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The exon sequence TAGG can inhibit splicing

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ABSTRACT

The fibroblast growth factor receptor-2 gene contains a pair of alternative exons, K-SAM and BEK, which are spliced in a cell type specific manner. We have shown previously that a 10 nucleotide sequence within the K-SAM exon exerts a negative effect on K-SAM exon splicing independent of cell type. We demonstrate here that this sequence works autonomously, as it can repress splicing of a heterologous exon, the EIIIb alternative exon of the rat fibronectin gene. By introducing point mutations into the 10 nucleotide sequence, we have shown that the functional portion is limited to 4 nucleotides, TAGG, the dinucleotide AG of which is particularly important. This short sequence may participate in the control of splicing of exons carrying it, provided that they carry weak splice sites.

INTRODUCTION

Different mRNAs can be obtained from a single pre-mRNA by alternative splicing (1,2). Several protein isoforms can thus be obtained from a single gene. Their production can be modulated in a tissue-specific manner by controlling splicing. This can be an important mechanism for controlling gene expression during development. For example, the fibroblast growth factor receptor-2 (FGFR-2) gene contains a pair of mutually exclusive exons, K-SAM (or IIIb) and BEK (or IIIc), whose splicing is controlled in this way (3–6). Epithelial cells splice the K-SAM exon with no detectable splicing of the BEK exon, while some other cell types make the opposite choice (7). The subset of fibroblast growth factor (FGF) family members (there are nine known to date) recognized by the resulting receptor depends on this splicing choice (8,9). Making the right choice is thus important to ensure an appropriate response to the growth factors available in a cell's environment. Indeed, an unprogrammed change in the splicing choice accompanies (and may participate in) malignant transformation in some cases (10).

Splicing is a multi-step process involving many proteins and small nuclear ribonucleoproteins (snRNPs), which assemble on the pre-mRNA to form spliceosomes (11). The exon definition model (12) proposes that an individual exon is recognized by the coordinated binding of splicing factors to its 3′ splice site (for example U2AF and U2 snRNP) and 5′ splice site (for example ASF/SF2 and U1 snRNP). Clearly any molecule which helps or hinders these binding events could control the efficiency of exon splicing. Thus in drosophila sex determination, the female-specific protein sxl binds to the polypyrimidine tract of a male-specific 3′ splice site on the tra pre-mRNA. This prevents binding of U2AF, and use of the male-specific splice site (13). Subsequently, in females, a complex composed of tra, tra-2 and several proteins of the SR family binds to a repeated exonic sequence on the dsx pre-mRNA to activate use of a female-specific 3′ splice site (14).

In mammalian systems, purine-rich exon splicing enhancers have been described (15–18) which function by binding SR proteins. These proteins presumably then help to stabilize splicing factor binding to the exon's splice sites. Intron sequences (often repeats of a short sequence motif) have also been reported to control splicing of nearby exons, although their mechanism of action remains to be determined (19–23). A few exon sequences which repress splicing have been described (24–31), although the mechanism involved is only clear for a very few cases. An inhibitory sequence close to the 3′ splice site of a beta-tropomyosin alternative exon participates in a secondary structure which represses use of the splice site (27,28). Another exon sequence represses splicing of a drosophila P element intron in somatic cells. Here, a protein complex binds together with U1 snRNP to pseudo-5′ splice sites within the exon and stops U1 snRNP binding to the *bona fide* 5′ splice site (29,30).

We have shown previously that the K-SAM exon of the FGFR-2 gene contains a sequence 5′-TAGGGCAGGC-3′ which represses splicing of the exon (31). Deleting this sequence allows efficient splicing of the K-SAM exon in HeLa cells, which normally splice the BEK exon. The sequence also has a negative effect on K-SAM exon splicing in SVK14 cells, which normally splice this exon. In this case the negative effect appears to be out-weighed by positive effects transmitted by downstream intron sequences. Given the dearth of information available concerning negatively acting exon sequences, we decided to undertake further analysis of the K-SAM sequence.

MATERIALS AND METHODS

Plasmid constructions

Minigenes RK3 and RK12 have been described elsewhere (31). Minigenes S10+8nt and S10+50nt were made by ligation of double-stranded oligonucleotides carrying the S10 sequence (5′-TAGGGCAGGC-3′) with appropriate overhangs into the *Ava*I and *Eco*RI sites respectively of RK3CAT. Minigene S10+97nt was made from RK12 by replacing the *Eco*RV–*Sal*I CAT sequence by the sequence 5′-GATATCGGATAGTG**TAGGGCAGGC**ATCGAT-3′ in double-strand form in which the S10 sequence (bold type) is flanked by *Eco*RV and *Cla*I sites. Minigenes containing mutated versions of the S10 sequence were made from minigene S10+97nt by replacing the *Eco*RV–*Cla*I

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fragment with mutated versions using synthetic oligonucleotides. Fibronectin minigenes containing the S10 sequence were made by ligating oligonucleotides containing the S10 sequence with *Bam*HI overhangs into the *Bam*HI site of the EIIIb alternative exon of plasmid ΔGATP3 (23).

RNA analysis

Cells and transfection of cells, harvesting of RNA and RT–PCR analysis were as described previously (31,32). Care was taken to remain within the exponential range of amplification (20 cycles or less of amplification were routinely used; however, the results obtained remained essentially unchanged if 30 cycles of amplification were carried out). In consequence, Southern blotting (31,32) was needed to visualize the PCR products. As demonstrated previously (31), RT–PCR results in our system correlate well with results obtained by Northern blotting. For fibronectin minigenes, PCR primers were as follows: 5′-ATGCCGATCAGAGTTCC-TGC-3′ and 5′-GGCGGTGACATCAGAAGAATCAAAA-3′.

RESULTS

A short K-SAM exon sequence represses splicing autonomously

We have described previously the RK3 minigene in which the alternative exons K-SAM and BEK together with flanking exons C1 and C2 lie between the Rous sarcoma virus (RSV) long terminal repeat and a bovine growth hormone (BGH) polyadenylation signal (31). To study splicing, this minigene is transfected into cells and an RT–PCR protocol with primers P1 in the C1 exon and P2 in the BGH sequence is used to analyse RNA transcribed from the minigene. In principle, three amplified fragments could be obtained: a 0.46 kb fragment with a *Hpa*I site (BEK spliced to C1 and C2); a 0.46 kb fragment with an *Ava*I site (SAM spliced to C1 and C2); and a 0.61 kb fragment with both *Ava*I and *Hpa*I sites (SAM spliced to BEK and C1, BEK spliced to SAM and C2). These fragments can be distinguished by restriction enzyme mapping, and this allows determination of which exons have been spliced. In particular, generation of a 0.20 kb *Ava*I fragment is associated with splicing of the K-SAM exon.

As described previously (31,32), the BEK exon is spliced when the RK3 minigene is transfected into HeLa cells, but when the bulk of the K-SAM exon internal sequences are replaced by bacterial CAT sequences of the same length (Fig. 1A), splicing of the K-SAM/CAT exon (which we shall henceforth refer to as the CAT exon) is activated. The RT–PCR protocol (Fig. 1B) now yields a mixture of 0.46 kb fragments (of which about half have a *Hpa*I site and half an *Ava*I site, and which correspond to BEK and CAT transcripts respectively, Fig. 1C) and 0.61 kb fragments with both *Ava*I and *Hpa*I sites. The latter correspond to CATBEK transcripts (Fig. 1C). Reintroduction of the K-SAM exon S10 sequence (5′-TAGGGCAGGC-3′) into the CAT exon so that its location relative to the 3′ and 5′ splice sites is the same as in the K-SAM exon [97 nucleotides (nt) downstream from the 3′ splice site, S10+97 nt, Fig. 1B] is sufficient to repress markedly splicing of the CAT exon. The RT–PCR protocol detects a 0.46 kb fragment with a *Hpa*I site (Fig. 1B), reflecting splicing of the BEK exon (Fig. 1C).

We wished to determine whether the S10 sequence could act in a position-independent manner, and introduced the S10 sequence into the CAT exon in two different sites. Placed 8 nt downstream from the 3′ splice site, the S10 sequence no longer represses splicing of the CAT exon (Fig. 1B and C, S10+8 nt: compare to CAT). Note that for the S10+8 nt minigene, the *Ava*I site has served for introduction of the S10 sequence and no longer exists. This explains why the 0.61 kb fragment is not cleaved by *Ava*I in this experiment. However, placed 50 nt downstream from the 3′ splice site, the S10 sequence represses splicing of the CAT exon as efficiently as in its ‘normal’ position (Fig. 1 and C, S10+50 nt: compare to S10+97 nt). Some flexibility in the position of the S10 sequence relative to the exon’s splice sites is thus possible.

Encouraged by this result, we set out to test whether the S10 sequence could work in a heterologous exon, and chose to exploit the rat fibronectin gene alternative exon EIIIb (23) in the minigene ΔGATP3 (Fig. 2A). With this minigene, the EIIIb exon

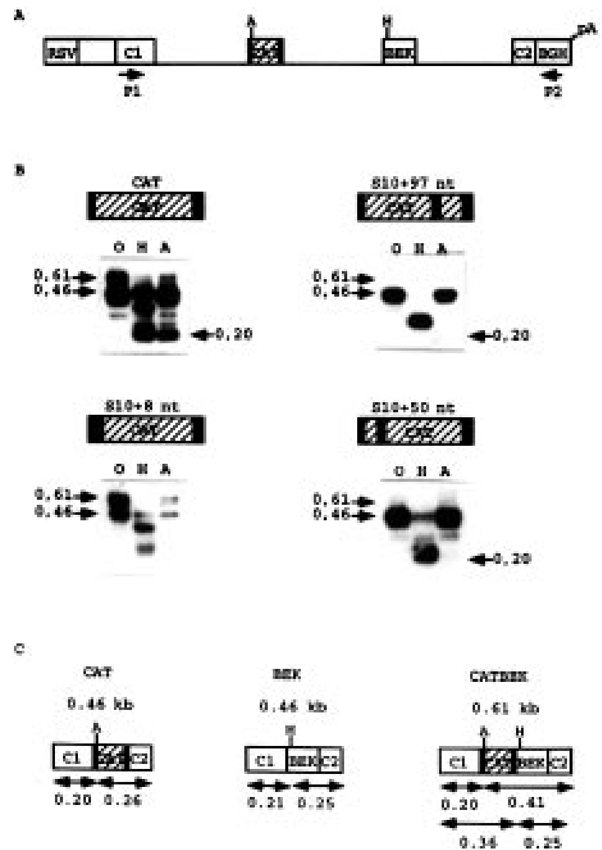

Figure 1. The negative effect on K-SAM exon splicing of the S10 sequence is not strictly position-dependent. (**A**) Schematic representation of the RK12 (31) minigene. RSV, Rous sarcoma virus long terminal repeat promoter; BGH, bovine growth hormone polyadenylation signal; BEK, C1 and C2, BEK exon and flanking exons. Most of the K-SAM internal exon sequences have been replaced by bacterial chloramphenicol acetyl transferase (CAT) gene sequences (diagonal dotted lines). Remaining K-SAM exon sequences are represented in black. A, *Ava*I; H, *Hpa*I; P1 and P2 represent primers used for RT–PCR. (**B**) RK12 and RK12-based minigenes containing the S10 sequence at different locations were transfected into HeLa cells, RNA was harvested, and subjected to RT–PCR using primers P1 and P2. Amplified products were subjected to Southern blotting (31,32) before (0) and after digestion with *Hpa*I (H) or *Ava*I (A). Note that for the S10+8nt minigene, the *Ava*I site has served for introduction of the S10 sequence and no longer exists. No digestion of PCR products by *Ava*I is expected. Sizes of some fragments are indicated in kb. (**C**) Restriction enzyme maps of various amplified products. Sizes of fragments are in kb.

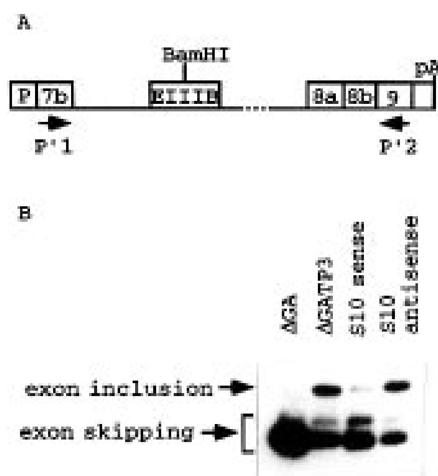

Figure 2. The S10 sequence can repress splicing of a fibronectin gene alternative exon. (**A**) Schematic representation of the ΔGATP3 minigene (23). P represents a human actin gene promoter, pA the polyadenylation signal of a human growth hormone gene. 7b, 8a, 8b and 9 are constitutively spliced exons, EIIIb is the alternative exon. The dotted line represents the TP3 activating sequence. P′1 and P′2 show the locations of primers used for RT–PCR. The S10 sequence was introduced into the *Bam*HI site shown in both possible orientations. (**B**) Minigenes were transfected into HeLa cells, RNA was harvested, and subjected to RT–PCR using primers P′1 and P′2. Amplified products were subjected to Southern blotting (31,32).

is spliced with ~20% efficiency in HeLa cells, thanks to the presence of the TP3 activating sequence (23). Removal of the TP3 sequence (minigene ΔGA) is sufficient to abolish splicing of the EIIIb exon (23). We introduced the S10 sequence in both orientations into the *Bam*HI site of the EIIIb exon in ΔGATP3, placing it 132 nt downstream from the 3′ splice site, and 141 nt upstream from the 5′ splice site (minigenes S10 sense and anti-sense). These minigenes, together with ΔGA and ΔGATP3, were transfected into HeLa cells, and RNA transcribed from them analyzed by an RT–PCR protocol using rat gene-specific primers within flanking exons 7b and 9 (P′1 and P′2, respectively, Fig. 2A). As expected, the EIIIb exon is skipped with ΔGA, and partially included with ΔGATP3 (Fig. 2B). The S10 sequence in the sense orientation (but not in the anti-sense orientation) reduces EIIIb exon inclusion significantly, showing that this sequence can act in a heterologous gene. Indeed, the S10 sequence appears to function autonomously.

TAGG is the active part of the S10 sequence

To determine which part of the sequence 5′-TAGGGCAGGC-3′ is important in repressing splicing, short blocks of the sequence (TAG, GGC or AGGC) were mutated in the S10+97 nt minigene as indicated in Figure 3, before transfection of mutated minigenes into HeLa cells. Mutation of the TAG or GGC blocks lifts repression of CAT exon splicing (presence of 0.61 kb CATBEK fragments and 0.46 kb CAT fragments with *Ava*I sites). The experiment where the AGGC block is mutated identifies 5′-TAGGGC-3′ as sufficient to repress splicing (absence of the 0.61 kb CATBEK fragment, presence of 0.46 kb BEK fragments with *Hpa*I but not *Ava*I sites). Individual nucleotides of the 5′-TAGGGC-3′ sequence were next mutated (we used the minigene where the AGGC block was already mutated to CTTG), and the effect of these mutations on splicing tested. As shown in Figure 3, only mutations which change the nucleotides 5′-TAGG-3′ of the sequence 5′-TAGGGC-3′ abolish repression of CAT exon splicing.

If all four nucleotides of the TAGG sequence are required for complete repression of K-SAM exon splicing, the AG dinucleotide seems to be of particular importance. Mutation of either residue of the dinucleotide leads to production of 0.61 and 0.46 kb fragments, with the majority of the latter fragments having *Ava*I sites. The K-SAM exon is now clearly preferred to the BEK exon. In contrast, mutation of the flanking T leads to production of significantly higher amounts of 0.46 kb fragments with *Hpa*I sites. Thus while this mutation still provokes splicing of the K-SAM exon, the BEK exon is now spliced at least as often. Mutation of the flanking G has an even smaller effect on derepression of the K-SAM exon. It is also interesting to compare the effects of mutating the TAGG sequence to TAAG (Fig. 4) and to TACG (Fig. 3). The former mutation, which retains an AG dinucleotide (although not in the original context), results in less lifting of the repression of the K-SAM exon than the latter mutation (as judged by the relative amounts of 0.46 kb fragments with a *Hpa*I site).

Why does the TAGG sequence repress splicing?

The sequence TAGG contains a stop codon, and also represents part of the 3′ splice site consensus YAGG. Several cases have been described where in-frame stop codons reduce the efficiency of exon splicing (33 and references therein). The TAG of interest here is not an in-frame stop codon for the K-SAM exon. However, as nothing is known about the mechanism which allows stop codons to influence splicing, we decided to test whether the sequence TAGG represses splicing because it contains a stop codon. Minigenes based on S10+97 nt were made in which the TAGG sequence was mutated to TAAG or TGAG, and they were transfected into HeLa cells (Fig. 4). In neither case was CAT exon splicing repressed (we detected 0.61 kb CATBEK fragments and 0.46 kb CAT fragments with *Ava*I sites in both cases). We conclude that the TAG in our sequence does not function exclusively as a stop codon.

Does the exonic TAGG sequence work as a decoy 3′ splice site, turning the attention of the splicing apparatus away from the true 3′ splice site to a non-functional one? It is a better fit to the consensus than the K-SAM exon's 3′ splice site, uagC (intron sequence in lower case letters, exon sequence in upper case letters), and could represent a good competitor. Indeed, when the K-SAM exon's 3′ splice site is mutated to TAGG, the exon sequence TAGG is no longer capable of repressing splicing of the K-SAM exon in HeLa cells (Fig. 4; the RT–PCR analysis detects 0.61 kb CATBEK fragments and 0.46 kb CAT fragments with *Ava*I sites). However, if the TAGG sequence functions simply as a decoy, one would imagine that the sequence CAGG would work at least as well. As shown in Figure 4, this is not the case. If the TAGG sequence competes with the K-SAM exon's 3′ splice site, it must be for a factor which prefers the sequence TAGG.

DISCUSSION

We showed previously that the K-SAM exon S10 sequence TAGGGCAGGC represses splicing of this exon, and also of an artificial CAT exon joined to K-SAM splice sites (31). The question remained open as to whether this sequence works exclusively in the context of a K-SAM exon (perhaps reflecting particular steric

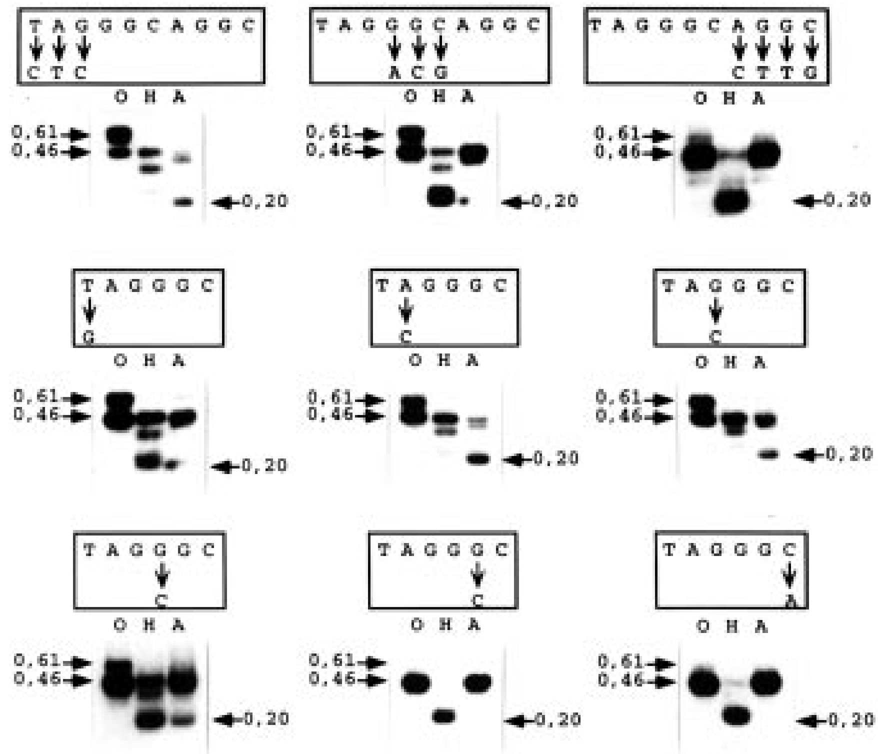

Figure 3. Identification of TAGG as the functional part of the S10 sequence. The S10 portion of the minigene S10+97nt was mutated as indicated by the arrows. The effect of mutations was analysed as in the legend to Figure 1C.

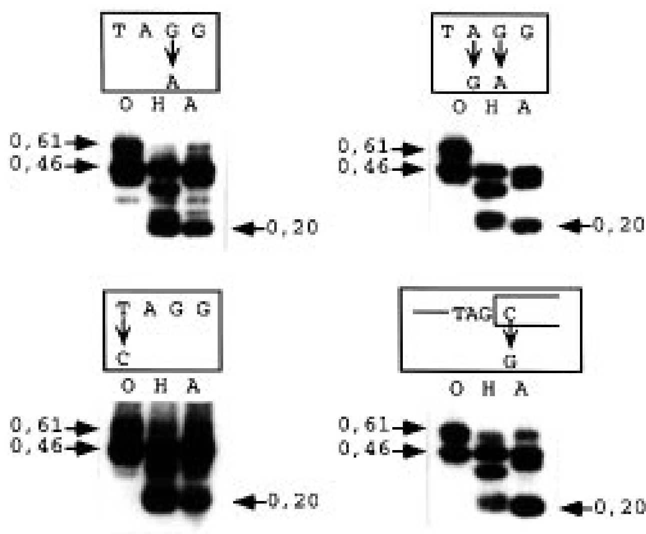

Figure 4. Functional analysis of the TAGG sequence. The S10 portion of the minigene S10+97nt was mutated as indicated by the arrows. The RK3 minigene was mutated to replace the first K-SAM exon base (C) by G. The effect of mutations was analysed as in the legend to Figure 1C.

constraints unique to this exon), or whether it functions autonomously, and represents a phenomenon of more general interest. We show here that the S10 sequence can function to repress splicing of a rat fibronectin gene alternative exon, and thus appears to act autonomously. We also show that the functional part of the S10 sequence is limited to the first four residues, TAGG, of which the AG dinucleotide is of particular importance.

That such a short sequence can repress splicing may seem surprising *a priori*, in as far as it is present in many constitutively spliced exons. However, mutations which bring the K-SAM exon's 3′ or 5′ splice sites closer to the corresponding consensus sequences render the TAGG sequence incapable of repressing splicing of the exon, and introducing the TAGG sequence into a constitutively spliced globin gene exon has no effect on splicing of the exon (our unpublished results). Thus the TAGG sequence is only capable of repressing splicing of exons associated with weak splice sites. This is the case for the rat fibronectin gene EIIIb exon (23), which is inefficiently spliced in many cell types. Indeed, exons undergoing alternative splicing are frequently associated with weak splice sites, and so the sequence TAGG may participate in the control of splicing of many alternative exons which carry it.

Some other alternatively spliced exons are associated with exon inhibitory sequences which contain motifs related to the TAGG sequence. The human fibronectin EDA exon contains the sequence CAAGG (note the AG dinucleotide), deletion of which leads to constitutive splicing of the otherwise partially spliced exon (25). Exon splicing silencers within human immunodeficiency virus type 1 tat exon 2 and tat-rev exon 3 have been narrowed down to a 20 nt stretch which for the tat exon 2 has the sequence <u>AGATCC</u>TA<u>GA</u>C<u>TAG</u>A<u>G</u>CCCT (the underlined residues are identical in the two viral silencers (24). A decoy strategy is used to inhibit splicing of a drosophila P element third intron in somatic cells (29,30). Inhibition requires binding of a multiprotein complex to a 5′ exon inhibitory element, leading to U1 snRNP binding to a

pseudo-5′ splice site (UAAGUAUAG) within the exon rather than to the *bona fide* 5′ splice site. The multiprotein complex contains hrp48 (a protein similar to the mammalian splicing factor hnRNP A1) which binds to specific nucleotides (underlined) in the sequence UAGGUUAAG.

Could the binding of mammalian hnRNP A1 (or a similar protein with overlapping binding specificity) to the TAGG motif be involved in repressing K-SAM exon splicing? If mammalian hnRNP A1 binds with high affinity to the sequence UAGGGA/U, it also binds to related sequences such as the globin gene 5′ and 3′ splice sites with lower affinity (34). Several experiments have suggested a link between high levels of hnRNP A1 and exon skipping (35–37). As with the TAGG motif, hnRNP A1 provokes exon skipping most effectively with short exons or exons which have weak splice sites (36). Perhaps binding of hnRNP A1 to the UAGG motif recruits other proteins or RNPs which hinder splicing by impeding binding of splicing factors to splice sites, or by blocking the dialogue between 5′ and 3′ splice sites needed to define an exon. This could suffice to repress splicing of an exon with weak splice sites.

The TAGG sequence contains a stop codon (out of frame for the K-SAM exon), but our results show that it is the sequence TAG rather than the sequence of a stop codon which is functionally important. Nevertheless, it is interesting to note that mutations which create in-frame stop codons can lead to exon skipping and be involved in human disease. For example, a nonsense mutation (TAT G to TAG G) leads to skipping of exon 51 of the fibrillin gene in cases of Marfan syndrome (38). Mutating *in vitro* TAT G to TAA G or TGA G also leads to exon skipping. However, while moving the TAA and TGA stop codons out of frame by adding two base pairs to exon 50 restores completely splicing of exon 51, mutations which place the TAG stop codon out of frame, but still in the context TAGG, only partially restore exon 51 splicing. This is consistent with the notion that the sequence TAGG exerts a negative effect on splicing. It also suggests that mutations which create in-frame stop codons of the type TAG G will be particularly effective in provoking exon skipping, cumulating the effects of a stop codon with those linked to the creation of a splicing repression sequence.

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