A mechanism for exon skipping caused by nonsense or missense mutations in *BRCA1* and other genes

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Point mutations can generate defective and sometimes harmful proteins. The nonsense-mediated mRNA decay (NMD) pathway minimizes the potential damage caused by nonsense mutations[1–4]. In-frame nonsense codons located at a minimum distance upstream of the last exon-exon junction are recognized as premature termination codons (PTCs), targeting the mRNA for degradation. Some nonsense mutations cause skipping of one or more exons, presumably during pre-mRNA splicing in the nucleus; this phenomenon is termed nonsense-mediated altered splicing (NAS), and its underlying mechanism is unclear[1,2,5,6]. By analyzing NAS in *BRCA1*, we show here that inappropriate exon skipping can be reproduced *in vitro*, and results from disruption of a splicing enhancer in the coding sequence. Enhancers can be disrupted by single nonsense, missense and translationally silent point mutations, without recognition of an open reading frame as such. These results argue against a nuclear reading-frame scanning mechanism for NAS. Coding-region single-nucleotide polymorphisms[7] (cSNPs) within exonic splicing enhancers or silencers may affect the patterns or efficiency of mRNA splicing, which may in turn cause phenotypic variability and variable penetrance of mutations elsewhere in a gene.

Several models have been proposed to explain the link between nonsense mutations and RNA-processing alterations[1–6]. Proposed mechanisms for NMD include scanning of ORFs by a hypothetical nuclear machinery; nonsense-codon recognition during translocation of partially spliced mRNA through nuclear pores; and tagging of exon–exon junctions, immediately after nuclear splicing, by putative factors that are later recognized during the first round of cytoplasmic translation. These proposed mechanisms may also be relevant to NAS, which has additionally been attributed to disruption of sequences or secondary structures involved in exon definition, to instability of the exon-included form of the mRNA due to NMD, or to RNA assay artifacts[1,2,6,8–11]. Besides nonsense mutations, some missense and silent mutations also cause skipping of constitutive exons[6,8,12,13].

Exon sequences comprise *cis*-acting elements that influence the use of flanking splice sites. For example, exonic splicing enhancers (ESEs) are present in constitutive or alternative exons of certain genes, and are required for efficient splicing of those exons[8–14]. The ESEs in pre-mRNAs are recognized by serine/arginine-rich (SR) proteins, a family of essential splicing factors that also regulate alternative splicing[15,16]. Each SR protein recognizes a distinct, albeit degenerate, functional sequence motif under splicing conditions[17–19]. Score matrices for four SR proteins, derived from their functional consensus sequences, were recently used to show that high-score SR protein motifs are enriched in exons, especially in regions corresponding to known, natural ESEs (refs. 17,19).

An amber (TAG) nonsense mutation (Glu1694Ter), consisting of a G→T transversion at position 6 of exon 18 of the breast cancer susceptibility gene *BRCA1*, causes inappropriate skipping of the entire constitutive exon *in vivo*[20]. This mutation was found in a family with eight cases of breast or ovarian cancer, and five other independent cases were reported in the BRCA1 Information Core Database. Skipping of exon 18 results in retention of the same reading frame and removal of 26 amino acids, disrupting the first BRCT (for BRCA1 C terminus) domain of BRCA1 (ref. 20). How nonsense mutations cause exon skipping has been unclear. ESE disruption is one proposed mechanism[2,6,8–10], although inactivation of known ESEs typically requires deletions or multiple substitutions. Because most characterized ESEs are purine-rich, and the Glu1694Ter mutation does not occur in a purine-rich segment, ESE inactivation was not favored as a mechanism[20]. But certain non-purine-rich segments can enhance splicing[17,19,21,22]; therefore, we tested whether this mutation in *BRCA1* inactivates an ESE.

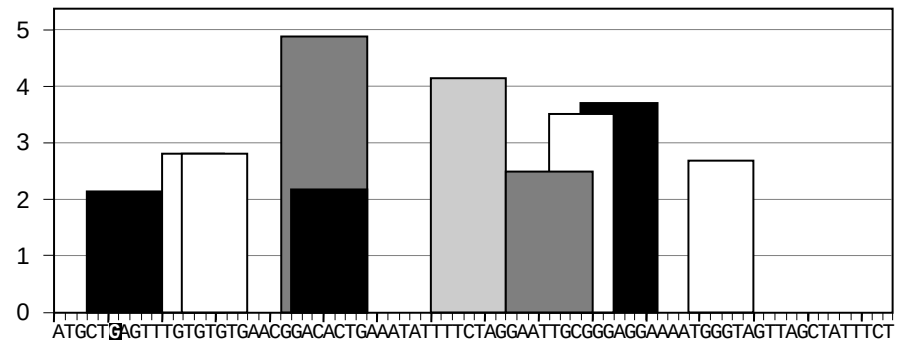

Fig. 1 High-score SR protein motifs in *BRCA1* exon 18. We searched the 78-nt sequence of wild-type exon 18 with four nucleotide-frequency matrices derived from pools of functional enhancer sequences selected *in vitro*[17,19]. Motif scores reflect the extent of matching to a degenerate consensus, and only the scores above the threshold for each SR protein are shown. High-score motifs are shown in black for SF2/ASF, dark gray for SC35, light gray for SRp40 and white for SRp55. The width of each bar reflects the length of the motif (6, 7 or 8 nt); the placement of each bar along the *x* axis, the position of a motif along the wild-type exon DNA sequence; and the height of the bar, the numerical score on the *y* axis. The thresholds and maximal values are different for each SR protein. The G at position 6 (wild type) is highlighted. The nonsense mutation that changes this G to a T only affects the first SF2/ASF motif, reducing the score from 2.143 to 0.079 (below the threshold).

We used SF2/ASF, SC35, SRp40 and SRp55 motif-scoring matrices[17,19] to analyze exon 18 of wild-type and mutant *BRCA1*. Multiple high-score motifs of each type are distributed throughout this exon (Fig. 1). The mutation specifically disrupts the first of three high-

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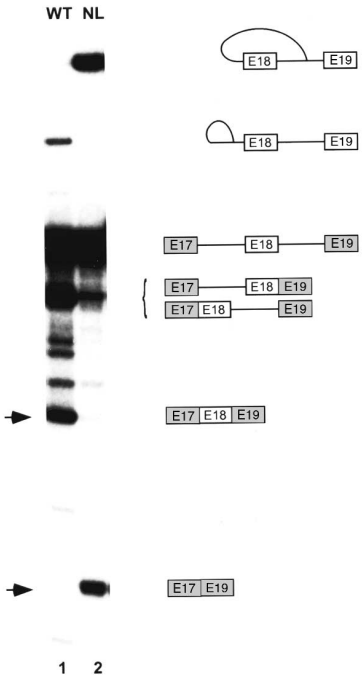


Fig. 2 *In vitro* splicing of *BRCA1* minigene transcripts reproduces the exon-skipping phenotype of a nonsense mutation. We generated wild-type and mutant *BRCA1* minigene transcripts by PCR and *in vitro* transcription. We deleted an internal portion of each intron—away from the splice sites and branch site—to generate pre-mRNAs of adequate length for *in vitro* splicing. We spliced wild-type (WT, lane 1) and nonsense mutant with low SF2/ASF score (NL, lane 2) radiolabeled transcripts in HeLa cell nuclear extract, and analyzed the products of the reaction by denaturing PAGE and autoradiography[23,24]. The identity of each band is indicated schematically on the right. Exons 17 and 19 are shown as gray boxes, exon 18 as a white box, and the shortened introns as lines. The arrows indicate the mRNAs generated by exon 18 inclusion or skipping. Although the extent of exon inclusion and skipping varied with different extract preparations or buffer conditions, the ratio of exon skipping over inclusion was reproducibly greater with the mutant pre-mRNA.

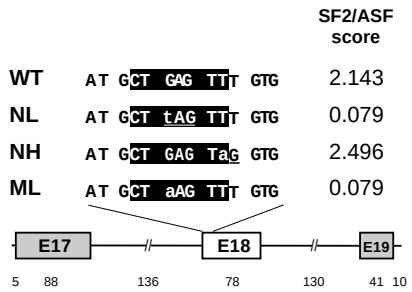
score SF2/ASF motifs. To study the exon-skipping mechanism, we constructed wild-type and mutant minigenes comprising exons 17–19 and shortened introns 17 and 18. Transcripts from these minigenes were spliced *in vitro*[23,24] (Fig. 2). The two pre-mRNAs were spliced in different ways: wild-type exon 18 was efficiently included (lane 1), whereas mutant exon 18 was predominantly skipped (lane 2). The overall RNA recovery was unaffected by the mutation (Fig. 2); hence, differential mRNA stability cannot explain the different splicing patterns. The correlation between the splicing patterns and the SF2/ASF high-score motif distribution suggests that the nonsense mutation disrupted an ESE.

There is no a priori reason why ESE inactivation should result preferentially from in-frame nonsense mutations, as opposed to other types of base substitution. To examine the requirement for a nonsense mutation, we designed two additional *BRCA1* substrates (Fig. 3*a*). One pre-mRNA, dubbed 'ML', has a G→A transition at the same position as the original mutation; this missense mutation also eliminates the high-score SF2/ASF motif. The other pre-mRNA, 'NH', has an amber nonsense mutation in the following codon, but maintains a high-score SF2/ASF motif. We compared splicing of the wild-type and mutant transcripts *in vitro* (Fig. 3*b*). The NH pre-mRNA spliced predominantly by means of exon 18 inclusion, whereas the ML pre-mRNA spliced primarily by means of exon 18 skipping. Therefore, exon inclusion correlates with a high-score SF2/ASF motif, and an in-frame nonsense mutation is neither necessary nor sufficient for exon skipping.

To further investigate the functional significance of the SF2/ASF motifs, we tested four additional high-score heptamers. We replaced the wild-type CTGAGTT sequence with the other two SF2/ASF heptamers in exon 18 (gacAcTg, score 2.164; ggGAGga, score 3.714), a maximum-score heptamer (CacAcga, score 6.589) or an *in vitro*-selected SF2/ASF-dependent ESE motif[17] (gacccgg, score 3.291). The motifs promoted exon 18 inclusion *in vitro* at levels similar to those in wild type; in contrast, a control heptamer (taGAcaa, score below threshold) caused skipping of exon 18 (data not shown). We conclude that an SF2/ASF heptamer recognition sequence is necessary and sufficient for efficient exon 18 inclusion. Moreover, the effectiveness of multiple SF2/ASF motifs with 4–7-base substitutions, compared with the wild-type sequence, indicates that secondary structure disruption[11,25] is not the mechanism of mutation-induced exon skipping in *BRCA1* exon 18.

To determine the general significance of these findings, we examined whether point mutations in other genes also disrupt ESEs. We analyzed a database of 50 single-base substitutions in human genes that cause exon–skipping *in vivo*[6]. We compared the wild-type and mutant sequences of each gene using the four SR protein motif-scoring matrices and threshold values. More than one-half of the single-base substitutions reduced or eliminated at least one high-score motif for one or more of these SR proteins (Table 1). The wild-type set of sequences had a statistically significant excess of high-score motifs compared with the mutant set. Hence, aberrant exon skipping resulting from missense, nonsense or translationally silent single-base substitutions is frequently, if not always, caused by disruption of a critical ESE. Small insertions or deletions within exons should have similar effects.

a



b

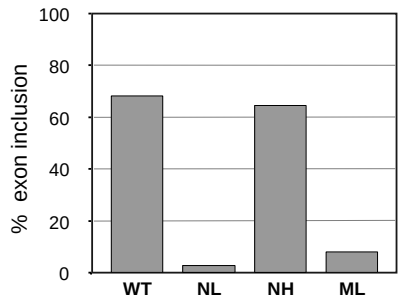


Fig. 3 Exon skipping correlates with the SF2/ASF enhancer motif score and not with reading frame disruption. ***a***, Diagram of the *in vitro*-transcribed portions of wild-type and mutant *BRCA1* minigenes. The relevant portion of the exon 18 sequence is shown above the diagram, beginning at position 1 and with the triplet grouping indicating the reading frame. The heptamer sequence corresponding to the first SF2/ASF motif in Fig. 1 is highlighted. The mutated nucleotides are shown in lower-case, and the in-frame nonsense codons are underlined. WT, wild type; NL, original nonsense mutant with a low SF2/ASF motif score; NH, nonsense mutant with a high score; ML, missense mutant with a low score. The calculated scores for the highlighted heptamers are shown on the right. The sizes of the exons and truncated introns, including 5 nt of T7 sequence and 10 nt of intron 19, are shown below the diagram. ***b***, Quantitation of the relative extent of exon 18 inclusion. We spliced WT, NL, NH and ML pre-mRNAs *in vitro* as in Fig. 2, measured the intensities of the mRNA bands arising from exon 18 inclusion or skipping, and calculated the percent inclusion on a molar basis.

When missense mutations are identified in genomic DNA—especially for routinely sequenced, clinically important genes—the usual assumption is that the affected amino acid is critical for the function of the encoded protein. Moreover, mutation hot spots within exons are presumed to correspond to important functional domains of the protein. If these mutations disrupt an ESE, however, the transcripts may be spliced incorrectly, effectively deleting an entire exon-encoded segment of the protein. Mutations in one exon sometimes cause multiple consecutive exons to be skipped[6]. If the length of the skipped exon(s) is not a multiple of three nucleotides, the frameshift may cause downregulation of the aberrantly spliced mRNA by NMD. Therefore, using genomic sequence to infer the importance of single amino acids may be misleading. To correctly interpret the molecular nature of mutations, splicing patterns should be compared between wild-type and mutant samples, for example, by cDNA sequencing. The SR protein score matrices also provide an indication of putative ESEs that may be disrupted by mutations.

Mutations that affect the intron consensus sequences at the splice and branch sites—sometimes activating cryptic sites—and mutations that create ectopic splice sites account for 15% of point mutations associated with human genetic disease[26]. Additional splicing mutations whose phenotype results primarily or exclusively from ESE disruption may be even more prevalent. Indeed, characterization of numerous mutations in the *NF1* neurofibromatosis gene[27] and the *ATM* ataxia-telangiectasia gene[28] demonstrated that 43–48% of these mutations caused splicing alterations.

Intragenic polymorphisms frequently affect gene function[7]. Because individual exons comprise multiple positive and negative *cis* elements that affect splicing, some exonic SNPs may influence splicing efficiency or accuracy, and therefore gene expression levels. cSNPs within splicing enhancer or silencer elements in a given gene may account for the variable penetrance of particular mutations elsewhere in that gene, and for the variable severity of clinical phenotypes.

Our observations support the notion that ESEs are very prevalent, being present in most, perhaps all, exons, including constitutive exons[17–19,21,22,29,30]. Although some exons have redundant ESEs, and are therefore resistant to point mutations, in cases such as exon 18 of *BRCA1*, single point mutations can disrupt a critical ESE, causing partial or complete inappropriate exon skipping. Thus, the evolution of exon sequences is constrained not only by protein function and codon usage requirements, but also by the presence of signals critical for proper pre-mRNA splicing in the nucleus.

Table 1 • Alteration of enhancer motif scores by point mutations

Gene	Mut.	Sub.	Exon	Type	SF2/ASF	SRp40	SRp55	SC35
CFTR	E60X	G→T	3	N				↓
CFTR	R75X	C→T	3	N			↓	
CFTR	R553X	C→T	11	N				↑
CFTR	W1282X	G→A	20	N	↓↓	↓		
F8	E1987X	G→T	19	N	↓	↓		
F8	R2116X	C→T	22	N			↑	
FANCC	R185X	C→T	6	N	↓	↓↓		
FBN1	Y2113X	T→G	51	N		↓	↓	
HMGCL	E37X	G→T	2	N			↑	
HPRT1	E30X	G→T	2	N	↓		↓↑	
HPRT1	E47X	G→T	3	N		↑		
HPRT1	R51X	C→T	3	N		↓		
HPRT1	C66X	T→A	3	N		↑	↓	
HPRT1	K103X	A→T	3	N	↓		↑	↓
HPRT1	L125X	T→G	4	N		↓		
HPRT1	E197X	G→T	8	N		↑	↓	
HPRT1	Y198X	C→G	8	N		↑↓	↓	
IDUA	Y64X	C→A	2	N			↓	
ATP7A	R645X	C→T	8	N		↓		
NF1	Y2264X	C→A	37	N		↓		
NF1	Y2264X	C→G	37	N		↓		
OAT	W178X	G→A	6	N		↓		↓
PROS1	S62X	C→G	4	N				↑
WAS	Q99X	C→T	3	N	↓			
ADA	A215T	G→A	7	M	↑↑	↑↑		
HEXB	P404L	C→T	11	M	↓		↓	
HPRT1	G40V	G→T	2	M	↑		↑	
HPRT1	R48H	G→A	3	M			↓	
HPRT1	A161E	C→A	6	M	↓	↓↑		↓
HPRT1	P184L	C→T	8	M	↓	↑		↓
HPRT1	D194Y	G→T	8	M		↑		↓
HPRT1	E197K	G→A	8	M			↓	
HPRT1	E197V	A→T	8	M			↑	
FBN1	I2118I	C→T	51	S				↑
HPRT1	F199F	C→T	8	S		↓	↓	
HMBS	R28R	C→G	3	S			↓	↓

Downward arrows denote a reduction or elimination of the motif score as a result of the mutation. Upward arrows denote a higher score in the mutant than in the wild type. Sequence motifs for the same or for a different SR protein can overlap. We considered only the wild-type or mutant sequence motifs with scores greater than or equal to the threshold for the corresponding SR protein. The following 14 mutations, which do not fall within or create high-score motifs for SF2/ASF, SRp40, SRp55 or SC35, are not shown: *ADA* R142X, *DMD* E1211X, *HPRT1* K55X, *HPRT1* G119X, *HPRT1* G180X, *HPRT1* G180E, *HPRT1* G180V, *HPRT1* E182X, *HPRT1* E182K, *HPRT1* D201V, *ATP7A* G1302R, *OAT* W275X, *PDHA1* G185G, *TG* R717X. We found that 36 of the 50 mutations[6] fell within or created 1 or more high-score motifs, and 27 of these mutations reduced or eliminated at least 1 high-score motif. There are over twice as many downward arrows (43) as upward arrows (21), which is a statistically significant difference ($P<0.01$, binomial exact test). The type of mutation is shown: N, nonsense mutation; M, missense mutation; S, synonymous mutation. The exon with the mutation, which is also the exon skipped during splicing, is indicated (column labeled "Exon"). The specific mutations are identified by the wild-type amino acid in the one-letter code, followed by the residue number in the protein sequence and the mutant amino acid (X denotes one of the three nonsense codons) as it would be in the absence of exon skipping (column labeled "Mut."). The base substitution is shown (column labeled "Sub."). Genes and their encoded proteins are as follows: *ADA*, adenosine deaminase; *CFTR*, cystic fibrosis transmembrane conductance regulator; *DMD*, dystrophin; *F8*, coagulation factor VIII; *FANCC*, Fanconi anemia, complementation group C; *FBN1*, fibrillin 1; *HEXB*, hexosaminidase B, β-polypeptide; *HMGCL*, 3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase; *HPRT1*, hypoxanthine phosphoribosyltransferase 1; *IDUA*, α-L-iduronidase; *ATP7A*, ATPase, Cu^{++} transporting, α-polypeptide; *NF1*, neurofibromin 1; *OAT*, ornithine aminotransferase; *HMBS*, hydroxymethylbilane synthase; *PDHA1*, pyruvate dehydrogenase (lipoamide) α1; *PROS1*, protein S-α; *TG*, thyroglobulin; *WAS*, Wiskott-Aldrich syndrome.

Methods

BRCA1 DNA templates. We amplified a portion of the wild-type human *BRCA1* gene using PCR from human genomic DNA (Promega) with primers T7P1 (5′–TAATACGACTCACTATAGGGAGATGCTCGTGTACAAGTTTGC–3′) and P6 (5′–AAGTACTTACCTCATTCAGC–3′). The amplified DNA was then used as a template for three separate PCR amplifications to synthesize intron-truncated DNA fragments: the first PCR amplified exon 17 and the 5′ part of intron 17 using primers T7P1 and P2 (5′–TAAGAAGCTAAAGAGCCTCACTCATGTGGTTTTATGCAGC–3′); the second PCR amplified the 3′ part of intron 17, exon 18, and the 5′ part of intron 18 using primers P3 (5′–TGAGGCTCTTTAG CTTCTTA–3′) and P4 (5′–AGATAGAGAGGTCAGCGATTTGCAA

TTCTGAGGTGTTAAA–3′); the third PCR amplified the 3′ part of intron 18, exon 19 and the first 10 nt of intron 19 using primers P5 (5′–AATCGCTGACCTCTCTATCT–3′) and P6. We then combined the three PCR products and amplified them with primers T7P1 and P6. This overlap-extension PCR generated a *BRCA1* minigene (WT) with shortened introns, but with otherwise natural intronic splicing signals, wild-type exons 17, 18 and 19, and a T7 bacteriophage promoter. We constructed the mutant *BRCA1* minigene NL by overlap-extension PCR with primers T7P1 and P6, using as the template the products of two combined PCR amplifications of WT DNA: the first PCR used primers T7P1 and Pna (5′–CACACACAAACTAAGCATCTGC–3′); the second PCR used primers Pns (5′–GCAGATGCTTAGTTTGTGTGTG–3′) and P6. Similar construction of the mutant *BRCA1* minigenes ML and NH involved replacing primers Pna and Pns with primers Pla (5′–CACACAC AAACTTAGCATCTGC–3′) and Pls (5′–GCAGATGCTAAGTTTGTGT GTG–3′), or primers Pha (5′–CACACACCTACTCAGCATCTGC–3′) and Phs (5′–GCAGATGCTGAGTAGGTGTGTG–3′), respectively.

In vitro transcription and splicing. We uniformly labeled T7 runoff transcripts with ^{32}P-GTP or UTP, purified them by denaturing PAGE, and spliced them in HeLa cell nuclear extracts as described[23,24]. We incubated ^{32}P-labeled, m^{7}G(5′)*P*(5′)G-capped T7 transcripts (20 fmol) in 25-µl splicing reactions containing nuclear extract (5 µl) in buffer D (ref. 24) and $MgCl_2$ (4.8 mM). After incubation at 30 °C for 1 h, we extracted the RNA and analyzed it on 12% denaturing polyacrylamide gels, followed by autoradiography.

High-score motif analysis. We analyzed wild-type or mutant exon sequences from *BRCA1* and from the genes in Table 1 using SR protein score matrices, essentially as described[17,19], except for the use of slightly revised nucleotide frequency matrices and threshold values (unpublished data). For each SR protein, we calculated the highest score for each sequence in a random-sequence pool, and we set the median of these high scores as the threshold value for that SR protein. The threshold values were as follows: SF2/ASF heptamer motif, 1.956; SRp40 heptamer motif, 2.670; SRp55 hexamer motif, 2.676; SC35 octamer motif, 2.383.

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