Regulated Unproductive Splicing and Translation (RUST)

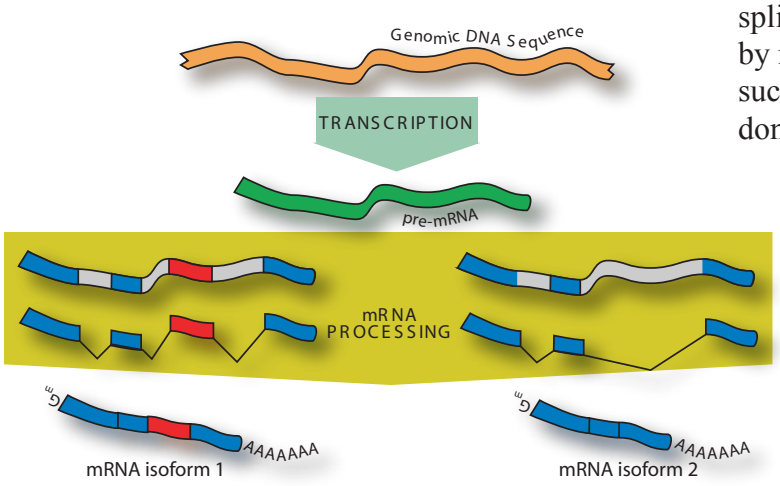
This page is intended to serve as background and complement for the manuscript:

Lewis BP, Green RE, Brenner SE. 2003. Evidence for the widespread coupling of alternative splicing and nonsense-mediated mRNA decay in humans. *Proceedings of the National Academy of Sciences of the United States of America 100*:189-192. [PDF].

Alternative splicing has been shown to affect more than one-third of all human genes. We have found that many alternative isoforms are apparent targets of nonsense-mediated mRNA decay (NMD), an mRNA surveillance system. The coupling of alternative splicing with NMD is intriguing and could provide a general means of regulating gene expression.

Alternative splicing increases mRNA diversity

Alternative splicing is the process whereby a single genetic locus can be transcribed and processed to generate multiple, distinct isoforms[1-3]. Recent reports have shown that more than one third of all human genes may be affected by alternative splicing[4-9]. The presence, absence, abundance and activity of splicing factors can effect which regions of the pre-mRNA will be included in the mature mRNA. How alternative splicing (or splicing in general) is regulated remains poorly understood.

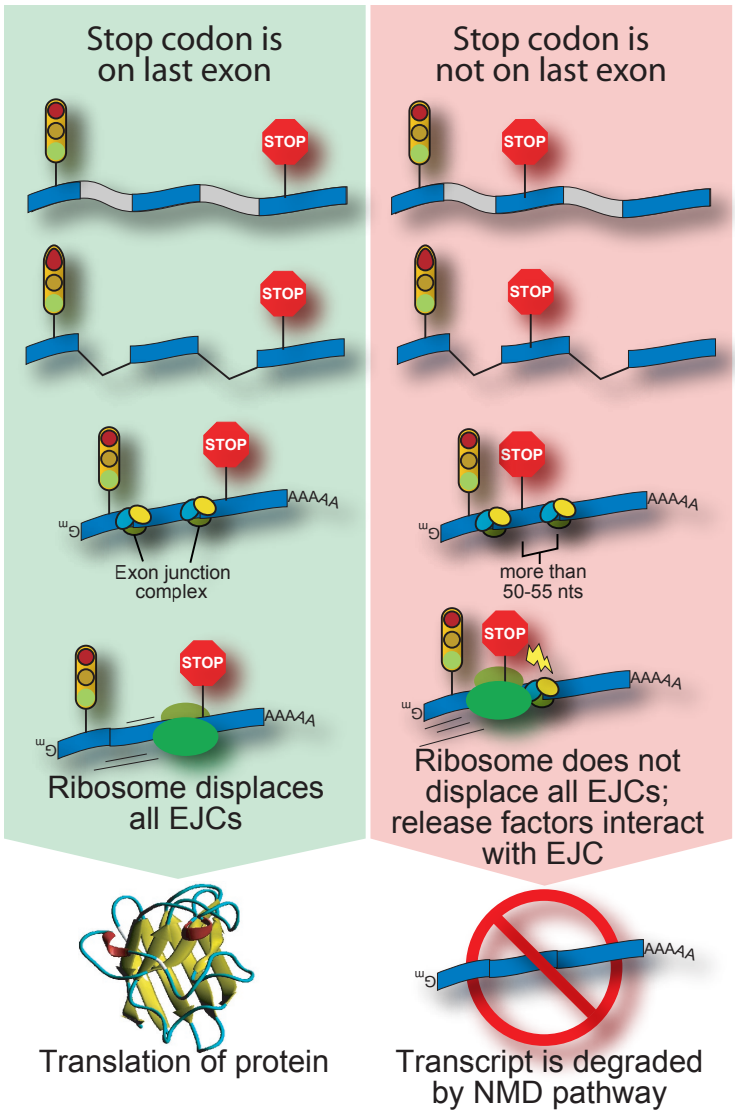


Differences between isoforms of an alternatively spliced gene may be subtle or profound. For example, the human Bcl-x gene can be processed to generate several isoforms with markedly different activities. The Bcl-x(L) isoform inhibits apoptosis, whereas Bcl-x(S) can induce apoptosis[10]. Many other genes are alternatively spliced to produce isoforms whose differences are only in non-coding regions; indeed, a recent study of alternative isoforms in mice showed that 21% of splice variations do not affect coding potential[14, 15]. Because of the prevalence of alternative splicing, researchers would like to know the regulatory mechanisms that control it and the functional consequences of the isoforms that are produced. To these ends, several groups have classified and catagorized known alternative isoforms in terms of changes in gene structure between alternative isoforms or by the functional classes of the genes that are involved[4, 16]. The only general conclusion that can be drawn from these analyses, however, is that alternative splicing affects genes of nearly every functional class by modifying gene structure in every conceivable way, such as using mutually exclusive exons or alternative donor sites.

After mRNA processing, most transcripts are exported to the cytoplasm for translation into protein. Each mRNA transcript can serve as template for repeated translation into protein by ribosomes. The number of protein products produced by any single mRNA can vary widely. This number is a function of, among other things, the life span of the mRNA. In the cytoplasm, mRNAs gradually loose their poly-adenosine tails. Once this tail has been reduced to a threshold length, the mRNA is digested by exonucleases. Specific signal sequences, AREs for example[17], can affect the rate at which the poly-adenosine tail is shortened. Some mRNAs, however, can be degraded almost immediately, by a process that is independent of poly-adenosine tail length.

Nonsense-mediated mRNA decay is an mRNA surveillance mechanism

It has long been known that mRNAs carrying a premature termination codon are highly unstable[18-24]. A process known as nonsense-mediated mRNA decay (NMD) recognizes these mRNAs and degrades them. Recently, the molecular details of this process have begun to be elucidated. During mRNA processing, a complex is deposited near sites of intron removal[25-30]. These exon-junction complexes are important both for facilitating export from the nucleus and for remembering gene structure[31]. That is, they mark the sites where the introns were spliced out. This relative positioning appears to be checked during the pioneering round of translation[32, 33]. The ribosome, as it traverses the mRNA, displaces any exon-junction complexes in its path. Upon arrival at the termination codon, release factors interact with any undisplaced exon-junction complexes[34]. This association triggers decapping of the transcript, followed by degradation[35].



In vertebrates, the location of the last exon-junction complex relative to the termination codon usually determines whether the transcript will be subjected to NMD or not. If the termination codon is downstream of or within about 50 nucleotides of the final exon-junction complex then the transcript is translated normally. However, if the termination codon is further than about 50 nucleotides upstream of any exon-junction complexes, then the transcript is down regulated by NMD.

There are several lines of evidence supporting this model. First, intron-less transcripts appear to be generally immune to NMD[36-38]. Second, tethering any of several components of the exon-junction complex downstream of a termination codon will cause the transcript to be degraded[35]. Finally, NMD is inhibited by *cis*-elements or chemical reagents that prevent efficient translation[23, 39].

This model of NMD has led to increased understanding of the formerly mystifying relationship between genotype and phenotype for many disease genes like dystrophin[40] and beta-globin[41, 42].

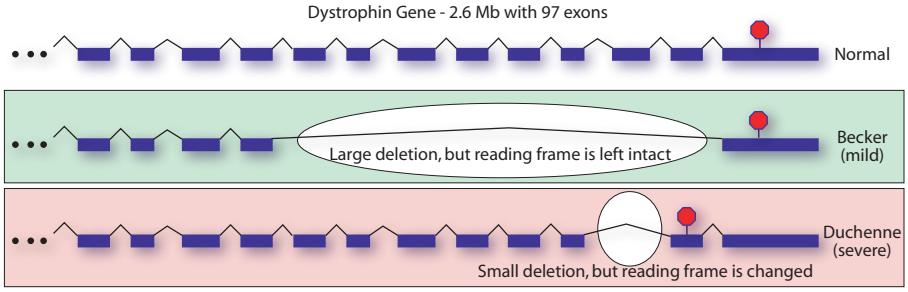
Analysis of the well characterized human genes in RefSeq reveals that the vast majority are not candidates for NMD[16, 49] . This is because their termination codons are on the last exon or within 50 nucleotides of it. This indicates that NMD is pervasive, as there appears to be selective pressure toward keeping the termination codon on the final exon. Start codons, on the other hand are commonly found downstream of the first intron.

The Dystrophin Story

NMD leads to new understanding of Muscular Dystrophy

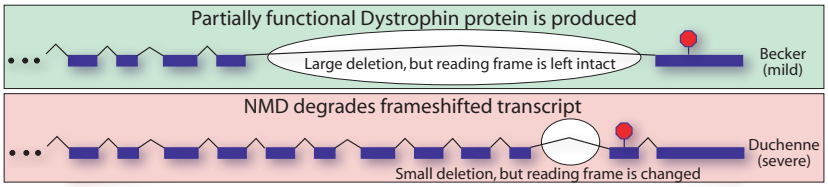
Muscular dystrophy (MD) refers to a group of genetic disorders whose major symptom is muscle wasting. There are two major forms of MD, differing in severity and age of onset. In Duchenne muscular dystrophy, symptoms are noticeable in early childhood and quickly become debilitating. Becker muscular dystrophy, on the other hand, is of later onset and less severe. Both forms of MD are caused by mutations in the dystrophin gene, a large (2.6Mb) gene comprised of 97 exons. The dystrophin protein plays an important structural role as part of a large complex in muscle fiber membranes. When dystrophin is missing or non-functional, the entire complex is compromised, leading to degeneration of muscle tissue. When the ability to regenerate the muscle is exhausted, muscle wasting occurs.

Once it was discovered that Duchenne MD and Becker MD were both forms of the same disease, researchers attempted to determine which regions of the dystrophin gene were most important by correlating genotype and phenotype. It was mystifying that several large deletions were present in patients with the mild, Becker, form, while smaller deletions were sometimes found in patients with Duchenne MD[1-3].



What seemed to be more important than the amount of coding sequence that was deleted was whether or not the offending mutation resulted in a frameshift or not. Researchers working on the mouse model of Duchenne MD, mdx mouse, found a similarly puzzling result: a C-terminally truncated version of dystrophin was competent to rescue the MD phenotype in these mice[4]. In 1988, it was entirely unclear how to explain the apparent disconnect between genotype and phenotype.

In light of NMD, these findings are comprehensible. Becker MD patients have a partially functional version of dystrophin that leads to less severe symptoms. Duchenne MD patients, however, have mutations that prevent any functional dystrophin production. Because of NMD, this includes mutations that introduce premature termination codons.



Appreciation of the role of NMD in MD has led to improved diagnostics and promising new treatment strategies. For example, the drug gentamicin, which causes translational read-through of stop codons, has shown some effectiveness for restoring dystrophin expression in cultured cells from mdx-mice[5]. Human clinical trials are currently underway.

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For more information about Muscular Dystrophy:

Muscular Dystrophy Association USA

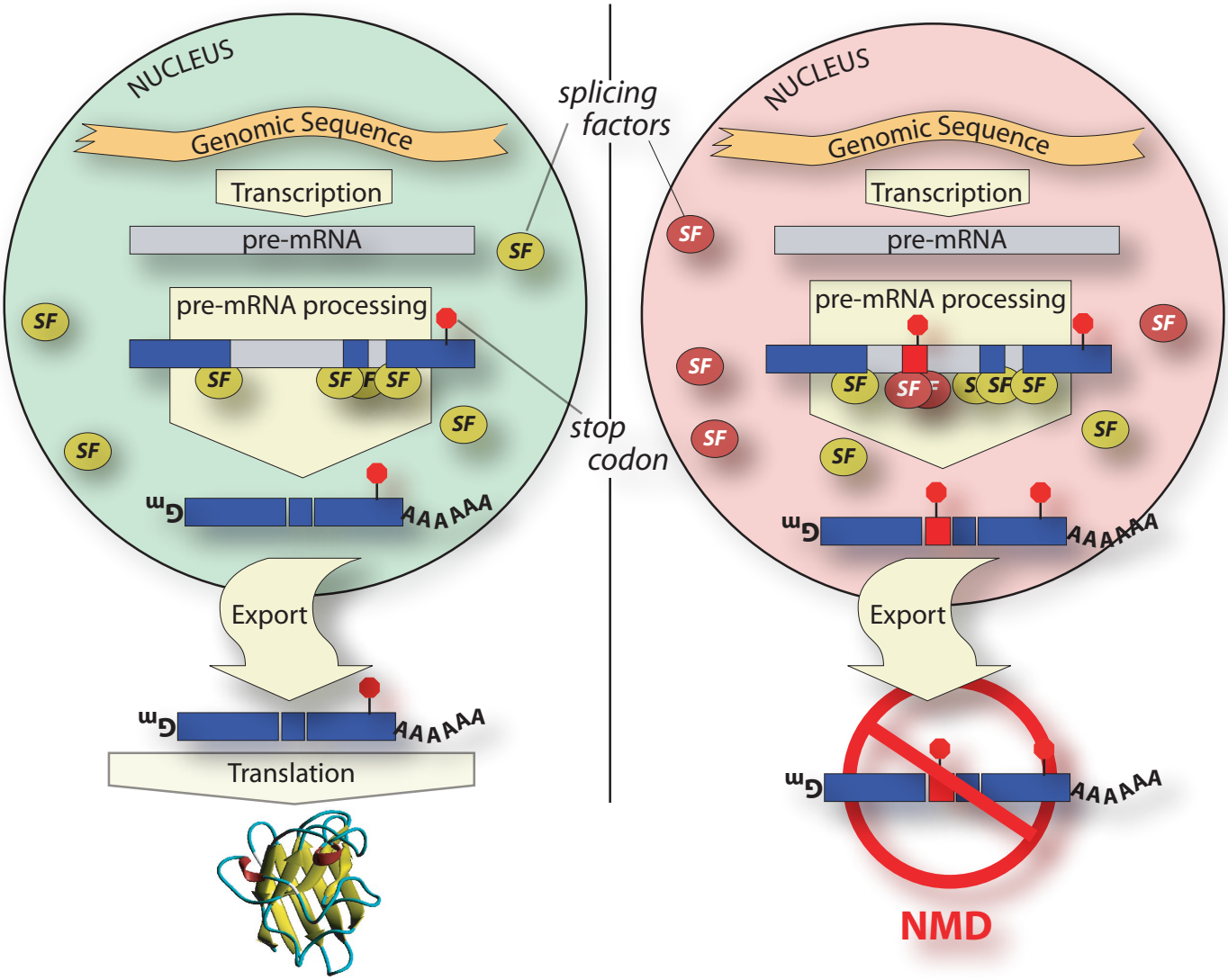
Duchenne Muscular Dystrophy:Research Approaches Toward a Cure (from Testlaboratorium Breitnau)

Alternative splicing can be coupled with NMD to regulate gene expression

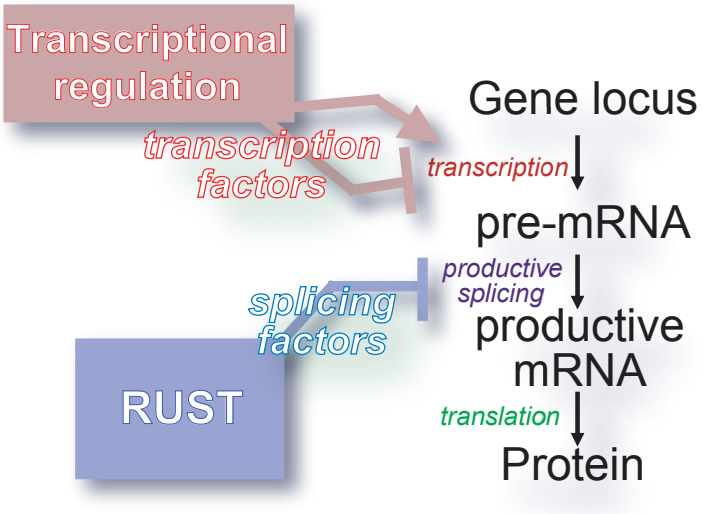
Alternatively spliced genes may have some isoforms that are candidates for NMD and others that are translated normally. By coupling alternative splicing to NMD, a cell could functionally down regulate expression of that gene under desired conditions. In these cases, the protein coding sequence of the alternative isoform is not nearly as important as the fact that its structure will cause it to be degraded by NMD. There are, in fact, cases in which alternative splicing does not affect the coding region at all. It only affects whether the isoform will be down regulated by NMD.

Regulation of this kind, which we term regulated unproductive splicing and translation (RUST), is mediated by the splice environment - the set of splicing factors present and active at a given time and place. Under certain conditions, one set of splice sites could be used that generate an isoform whose stop codon is on the last exon. This productive isoform would then be translated normally. Under different conditions or in a different cell, alternative splice sites could be used that introduce a premature termination codon, generating an unproductive isoform. This can be done by splicing in an alternative exon (as in the figure), causing a frameshift, or splicing out an intron downstream of the normal termination codon. This would shunt the gene from the normally translated pathway into the NMD pathway.

RUST is analogous to transcriptional regulation in that both *cis*-elements and *trans*-factors are involved. Under transcriptional regulation, transcription factors interact with the *cis*-control elements in the regulatory regions of target genes. The concentration, localization, and



activity of transcription factors determine which genes will be transcribed into pre-mRNA. RUST acts during pre-mRNA processing, the next step in gene expression. As with transcriptional regulation, the concentration, localization, and activity of *trans*-factors determines which genes will generate functional end products. In this case, however, the *trans*-factors are splicing factors and the *cis*-elements are the splicing signals present within the pre-mRNAs. Several well characterized signaling pathways have been shown to alter the splice environment by activating splicing factors[50,51]. Furthermore, the *cis*-elements needed for RUST are well conserved in several known RUST genes [52, 53]. In some cases, these are even more conserved than the protein coding sequence.

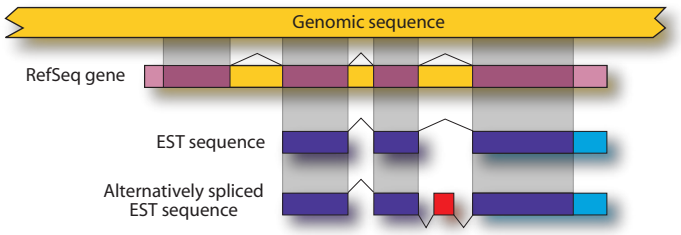


Recent studies by several independent research groups have uncovered genes whose expression appears to be influenced by RUST [13, 46, 47, 48]. One particularly interesting example is the splicing factor, SC35 [11-13], which autoregulates its own expression by coupling alternative splicing with NMD.

ESTs can be used to detect alternative splicing

Although some alternative isoforms are described in RefSeq[43] and other databases, the majority are not. The most comprehensive data sources for alternative splicing are the EST databases, such as dbEST[44]. Several groups have shown that it is possible to cluster EST sequences with one another or with known gene sequence to learn which transcripts are alternatively spliced and what these alternative isoforms look like.

With human genome sequence available, it is then possible to compare these alternative isoforms with their genomic regions to determine their underlying



gene structures. This information can then be used to predict which isoforms of a given gene do not follow the 50 nucleotide rule and are therefore candidates for NMD.

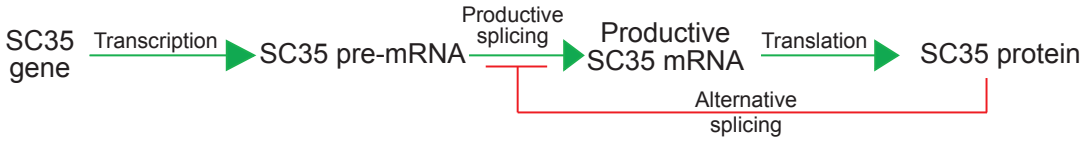
Alternative splicing frequently generates NMD-candidate isoforms

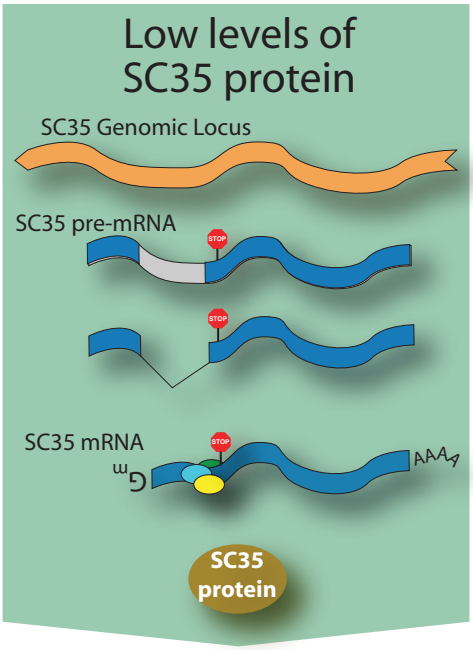
To determine the extent to which alternative splicing generates NMD-candidate isoforms, we aligned RefSeq sequences to the human genome[45] to determine their gene structures. To the coding region of these alignments, we then aligned EST sequences to reveal patterns of alternative splicing. If the EST sequences showed a different splicing pattern than the RefSeq sequence, it was taken as evidence for an alternatively spliced isoform.

Many filters were applied to ensure reliability. For example, we disregarded cases of intron retention as these are indistinguishable from incompletely processed transcripts, a common EST database contaminant. We also restricted alignments to the coding regions of the RefSeq sequences to ensure alignments of the highest quality possible. Because the RefSeq isoforms are annotated with start and stop codon positions, it was then possible to determine which isoforms obeyed the 50 nucleotide NMD rule.

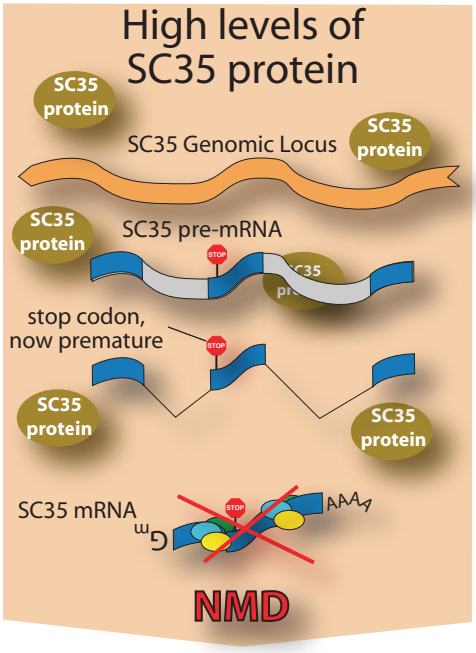
The SC35 Story

SC35 is autoregulated by RUST





SC35 is an RRM-containing SR protein that plays a role in alternative splicing. It was shown recently that SC35 can autoregulate its own expression. This is accomplished via a feedback loop that connects the level of SC35 protein to the productive splicing of the SC35 transcript.



Increased concentrations of SC35 protein change the splice pattern of the SC35 transcript. The SC35 protein binds to its own pre-mRNA, uncovering splice sites downstream of the termination codon. All isoforms of SC35 share the same open reading frame and, therefore, encode the same protein. However, when these alternative splice sites in the 3' untranslated region are used, the normal termination codon becomes a premature termination codon based on the 50 nucleotide rule. NMD then degrades the transcript, down-regulating the expression of SC35. In this way, SC35 appears to auto-regulate its own production, by RUST.

Sureau A, Gattoni R, Dooghe Y, Stevenin J, Soret J.
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EMBO J. 2001 Apr 2;20(7):1785-96.

Fu XD, Maniatis T.
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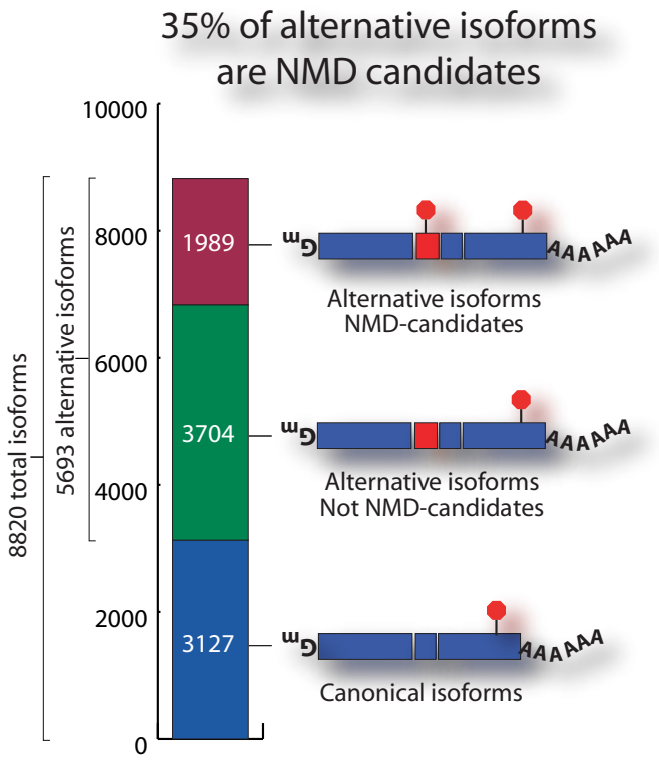
Science. 1992 Apr 24;256(5056):535-8.



We found that about one third of all alternative splicing events generate NMD candidate isoforms. Furthermore, about one third of all genes for which there is alternative splicing EST data generate at least one NMD candidate isoform. These numbers suggest that coupling of alternative splicing and NMD may be widespread. Because our analysis did not consider alternative splicing outside of coding regions and because destabilized transcripts may be under-represented in EST databases, it could be the case that this phenomenon is even more pervasive than our data suggest.

Discussion

Our finding that alternative splicing generates a large number of transcripts that may be destined to be degraded by NMD can be interpreted in several ways. It is possible that EST data, in sum, is of such poor quality that it can not be reliably used for studies such as this one. If this is the case, then the value of EST sequencing projects is called into question, as we found that even the alternative isoforms represented by multiple ESTs generated a large number of NMD-candidates. We discount this conclusion based on the findings of hundreds of independent researchers: EST sequences, when properly screened, can be a reliable resource of expressed gene sequence.

Another possible conclusion is that the process of splicing is not nearly as precise as one might imagine. Perhaps the process of finding and splicing small exons in a sea of large introns is so difficult that the splicing machinery is very error prone. If this is the case, then the splicing process may rely on the presence of the NMD pathway to dispose of incompletely or incorrectly spliced products to an extent not previously appreciated. We cannot presently rule out this possibility. Therefore, it is imperative that researchers who use the EST databases as a source of gene sequence must consider which isoforms are NMD candidates. We feel that this is especially prudent advice, as genes are commonly cloned as intronless cDNAs, immune to NMD, prior to further characterization.

It is also possible, and likely, that there are still gaps in our understanding of the NMD pathway. There are a handful of genes that generate isoforms that should be NMD substrate, based on the 50 nucleotide rule, that appear to be immune to NMD (the male-specific isoform of sex-lethal in drosophila, for example). Also, there are specific signal sequences that appear to be functionally equivalent to exon-junction complexes in triggering NMD. It is likely that there are caveats to the 50 nucleotide rule that, once discovered, can be used to refine our list of NMD-candidate isoforms.

A final, intriguing possibility is that the regulated coupling of alternative splicing and NMD represents a general mode of controlling gene expression. This interpretation is attractive in that it depends only on systems, NMD and alternative splicing, that are known to be pervasive. In the RUST process, splicing factors play a role analogous to transcription factors in that they regulate which genes are expressed. In addition to being attractive just for its ease of use, RUST would allow for a degree of temporal control of very large genes that take a long time to transcribe, that is unachievable with transcription factors. Several instance of RUST have already been discovered, like the splicing factors SC35[13] and AUF1[46].

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