusion of a “poison” exon into the *Tra2a* mRNA [9,23]. This poison exon inserts in frame stop codons into the *Tra2a* mRNA, thereby targeting it for nonsense mediated decay and preventing subsequent production of Tra2 α protein. Identification of this regulated poison exon has thus revealed a novel pathway of feedback control which operates between the vertebrate Tra2 proteins: over-expression of

Tra2 β protein leads to increased splicing inclusion of the *Tra2a* poison exon, and down-regulation of Tra2 α expression.

Both the *Nasp-T* and *Tra2a* exons are found in all available vertebrate genome sequences indicating strong selective pressure [24,25,26,27,28]. High conservation of the *Nasp-T* and *Tra2a* exons is particularly remarkable in the context of the testis, since many alternative exons included in this tissue are not conserved between species [24]. Functionally important exons also tend to be frequently included into mRNAs in at least some tissues. In mice the *Nasp-T* and *Tra2a* exons are spliced into mRNAs at high levels in testis, and also spliced into mRNAs in other adult tissues and in the embryo. Defects in *Nasp-T* and *Tra2a* splicing may therefore contribute to the phenotype of the *Sfrs10*^{-/-} mice. Since RNA binding proteins are global regulators defects resulting from their absence are likely to be widespread. Tra2 β can bind and potentially regulate thousands of exons in parallel across the transcriptome, many of which might contribute to the embryonic or brain phenotype when Tra2 β is missing [14,15].

Multiple binding sites are needed for Tra2 β -mediated splicing activation of *Nasp-T* and *Tra2a*

Both the *Nasp-T* and *Tra2a* poison exons have two key features relating to their particular dependence on Tra2 β for splicing inclusion:

(1) A high frequency of Tra2 β binding sites

Both the *Nasp-T* and *Tra2a* exons contain multiple Tra2 β binding sites inferred from transcriptome-wide analysis [15]. Within the transcriptome-wide dataset of RNA targets particular 6-mer sequences were identified as enriched compared to either the genomic or testis specific transcriptomic backgrounds. Each of these most frequently recovered 6-mers represent binding sites for Tra2 β recognised *in vivo* within the mouse transcriptome, and were subtle variations variants of AGAA-rich sequences very similar to the known Tra2 β binding site [11,12].

These frequently recovered 6-mers were used as a Tra2 β binding site matrix, with the most frequently recovered 6-mers representing the best physiological binding sites. When analysed according to preferred physiological binding sites, the *Nasp-T* exon contains approximately 37 Tra2 β binding sites (defined as containing sequences within the 25 most frequently recovered 6-mers in the transcriptome-wide dataset, with particular importance being placed on the top 5 recovered 6-mers); and the *Tra2a* exon contains approximately 12 binding sites [15]. Note that these numbers are approximations: some predicted Tra2 β binding sites overlap, making it difficult to be precise. Tra2 β binding sites clustered in the upstream and downstream portions of the *Nasp-T* exon (overlapping upstream and downstream clusters of binding sites identified by CLIP are shown in Figure 1).

(2) Highly efficient Tra2 β binding

Consistent with their high concentration in binding sites, both the *Nasp-T* and *Tra2a* exons bound Tra2 β protein very efficiently in gel shift experiments, and less stable complexes were formed after binding site mutagenesis [15].

By looking at the exon sequences of *Nasp-T* and *Tra2a*, it is possible to estimate when splicing regulation by Tra2 β might have evolved. Superimposition of binding sites and detailed phylogenetic comparisons of exon sequence show the *Tra2a* poison exon is likely to be controlled by Tra2 β across all vertebrates (all of which contain multiple binding sites), while Tra2 β control of *Nasp-T* is more likely to have evolved in mammals [15].

Models for physiological splicing control by Tra2 β

The *Sfrs10* gene is expressed fairly evenly in different mouse tissues [15]. This raises the important question: how does Tra2 β regulate tissue-specific splicing patterns? Two conceptually different (but not mutually exclusive) models might explain how Tra2 β protein operates as a tissue-specific splicing factor.

(1) Tissue-specific patterns of splicing may be controlled by differences in the cellular concentration of Tra2 β (Figure 2A)

Subtle but important differences in Tra2 β protein concentration might occur both between and within tissues to drive tissue-specific splicing patterns. For example within the mouse testis Tra2 β protein is low in spermatogonia (a cell population including the stem cells) but expression is higher in spermatocytes (the meiotic cells) [15]. Tra2 β protein levels then fall again in round spermatids (the post-meiotic haploid cells). The splicing inclusion of exons like *Nasp-T* and *Tra2a* thus might peak in spermatocytes, which have the highest Tra2 β protein concentration to activate splicing inclusion. When compared between different tissues, total RNA and protein preparations would conceal these intrinsic gradients of Tra2 β protein concentration. Another piece of evidence argues for this kind of differential splicing control within tissues. Tra2 β protein regulates its own level through regulating splicing inclusion of a poison exon into its own mRNA [29], and splicing levels of this poison exon are highest in the testis, possibly also in meiotic cells expressing maximum levels of Tra2 β protein. Muscle also shows high levels of inclusion of this poison exon [15].

(2) Tra2 β might regulate specific exons which are de-repressed in particular tissues or cell types and thus available for activation (Figure 2B)

In this model alternative splicing changes might be driven not directly by changes in Tra2 β protein concentration *per se*, but rather in changes in the concentration of splicing repressors which antagonise Tra2 β . Most exons are under combinatorial control, meaning they are influenced by a combination of both activating and repressive activities of splicing factors [30]. Particular tissue-specific exons which contain Tra2 β protein binding sites might be strongly repressed in some tissues due to high concentrations of these splicing repressors, but more weakly repressed in other tissues or cell types where the expression of these repressors is weaker. In tissues containing lower levels of splicing repressors, Tra2 β might operate as a tissue-specific activator of exons which contain Tra2 β binding sites, even though its actual expression level may not be different compared to the former tissues. For example, hnRNP A1 protein (a splicing repressor) is down-regulated over the course of germ cell development [31], so de-repressed exons which contain Tra2 β binding sites might be co-ordinately activated at this time.

Irrespective of the actual model which explains tissue-specific splicing of individual exons by Tra2 β (referred to as “activator led” or “repressor led” in Figure 2), experimental evidence directly shows multiple adjacent Tra2 β binding sites are important to activate tissue-specific exons (Figure 2) [15]. Since RNA-protein interactions are likely to be dynamic in the nucleus and have both on and off rates, multiple adjacent binding sites might simply act to increase the probability of occupancy by a single Tra2 β protein at any one time to activate exon splicing. It is however difficult to see how mutation of just two binding sites out of a total of ~37 potential sites in *Nasp-T* could explain the ~80% reduction in splicing inclusion using this kinetic probability model [15].

A more likely scenario than the probability model is that adjacent RNA binding sites might act as a platform to assemble multiple Tra2 β proteins into a splicing activator complex [9]. Gel shifts show large protein complexes assembling on these exons *in vitro* [9,15]. For some

exons like *Nasp-T*, a version of Tra2 β unable to directly bind RNA by itself can still co-activate splicing *in cellulo*, possibly by attaching to splicing complexes already nucleated by wild type endogenous Tra2 β protein on the regulated exons [9,15]. Binding site mutagenesis of *Nasp-T* also resulted in large differences in splicing inclusion levels between individual double mutants, indicating that the organisation of available Tra2 β binding sites is also important as well as the number of sites available.

Over-expressed Tra2 β protein might also play a key role in disease

The above studies are starting to address the role of Tra2 β protein in normal development. Tra2 β might have additional roles when it is expressed above its normal cellular concentration, perhaps by enabling it to activate weaker target exons than would be normally regulated. One such important target exon is *SMN2* exon 7.

Splicing of *SMN2* exon 7 was not obviously affected in the *Sfrs10*^{-/-} mice [14] but was activated by over-expression of Tra2 β in transfected cells [32]. Regulation of *SMN2* makes over-expression of Tra2 β a potential therapeutic option to treat the developmental disease Spinal Muscular Atrophy (SMA). SMA is caused by deletion of the *SMN1* gene. Expression of the adjacent *SMN2* gene is normally very low, since exon 7 is poorly spliced into the *SMN2* mRNA, resulting in an unstable protein product. By improving exon 7 splicing, Tra2 β could improve expression from the *SMN2* gene and ameliorate disease in some patients. As well as potentially useful roles in therapy, Tra2 β has also been implicated as a more sinister modifier of other diseases, including breast cancer [33] where over-expression leads to increased inclusion of *CD44* alternative exons associated with metastasis.

Conclusions

Recent research has identified new physiological target RNAs for Tra2 β in development, and revealed it as an important developmental splicing regulator. Gradients in Tra2 β protein concentrations may drive exon inclusion patterns over development leading to the regulated production of developmentally important protein isoforms. Alternatively, Tra2 β might provide a level of intrinsic background activation which is enough to activate available de-repressed exons which contain Tra2 β -binding sites and generate tissue-specific splicing profiles. Future work will continue to elucidate the role of Tra2 β both in splicing regulation, as well as in the metabolism of other kinds of cellular RNA target.

Acknowledgments

This work was supported by the Wellcome Trust (Grant numbers WT080368MA and WT089225/Z/09/Z to DJE), the BBSRC (Grant numbers BB/D013917/1 and BB/I006923/1 to DJE), and the Breast Cancer Campaign (to DJE).

References

1. Stamm S. Signals and their transduction pathways regulating alternative splicing: a new dimension of the human genome. *Hum Mol Genet.* 2002; 11:2409–2416. [PubMed: 12351576]
2. Shepard PJ, Hertel KJ. The SR protein family. *Genome Biol.* 2009; 10:242. [PubMed: 19857271]
3. Long JC, Caceres JF. The SR protein family of splicing factors: master regulators of gene expression. *Biochem J.* 2009; 417:15–27. [PubMed: 19061484]
4. Beil B, Screaton G, Stamm S. Molecular cloning of htra2-beta-1 and htra2-beta-2, two human homologs of tra-2 generated by alternative splicing. *DNA Cell Biol.* 1997; 16:679–690. [PubMed: 9212162]
5. Dauwalder B, Amaya-Manzanares F, Mattox W. A human homologue of the *Drosophila* sex determination factor transformer-2 has conserved splicing regulatory functions. *Proc Natl Acad Sci U S A.* 1996; 93:9004–9009. [PubMed: 8799144]

6. Baker BS. Sex in flies: the splice of life. *Nature*. 1989; 340:521–524. [PubMed: 2505080]
7. Hoshijima K, Inoue K, Higuchi I, Sakamoto H, Shimura Y. Control of doublesex alternative splicing by transformer and transformer-2 in *Drosophila*. *Science*. 1991; 252:833–836. [PubMed: 1902987]
8. Belote JM, Baker BS. The dual functions of a sex determination gene in *Drosophila melanogaster*. *Dev Biol*. 1983; 95:512–517. [PubMed: 6402401]
9. Grellscheid SN, Dalglish C, Rozanska A, Grellscheid D, Bourgeois CF, et al. Molecular design of a splicing switch responsive to the RNA binding protein Tra2 β . *Nucleic Acids Res*. 2011; 39:8092–104. [PubMed: 21724598]
10. Venables JP, Bourgeois CF, Dalglish C, Kister L, Stevenin J, et al. Up-regulation of the ubiquitous alternative splicing factor Tra2 β causes inclusion of a germ cell-specific exon. *Hum Mol Genet*. 2005; 14:2289–2303. [PubMed: 16000324]
11. Tsuda K, Someya T, Kuwasako K, Takahashi M, He F, et al. Structural basis for the dual RNA-recognition modes of human Tra2 β RRM. *Nucleic Acids Res*. 39:1538–53. [PubMed: 20926394]
12. Clery A, Jayne S, Benderska N, Dominguez C, Stamm S, et al. Molecular basis of purine-rich RNA recognition by the human SR-like protein Tra2-beta1. *Nat Struct Mol Biol*. 18:443–50. [PubMed: 21399644]
13. Tacke R, Tohyama M, Ogawa S, Manley JL. Human Tra2 proteins are sequence-specific activators of pre-mRNA splicing. *Cell*. 1998; 93:139–148. [PubMed: 9546399]
14. Mende Y, Jakubik M, Riessland M, Schoenen F, Rossbach K, et al. Deficiency of the splicing factor Sfrs10 results in early embryonic lethality in mice and has no impact on full-length SMN/ Smn splicing. *Hum Mol Genet*. 19:2154–2167. [PubMed: 20190275]
15. Grellscheid S, Dalglish C, Storbeck M, Best A, Liu Y, et al. Identification of evolutionarily conserved exons as regulated targets for the splicing activator tra2 β in development. *PLoS Genet*. 2011; 7:e1002390. [PubMed: 22194695]
16. Gabut M, Chaudhry S, Blencowe BJ. SnapShot: The splicing regulatory machinery. *Cell*. 2008; 133:192–e191. [PubMed: 18394998]
17. Licatalosi DD, Mele A, Fak JJ, Ule J, Kayikci M, et al. HITS-CLIP yields genome-wide insights into brain alternative RNA processing. *Nature*. 2008; 456:464–469. [PubMed: 18978773]
18. Richardson RT, Alekseev OM, Grossman G, Widgren EE, Thresher R, et al. Nuclear autoantigenic sperm protein (NASP), a linker histone chaperone that is required for cell proliferation. *J Biol Chem*. 2006; 281:21526–21534. [PubMed: 16728391]
19. Fujita PA, Rhead B, Zweig AS, Hinrichs AS, Karolchik D, et al. The UCSC Genome Browser database: update 2011. *Nucleic Acids Res*. 39:D876–882. [PubMed: 20959295]
20. Alekseev OM, Richardson RT, O'Rand MG. Linker histones stimulate HSPA2 ATPase activity through NASP binding and inhibit CDC2/Cyclin B1 complex formation during meiosis in the mouse. *Biol Reprod*. 2009; 81:739–748. [PubMed: 19553603]
21. Osakabe A, Tachiwana H, Matsunaga T, Shiga T, Nozawa RS, et al. Nucleosome formation activity of human somatic nuclear autoantigenic sperm protein (sNASP). *J Biol Chem*. 285:11913–11921. [PubMed: 20167597]
22. Richardson RT, Batova IN, Widgren EE, Zheng LX, Whitfield M, et al. Characterization of the histone H1-binding protein, NASP, as a cell cycle-regulated somatic protein. *J Biol Chem*. 2000; 275:30378–30386. [PubMed: 10893414]
23. Lareau LF, Inada M, Green RE, Wengrod JC, Brenner SE. Unproductive splicing of SR genes associated with highly conserved and ultraconserved DNA elements. *Nature*. 2007; 446:926–929. [PubMed: 17361132]
24. Kan Z, Garrett-Engel PW, Johnson JM, Castle JC. Evolutionarily conserved and diverged alternative splicing events show different expression and functional profiles. *Nucleic Acids Res*. 2005; 33:5659–5666. [PubMed: 16195578]
25. Lareau LF, Green RE, Bhatnagar RS, Brenner SE. The evolving roles of alternative splicing. *Curr Opin Struct Biol*. 2004; 14:273–282. [PubMed: 15193306]
26. Sorek R, Shamir R, Ast G. How prevalent is functional alternative splicing in the human genome? *Trends Genet*. 2004; 20:68–71. [PubMed: 14746986]

27. Yeo GW, Van Nostrand E, Holste D, Poggio T, Burge CB. Identification and analysis of alternative splicing events conserved in human and mouse. *Proc Natl Acad Sci U S A*. 2005; 102:2850–2855. [PubMed: 15708978]
28. Keren H, Lev-Maor G, Ast G. Alternative splicing and evolution: diversification, exon definition and function. *Nat Rev Genet*. 11:345–355. [PubMed: 20376054]
29. Stoilov P, Daoud R, Nayler O, Stamm S. Human tra2-beta1 autoregulates its protein concentration by influencing alternative splicing of its pre-mRNA. *Hum Mol Genet*. 2004; 13:509–524. [PubMed: 14709600]
30. Smith CW, Valcarcel J. Alternative pre-mRNA splicing: the logic of combinatorial control. *Trends Biochem Sci*. 2000; 25:381–388. [PubMed: 10916158]
31. Kamma H, Portman DS, Dreyfuss G. Cell type-specific expression of hnRNP proteins. *Exp Cell Res*. 1995; 221:187–196. [PubMed: 7589244]
32. Hofmann Y, Lorson CL, Stamm S, Androphy EJ, Wirth B. Htra2-beta 1 stimulates an exonic splicing enhancer and can restore full-length SMN expression to survival motor neuron 2 (SMN2). *Proc Natl Acad Sci U S A*. 2000; 97:9618–9623. [PubMed: 10931943]
33. Watermann DO, Tang Y, Zur Hausen A, Jager M, Stamm S, et al. Splicing factor Tra2-beta1 is specifically induced in breast cancer and regulates alternative splicing of the CD44 gene. *Cancer Res*. 2006; 66:4774–4780. [PubMed: 16651431]

Figure 1. HITS-CLIP physically identifies multiple Tra2 β binding sites on the *Nasp-T* exon

This screenshot shows the location of *in vivo* binding sites for Tra2 β within a region of the mouse transcriptome corresponding to the *Nasp-T* exon and flanking intron sequences. The position of physical RNA-Tra2 β protein interactions identified by HITS-CLIP are annotated (blue boxes). Clusters of Tra2 β binding sites mapped to the upstream and downstream regions of the *Nasp-T* exon. Additional sites of Tra2 β binding were observed in the upstream constitutive exon. Additional tracks shown are alternative events (highlighting the *Nasp-T* exon, shown in purple); the Affymetrix exon array dataset corresponding to this position in the transcriptome, indicating tissue-specific RNA expression levels (notice high levels of *Nasp-T* exon inclusion are seen in the testis, while the upstream exon has similar levels of inclusion in other tissues including spleen and thymus); and conservation levels in mammals. This screenshot is modified from the UCSC mouse genome browser [19].

Figure 2. Local levels of splicing activation and repression may co-ordinately control tissue-specific *Nasp-T* splicing inclusion

(A) Activator-driven model of alternative splicing inclusion, in which changes in concentration of Tra2 β protein between cells determine the splicing outcome. In the left hand side concentration, a high concentration of Tra2 β binds to the RNA and activates splicing of the target exon (exon splicing). In the right hand concentration, a decreased cellular concentration is not sufficient to activate exon splicing (so the exon is skipped). (B) Repressor-driven model of alternative splicing activation. In this model, tissues might have similar expression levels of Tra2 β but vary in expression of splicing repressor proteins which repress Tra2 β -target exons. In the left hand example, binding of Tra2 β protein to the exon would activate splicing inclusion (no repressor is present). In the right hand example there is a higher concentration of splicing repressor protein present and bound around the target exon. In this case, the same amount of Tra2 β still binds to the target RNA but cannot overcome the repressive effects of locally bound negatively acting splicing repressors (usually hnRNP proteins). As a result of this the exon is skipped.