Mechanistic links between nonsense-mediated mRNA decay and pre-mRNA splicing in mammalian cells

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Nonsense-mediated mRNA decay (NMD) generally involves nonsense codon recognition by translating ribosomes at a position ~25 nts upstream of a splicing-generated exon junction complex of proteins. As such, NMD provides a means to degrade abnormal mRNAs that encode potentially deleterious truncated proteins. Additionally, an estimated one-third of naturally occurring, alternatively spliced mRNAs is also targeted for NMD. Given the extraordinary frequency of alternative splicing together with data indicating that naturally occurring transcripts other than alternatively spliced mRNAs are likewise targeted for NMD, it is believed that mammalian cells routinely utilize NMD to achieve proper levels of gene expression.

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Introduction

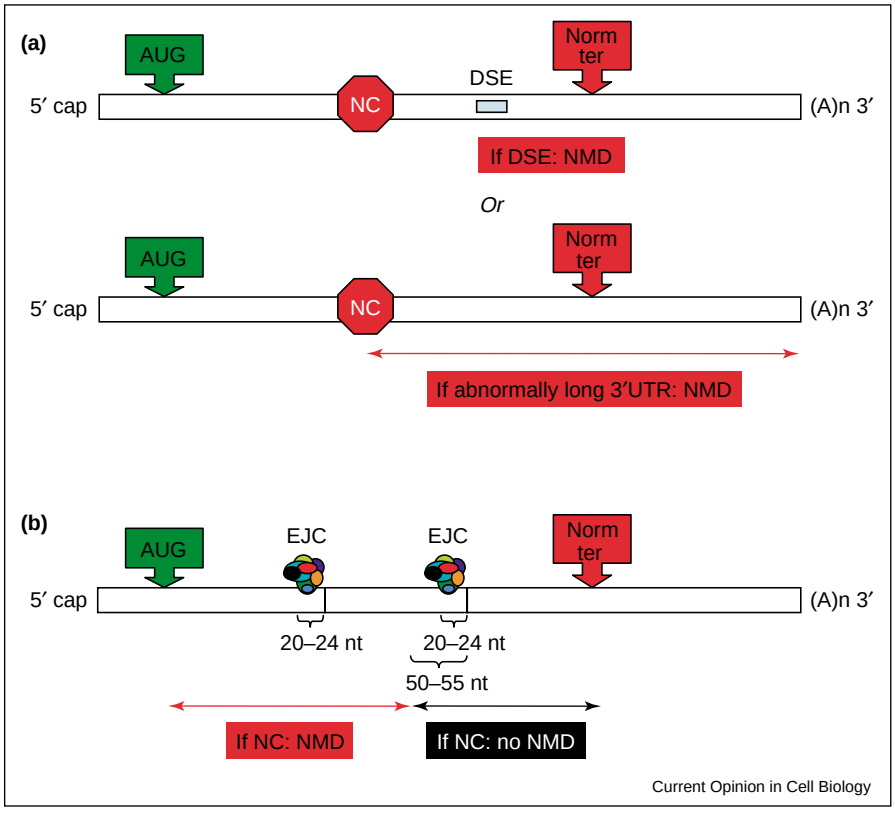
Nonsense-mediated mRNA decay (NMD) is recognized as an important cellular pathway, not only because it functions as a quality control mechanism to eliminate abnormal transcripts, but also because it modulates the levels of a variety of naturally occurring transcripts. In the case of quality control, different organisms have developed different pathways to distinguish between normal and premature termination codons (PTCs). For example, NMD in *Saccharomyces cerevisiae* has been attributed to either an abnormally long 3′ untranslated region (UTR) or a downstream sequence element (DSE) that recruits proteins required for the identification of a nonsense codon as a PTC ([1,2]; Figure 1a). Although *cis*-acting NMD elements in *Caenorabditis elegans*, *Drosophila melanogaster* or plants have yet to be characterized, they may be similar to those in *S. cerevisiae* since, as in yeast, NMD in these organisms can target mRNAs that derive from intronless genes (for a recent review see [3]). By contrast, NMD in mammalian cells generally occurs when a nonsense codon resides >50–55 nucleotides (nts) upstream of a splicing-generated exon–exon junction ([4]; Figure 1b). This junction can derive from the splicing of either U2- or U12-type introns [5]. Thus, although most PTC-containing spliced mRNAs are targeted for NMD, those harboring a PTC either <50–55 nts upstream of the 3′-most exon–exon junction or downstream of this junction are not targeted for NMD (Figure 1b). According to this rule, and the results of database and microarray analyses, an estimated one-third of naturally occurring, alternatively spliced mRNAs should also be targeted for NMD, which has led to the proposal that NMD is widely used by mammalian cells to achieve proper levels of normal gene expression [6•,7••]. This review aims to describe what is currently known about the link between NMD and pre-mRNA splicing in mammalian cells.

Nonsense-mediated decay generally requires an exon junction complex located downstream of a nonsense codon

Consistent with NMD being dependent on pre-mRNA splicing, studies of PTC-containing mRNA that encodes histone H4, heat shock protein 70 [8] or melanocortin 4-receptor [9] indicate that mRNAs from intronless genes are immune to NMD. The dependence of NMD on splicing reflects the need for an exon junction complex (EJC) of proteins downstream of a nonsense codon. This was most directly demonstrated using experiments that recapitulated NMD by artificially tethering any one of the three Upf NMD factors or certain constituents of the EJC downstream of a nonsense codon [10–16]. Nonsense codon recognition occurs after splicing, since mutating an amino-acid-encoding codon that spans two exons to a nonsense codon does not preclude NMD [17].

The EJC consists not only of the Upf NMD factors but also of proteins involved in pre-mRNA splicing, such as RNPS1, UAP56, SRm160 and Pnn/DRS; proteins involved in mRNA export, such as REF/Aly, Y14 and Magoh (reviewed in [18–21]); and proteins with incompletely known functions, such as eIF4AIII, Barentsz/MLN51 and probably PYM [14,15,22–26]. While the best characterized role of the EJC is its function in NMD, it has been also implicated in enhancing translation, but apparently not in mRNA export [27,28]. EJCs are detected only on mRNAs bound by the heteromeric cap binding complex (CBP) 80/20 and not mRNAs bound by eukaryotic initiation factor (eIF) 4E, which replaces CBP80/20 at the cap [29,30]. Consistent with this finding,

Figure 1



Mechanisms by which cells distinguish between nonsense codons that elicit NMD and nonsense codons that do not. **(a)** Nonsense codons (NCs) that elicit NMD in *S. cerevisiae*, and presumably *C. elegans* and *D. melanogaster*, are defined by a downstream *cis*-acting sequence that is arguably either a downstream element (DSE) or an abnormally long 3′UTR. **(b)** Nonsense codons in mammalian cells are recognized after splicing and generally elicit NMD provided there is at least one splicing-generated exon–exon junction >50–55 nts downstream. Dependence on a junction actually reflects dependence on an exon junction complex (EJC) of proteins that is deposited ~20–24 nts upstream of junctions. The EJC consists of at least 12 proteins, including the NMD factors Upf3 or Upf3X and Upf2. Data indicate that Upf1 also comprises the EJC but only transiently and in a way that depends on Upf3 or Upf3X and Upf2. Norm ter, normal termination codon.

NMD appears to be restricted to CBP80/20-bound mRNA as a consequence of nonsense codon recognition during the 'pioneer round' of translation [29–32]. Besides having an association with EJCs, pioneer translation initiation complexes differ from the eIF4E-bound translation initiation complexes in several ways, one of which is insensitivity to the eIF4E inhibitory protein 4E-BP1 [31,32].

Since the identification of the EJC in 2000 [22,33], numerous studies have focused on its assembly and how it connects pre-mRNA splicing to translation and NMD. For example, components of the EJC have been shown to concentrate at sites of transcription within the nuclei of human HeLa and mouse erythroleukemia cells [34•], which suggests that the EJC joins RNA co-transcriptionally. eIF4AIII, which is a member of the eIF4A family of RNA helicases, has been suggested to constitute the anchoring factor of the EJC, a proposal based on its ability to readily crosslink to spliced mRNA in the region of the EJC and on data indicating that related helicases span ~8–10 nts in an RNA-sequence-independent fashion, as does the EJC [15].

While EJC assembly appears to be stepwise, the order of assembly has yet to be detailed. Reichert *et al.* [35] used mass spectrometry to characterize *in vitro*-generated H and C splicing complexes, which are respectively the heterogeneous complexes present on pre-mRNA (or any largely single-stranded RNA) and catalytic complexes that form before exon ligation. Of the EJC components tested, only REF/Aly was detected in H complexes, indicating that only REF/Aly can bind pre-mRNA before splice-site cleavage. REF/Aly, together with Y14, Magoh, RNPS1 and SRm160, was detected in the C complex, suggesting that at least these components of the EJC associate before exon–exon ligation. Using immunoprecipitation and *in vitro* splicing, Kataoka and Dreyfuss [36] detected primarily REF/Aly but also other EJC components bound to pre-mRNA. RNPS1, SRm160 and Upf3X

(also called Upf3b) were found to associate primarily during or immediately after cleavage of exon 1 and intron-lariat formation, and Y14 and Magoh were found to join primarily during or after exon–exon ligation. Studies of the Upf NMD factors indicate that, at least in some cases, Upf3 (also called Upf3a) may functionally substitute for Upf3X. In fact, Upf3 may have a more predominant role in NMD, as suggested by its stronger interaction with Y14 [13]. Upf3X or Upf3 subsequently recruit Upf2, and Upf2 then recruits Upf1 [12,16]. Upf1 has recently been shown to interact with eukaryotic release factor (eRF)1 (F Lejeune and LE Maquat, unpublished) and eRF3 (G Singh and J Lykke-Andersen, personal communication). Considering that an EJC requires an upstream nonsense codon to elicit NMD, these findings suggest that Upf1 may interact with release factors before or simultaneously with EJC-associated Upf2.

Exceptions to the rule that connects nonsense-mediated decay and splicing

In some cases, NMD can occur when the distance between a nonsense codon and a downstream exon–exon junction is <50–55 nts or when splicing does not occur downstream of a nonsense codon. For example, within T-cell receptor (TCR)-β mRNA, PTCs that reside as close as 8–10 nts upstream of the 3′-most exon–exon junction or downstream of this junction can elicit NMD [37,38]. Other examples of what appears to be EJC-independent NMD derive from studies of β-hexosaminidase [39] or β-globin mRNA [40]. β-globin mRNA has been proposed to harbor a failsafe sequence that functionally substitutes for an EJC; however, failsafe sequence function appears to require a splicing-generated exon–exon junction upstream of the PTC, as PTC-containing β-globin mRNA that derives from an intronless gene is immune to NMD [40]. Like an EJC, a failsafe sequence is envisioned to function by recruiting NMD factors, although the mechanism for this is unknown.

β-globin mRNA manifests another unusual feature: PTCs within the first exon that reside close to the initiation codon fail to elicit NMD [41]. Failure is not due to translation reinitiation downstream of a PTC [42], which has been shown to abrogate NMD [43], or to effects of a PTC on β-globin pre-mRNA splicing [42]. While inserting sequences between the β-globin translation initiation codon and a PTC allows NMD to occur [41], proximity of a PTC to the initiation codon does not always preclude NMD, since a PTC located immediately downstream of the initiation codon within triosephosphate isomerase mRNA does elicit NMD [43]. As a final example of an exception to the rule, the editing of apolipoprotein B transcripts, which converts a CAA codon to a UAA codon at a position upstream of three splicing-generated EJCs, fails to result in NMD. Failure is the consequence of a mechanism that depends on formation of the editing complex downstream of the editing site [44].

Pre-mRNA splicing, nonsense codon production and nonsense-mediated decay

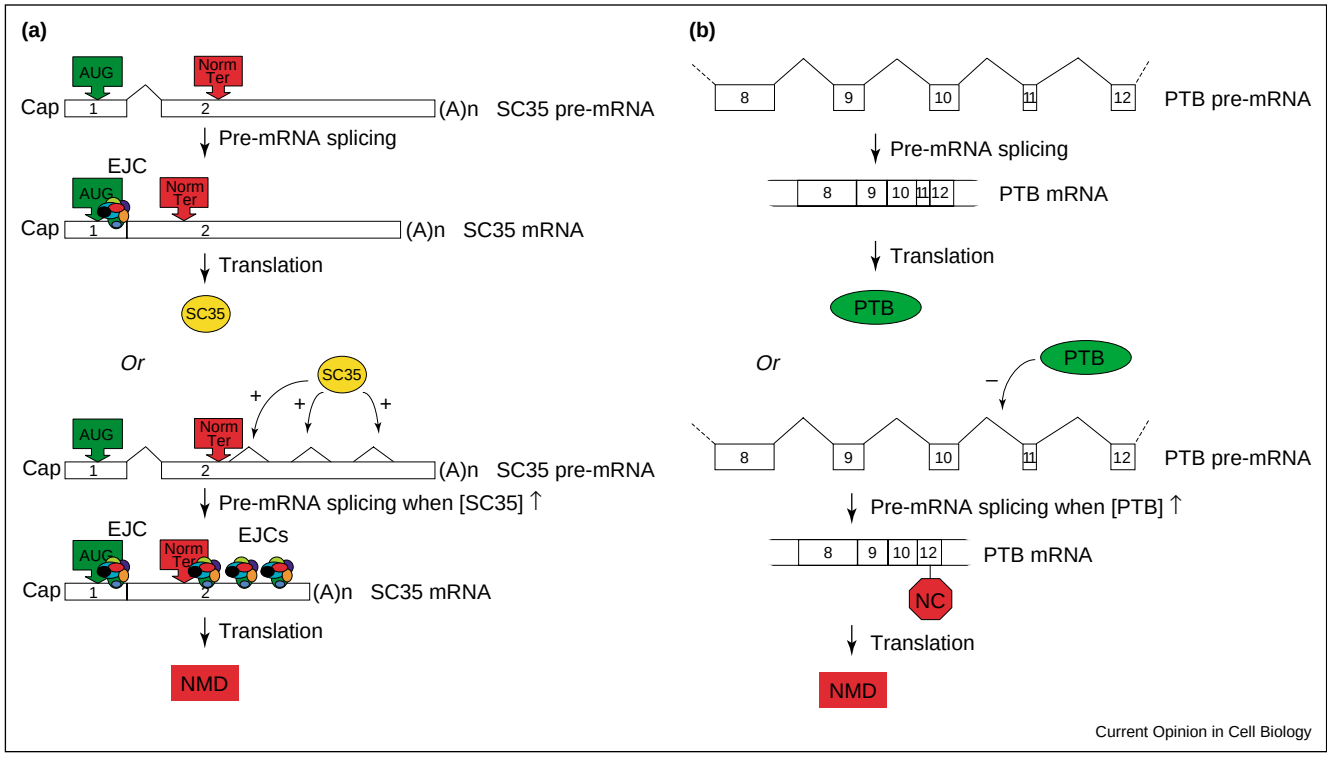
In theory, a nonsense codon could arise by mutation of a gene sequence, an error during transcription, the failure to incorporate selenocysteine at a UGA codon, the presence of an upstream open reading frame (ORF), or the insertion of a transposon or retrovirus. Nonsense codons could also arise as a consequence of alternative splicing or mis-splicing giving rise to an intron-derived nonsense codon or a shift in the ORF that generates a downstream nonsense codon. Results of microarray analyses have demonstrated that transcripts harboring nonsense codons that originated in many of these ways are indeed present at increased levels when NMD is inhibited by the down-regulation of Upf1 [7••].

One highly effective mechanism by which nonsense codons are generated occurs during the somatic cell hypermutations and rearrangements of T-cell receptor and immunoglobulin genes, which serve to generate immune diversity during normal lymphocyte development (reviewed in [45]). PTC-containing genes of this type are very effectively down-regulated by NMD as a result of a poorly characterized down-regulation-promoting element that overlaps with the VDJ exon as well as with parts of the two flanking introns and functions only if a nonsense codon is positioned downstream [46–48].

However, a primary source of nonsense codons that elicit NMD appears be alternative pre-mRNA splicing, which typifies the expression of an estimated 60% of human genes [49,50]. >30% of mRNAs that derive from alternative splicing have been estimated to contain a nonsense codon that elicits NMD [51,52••]. As an example, autoregulation of the SC35 splicing factor involves alternative SC35 pre-mRNA splicing that leads to the NMD-dependent decay of selective SC35 isoforms [53]. An increase in the amount of SC35 protein activates splicing within the 3′UTR of SC35 pre-mRNA, which in turn results in NMD when translation terminates at the normal termination codon as a consequence of the presence of downstream EJCs ([53]; Figure 2a). As another example, an increase in the amount of polypyrimidine tract binding protein (PTB) splicing factor leads to the skipping of exon 11 of PTB pre-mRNA, which in turn induces a frameshift and a PTC within exon 12 ([54•]; Figure 2b).

The remarkable observation that overexpression of serine/arginine-rich (SR) proteins increases the NMD of PTC-containing β-globin and glutathione peroxidase 1 mRNAs has uncovered an additional mechanistic connection between NMD and pre-mRNA splicing [55••]. Interestingly, NMD is activated to different extents by different SR proteins, which presumably reflects the extent and/or position of SR protein binding to the affected mRNA. Furthermore, activation requires the

Figure 2



Examples of gene regulation by NMD. **(a)** The splicing factor SC35 autoregulates by activating splicing events within the 3′UTR of SC35 pre-mRNA. As a consequence, splicing deposits EJCs downstream of the normal termination codon (Norm Ter) that elicit NMD when translation terminates normally. **(b)** The polypyrimidine tract binding protein (PTB) splicing factor autoregulates by inhibiting use of the 3′ splice site of exon 11. This leads to exon 11 skipping, which in turn results in a shift in the ORF that generates a nonsense codon within exon 12. EJCs located downstream of this nonsense codon elicit NMD. Boxes represent exons, and bent lines between boxes represent splicing events. Exon numbers are specified. Norm ter, normal termination codon.

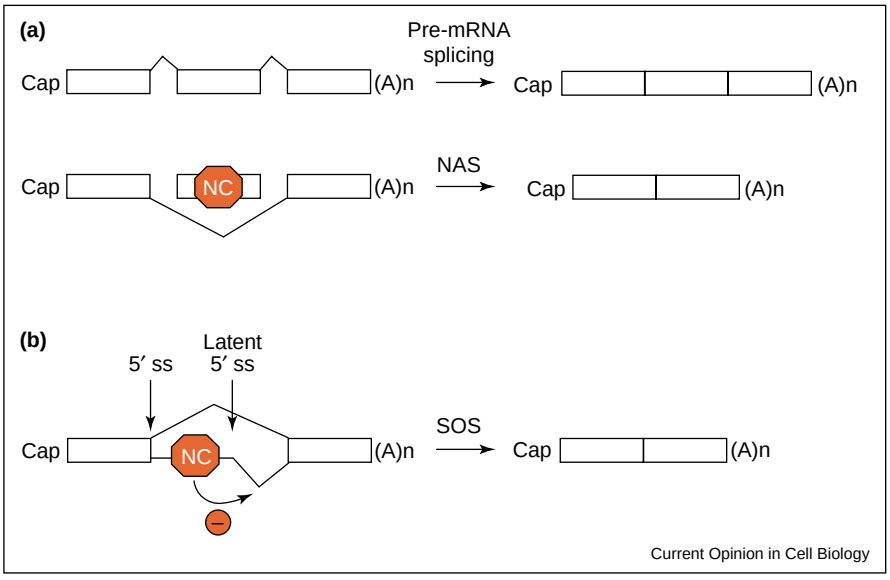
RS domain. These data augment the large number of roles that have been attributed to SR proteins, including roles in constitutive as well as alternative splicing (for a recent review see [56]), mRNA export [57–59] and translation [60]. The process by which SR proteins activate NMD remains to be characterized.

Nonsense-associated altered splicing and suppression of splicing

The presence of certain nonsense codons has been correlated with changes in the choice or efficiency of splice site usage in a controversial process called nonsense-associated altered splicing (NAS). For example, the presence of a nonsense codon within an exon can result in skipping of that exon under certain circumstances that are generally not well described (Figure 3a). Certain data indicate that it is not nonsense codon recognition by translating ribosomes but disruption of a *cis*-acting effector of splicing, such as an exonic splicing enhancer, by the nonsense mutation that elicits the change in splicing [61–63]. However, testing of the enhancer-disruption model by inserting PTCs at various sites that are distant from known enhancer elements within the VDJ exon of the T-cell receptor gene indicated that skipping cannot be a consequence of disrupting a splicing-regulatory element but is instead the consequence of nonsense codon recognition *per se* [38]. It has been proposed that nonsense codon recognition by translating ribosomes can influence splicing by a mechanism that involves Upf1 but not Upf2 [64]. Notably, an association of nonsense codons with changes in the choice or efficiency of splice site utilization is not general [65•,66].

The presence of certain nonsense codons, that is ones within an intron, has also been correlated with splice site choice in another controversial process called suppression of splicing (SOS). More than 90% of human genes surveyed have at least one in-frame nonsense codon between the normal 5′ splice site and an intronic 'latent' 5′ splice site, which according to analyses of product mRNAs are rarely used ([67]; Figure 3b). The finding that removing the nonsense codon activates the latent 5′ splice site in two unrelated transcripts has led to the proposal that nonsense codon recognition before splicing precludes latent 5′ splice site usage so as to preclude inclusion of a nonsense codon within the product mRNA [68].

Figure 3



Nonsense-associated altered splicing (NAS) and suppression of splicing (SOS). **(a)** NAS has been attributed to nonsense codon recognition before splicing. However, recent data indicate that NAS occurs via the effects of a nonsense codon on a *cis*-acting sequence that alters the choice or efficiency of splice site (ss) usage, such as an exonic-splicing enhancer. **(b)** SOS has been described as a translation-independent mechanism that recognizes nonsense codons before splicing. It has been proposed to select for use of the upstream-most of two 5′ splice sites so as to preclude inclusion of an intron-derived nonsense codon. However, recent data indicate that both 5′ splice sites can be utilized, and failure to detect use of the downstream-most site merely reflects NMD that results from use of this site. Boxes represent the exons, and bent lines between boxes represent splicing events.

Countering this proposal, results obtained using different transcripts demonstrated that SOS can, in fact, be attributable to the NMD of transcripts that derive from use of the latent 5′ splice site [55••]. Future studies will undoubtedly aim to resolve the mechanisms of NAS and SOS.

Conclusions

NMD is present in all eukaryotic organisms that have been studied. However, only mammals utilize splicing as a general means to differentiate between nonsense codons that elicit NMD and nonsense codons that do not. Mammals are also unique in having an extraordinarily high number of genes that direct pre-mRNA splicing, and a large fraction of these genes direct more than one splicing pathway. As a consequence, pre-mRNA splicing provides a main source of nonsense codon production either purposely, as a means to regulate gene expression, or accidentally, as a result of inaccurate or inefficient splicing. Therefore, splicing is a process by which many nonsense codons are generated as well as a process that is required for their elimination.

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