PERSPECTIVE

NAS_{ty} effects on fibrillin pre-mRNA splicing: another case of ESE does it, but proposals for translation-dependent splice site choice live on

Lynne E. Maquat¹

Department of Biochemistry and Biophysics, School of Medicine and Dentistry, University of Rochester, Rochester, New York 14642, USA

It is clear that steps in the pathway from gene transcription to protein synthesis are connected mechanistically even when they occur in distinct cellular compartments (Maniatis and Reed 2002). Mechanistic connections were initially evident when factors governing pre-mRNA capping, splicing and 3'-end formation were found to associate with the carboxyl-terminal domain of transcriptionally active RNA polymerase II. More recently, splicing factors have been shown to associate with the transcription elongation factor TAT-SF1, forming a complex that can stimulate both transcription and splicing (Fong and Zhou 2001). Furthermore, a conserved transcription/export (TREX) complex has been shown to couple transcription and mRNA export (Strasser et al. 2002), and pre-mRNA splicing is now known to influence both mRNA export and nonsense-mediated mRNA decay (Reed and Hurt 2002).

The intricate web of connections, in which an earlier step can impinge upon a later step, makes sense as a provision for cells to control the quality of mRNA function (Maquat and Carmichael 2001). Notably, however, studies of fibrillin (FBN1) RNA almost a decade ago were interpreted to suggest an entirely different type of quality control—a type in which splice site choice could be influenced by the translational reading frame in a way that favors translation termination at the normally used stop codon (Dietz et al. 1993; Dietz and Kendzior 1994). This interpretation was not readily explainable from a mechanistic perspective since dogma specified that splicing takes place in the nucleus and targets pre-mRNA while nonsense codon recognition takes place in the cytoplasm during the translation of fully spliced mRNA. As scientists studied other transcripts, a more readily envisioned explanation began to gain audience with growing evidence that nonsense mutations could elicit exon skipping by disrupting exonic splicing en-

hancers (ESEs) rather than the translational reading frame (Valentine 1998; Maquat 2001; Cartegni et al. 2002). Nevertheless, additional proposals that the translational reading frame can alter splicing have been put forth (e.g., see Gersappe et al. 1999; Mühlemann et al. 2001; Li et al. 2002; Wang et al. 2002), and recent data in support of protein synthesis in the nucleus (Iborra et al. 2001; Brogna et al. 2002) has been used to substantiate these proposals.

In this issue of *Genes & Development*, Caputi and co-workers (2002) re-examine the FBN1 transcript and conclude that, indeed, effects on an ESE explain how a nonsense codon can influence splice site choice. However, the possibility that translational reading frame influences splicing lives on in other nonsense-related observations.

NMD and NAS—A tale of two scenarios

It is well established that frameshift and nonsense mutations in mammalian cells can elicit nonsense-mediated mRNA decay (NMD; Fig. 1; Maquat 2002). NMD is dependent on pre-mRNA splicing and mRNA translation. Splicing deposits a complex of proteins ~20–24 nucleotides upstream of exon-exon junctions, and this exon junction complex (EJC) recruits the NMD factors Upf2 and Upf3/3X. If translation terminates more than 50–55 nucleotides upstream of an EJC-bearing junction, then one or more components of the translation termination complex (that presumably include the group I RNA helicase Upf1) interact with junction-bound Upf proteins to elicit NMD. Otherwise, translating ribosomes are thought to remove the EJCs and associated Upf proteins. While the polarity of NMD is currently unknown, data demonstrating that Upf1 immunopurifies with mammalian decapping protein suggest that NMD may be 5' to 3' (F. Lejeune, M. Kiledjian, and L.E. Maquat, unpubl.; J. Lykke-Andersen, pers. comm.).

NMD exemplifies one means by which cells control the quality of mRNA via the web of mechanistic inter-

¹E-MAIL lynne_maquat@urmc.rochester.edu; FAX (585) 271-2683.

Article and publication are at <http://www.genesdev.org/cgi/doi/10.1101/gad.1014502>.

Maquat

connections: Pre-mRNA splicing influences subsequent steps of mRNA translation and decay in a way that helps to ensure that nonsense-containing mRNAs are unstable and, therefore, ineffectively translated. For the majority

of mRNAs, NMD is evident in RNA purified from nuclei (Wilkinson and Shyu 2002). This might reflect nonsense codon recognition in the nucleoplasm, possibly consistent with recent evidence in support of translation at

(Figure 1 legend on facing page.)

sites of transcription (Iborra et al. 2001; Brogna et al. 2002; see below). However, while Upf3/3X and Upf1 are detected in nuclei (J. Mendell and H.C. Dietz, pers. comm.), Upf2 is not. Alternatively, it might reflect translation by cytoplasmic ribosomes during mRNA export. Recent data indicate that ~60% of mRNA that copurifies with mammalian cell nuclei is undergoing nuclear export (Zeng and Cullen 2002), suggesting that there may be opportunity for translation and NMD during export. For the remaining mRNAs, NMD is cytoplasmic and employs cytoplasmic ribosomes (Rajavel and Neufeld 2001). Regardless of the cellular site of NMD, the substrate for NMD has been shown to be fully spliced mRNA that is bound by the cap binding protein (CBP) 80/20 complex at the 5' end, poly(A) binding protein (PABP) 2 at the 3' end, as well as the EJCs bound by Upf3/3X and Upf2 (Belgrader et al. 1994; Ishigaki et al. 2001; Lejeune et al. 2002). These results indicate that NMD takes place after splicing during a "pioneer" round of translation. Once cap-bound CBP80/20 is replaced by eukaryotic initiation factor (eIF) 4E, which is the major cytoplasmic cap-binding protein, the EJCs and associated Upf proteins have also been removed so that the mRNA is immune to NMD (Ishigaki et al. 2001; Lejeune et al. 2002).

It is also established that frameshift and nonsense mutations in mammalian cells can elicit nonsense-associated altered splicing (NAS), including exon skipping, alternative splice site choice and intron retention (Fig. 2; Hentze and Kulozik 1999; Mendell and Dietz 2001; Cartegni et al. 2002). Recent studies of human genetic diseases, at least 15% of which are due to point mutations that affect splicing efficiency or accuracy (for review, see Krawczak et al. 1992; Cooper and Mattox 1997; Valentine 1998), have strengthened the view that NAS, unlike NMD, can be independent of translation. First and foremost, frameshift and nonsense mutations, as well as missense and silent mutations, are associated with alterations in splicing (for review, see Cooper and Mattox 1997; Valentine 1998). Since a significant fraction of these mutations do not coincide with splice junctions or induce cryptic splice sites, and since as many as 30%–60% of the estimated 30,000

human genes encode alternatively spliced transcripts (Brett et al. 2000; Lander et al. 2001; Modrek et al. 2001), it is likely that *cis*-acting effectors of splicing are not confined to canonical splice sites and intronic sequences. In fact, ESEs and exonic splicing silencers (ESSs) are thought to be present in most if not all alternatively as well as constitutively spliced exons (Cartegni et al. 2002). ESEs are discrete but degenerate sequences of approximately six to eight nucleotides that are recognized by effectors of splicing, including the serine- and arginine-rich (SR) protein splicing factors (Blencowe 2000; Graveley 2000). ESSs are less well understood (Fairbrother and Chasin 2000). Driving home the remarkable prevalence of ESEs, at least 10% of hexanucleotides within constitutively spliced exons have been predicted to possess ESE activity based on a multi-pronged approach that employed computational methods followed by assays for function *in vivo* (Fairbrother et al. 2002). NAS can also be attributable to disruption of an RNA secondary structure that either promotes or hinders splicing (Cartegni et al. 2002). Despite the prevalence of *cis*-acting sequences that influence splicing, reports of NAS are much less common than reports of NMD, consistent with the finding that NMD is elicited by the majority of nonsense codons within splicing-derived mRNAs but NAS is particular to only certain nonsense codons (Cartegni et al. 2002). Notably, nonsense codons associated with NAS often alter the use of weak splice sites, and strengthening these sites can eliminate NAS (see below).

Until Caputi et al. (2002), studies of exon 18 of *BRCA1* RNA offered the most detailed support of how nonsense mutations can effect splicing via effects on ESEs (Liu et al. 2001). The 78-nucleotide exon 18 was found to contain nine matches to the four types of ESEs examined, and a G → T nonsense mutation characteristic of six cancer-prone families was found to disrupt the 5'-most of these. Recapitulation of the associated exon 18 skipping using a mini-*BRCA1* gene and HeLa-cell nuclear extract active in splicing allowed for the analysis of different point mutations. A missense mutation predicted to disrupt ESE function caused exon skipping, while a nonsense mutation not predicted to disrupt ESE

Figure 1. Models for nucleus-associated and cytoplasmic NMD. (A) Nucleus-associated NMD. The majority of mammalian mRNAs are subject to NMD while associated with nuclei. Nucleus-associated NMD could take place in the nucleoplasm, in which case it is envisioned to involve nuclear ribosomes, or during mRNA export from the nucleus to the cytoplasm, in which case it could involve cytoplasmic ribosomes. NMD targets fully spliced mRNA and is dependent on an exon junction complex (EJC). The EJC recruits Upf3 or 3X, one of two mostly nuclear shuttling proteins involved in NMD. Upf3/3X then recruits Upf2, another protein involved in NMD that appears concentrated on the cytoplasmic side of the nuclear envelope. Notably, if NMD is nucleoplasmic, then Upf2 must also be nucleoplasmic even though it has only been detected in the cytoplasm. If translation terminates at a nonsense codon located more than 50–55 nucleotides upstream of an EJC-bearing junction (Pre ter), then one or more components of the translation termination complex (that presumably include the group I RNA helicase Upf1) interact with junction-bound Upf proteins to elicit NMD. Unlike Upf3/3X and Upf2, Upf1 is not detected as an mRNP protein but presumably interacts transiently with EJC-bound Upf2 when translation terminates more than 50–55 nucleotides upstream of an exon-exon junction. The polarity of NMD, while not known, is pictured as involving decapping and 5'–3' exonuclease activities (brown symbol) in view of evidence that Upf1 (not shown) immunopurifies with mammalian decapping protein. If translation terminates less than 50–55 nucleotides upstream of an exon-exon junction or downstream from a junction, then translating ribosomes are thought to remove the EJC and associated Upf proteins so that the mRNA becomes immune to NMD. (B) Cytoplasmic NMD. A small fraction of mammalian mRNAs is subject to NMD in the cytoplasm. Currently, there is no discernable mechanistic difference between nucleus-associated and cytoplasmic NMD.

Maquat

(Figure 2. Continued on facing page.)

function did not cause exon skipping. The conclusion that exon skipping correlates with ESE disruption rather than disruption of secondary structure was corroborated by analyzing the effects of replacing the ESE with a heptamer predicted to have ESE function or a heptamer predicted to lack ESE function. Offering additional support for a role of ESEs in NAS, a survey found that more than half of 50 nonsense, missense, and silent mutations known to cause exon skipping of transcripts other than *BRCA1* affect at least one predicted ESE (Valentine 1998).

The FBN1 transcript story pre-2002—translational reading frame effects on splicing

Mutations within FBN1 RNA are the cause of Marfan syndrome, an autosomally inherited connective tissue disorder that some people think afflicted Abraham Lincoln. Characterization of fibroblasts from one affected individual revealed a T → G nonsense mutation at nucleotide position 26 of exon 51 that resulted in exon 51 skipping (Dietz et al. 1993). Since exon 51 consists of 66 nucleotides, exon skipping generates mRNA that uti-

D TCR- β pre-mRNA **E**

Figure 2. Evidence for NAS. (A) Certain nonsense (and missense) mutations within exon 51 of FBN1 transcripts cause exon 51 skipping in a way that is independent of translation but dependent on disruption of a SC35-binding ESE. (ss, splice site). (B) A nonsense mutation within either the NS1/2 or NS2-specific exon of MVM transcripts causes retention of the intervening intron in a way that is abrogated when the adjacent splice site is strengthened. NAS elicited by the nonsense mutation within the NS1/2 exon is dependent on the AUG codon within this exon, while NAS elicited by the nonsense mutation within the NS2-specific exon is not. (C) Nonsense mutations within three different exons of Ig μ transcripts cause an approximately fivefold increase in the level of the two introns tested. (D) Two different nonsense mutations within the VDJ exon of TCR- β transcripts cause a twofold to fivefold increase in the level of the flanking introns in a way that is sensitive to a change in the translational reading frame that places the nonsense mutations out of frame. These same nonsense mutations also augment use of normally weak alternative (Alt) 5' and 3' splice sites so that the nonsense mutations are skipped and translation termination is restored to the normally used site. (E) Naturally occurring "nonsense codons" between a normally used 5' splice site and a downstream intronic splice site within CAD1 transcripts or artificially generated CAD2 transcripts (which harbor a single G deletion within exon N) preclude use of the intronic splice site in a way that is sensitive to mutations that shift the "nonsense codons" out of frame but insensitive to the translational inhibitors cycloheximide or G418.

lizes the proper termination codon. The transient expression of minigenes harboring exon 51 demonstrated that all three possible nonsense codons at position 26, unlike a T $\rightarrow$ C missense codon, induced in-frame exon 51 skipping that was for the most part abrogated when the nonsense codons were placed out of frame by generating a frameshift mutation upstream (Dietz et al. 1993; Dietz and Kendzior 1994). These data were interpreted as evidence for a nuclear mechanism that scans the translational reading frame within pre-mRNA and, if possible, alters pre-mRNA splicing so as to keep the reading frame open. However, the subsequent finding that a silent mutation located at nucleotide position 41 within exon 51 also induced exon skipping (Liu et al. 1997) suggested that positions 26 and 41 of exon 51 could reside within a *cis*-acting effector of splicing and that the missense mu-

tation at position 26, unlike the nonsense mutations at that position, may not disrupt this effector.

The FBN1 transcript story resolved—ESE all over again

Armed with this information, Caputi and co-workers (2002) used fibroblasts from a Marfan syndrome patient who carries the T $\rightarrow$ C nonsense mutation at position 26 to demonstrate that an anisomycin-induced block in translation abrogates NMD, that is, it increases the level of the nonsense-containing FBN1 transcript, but fails to affect the amount of exon 51 skipping. Therefore, while both NMD and exon skipping are dependent on the nonsense mutation, they otherwise operate by different mechanisms. Having demonstrated that the nonsense mutation elicits NMD in patient fibroblasts, Caputi and

co-workers argued that nonsense-containing transcripts produced from the transiently expressed minigene used by Dietz and co-workers (Dietz et al. 1993; Dietz and Kendzior 1994) were also likely to be subject to NMD except when the nonsense codon was placed out of frame. They went on to argue that since RT-PCR had been used to measure the relative levels of exon 51-skipped and exon 51-containing transcripts, the level of exon 51-containing transcripts when the nonsense codon was placed out of frame could have been sufficiently high to preclude detection of exon 51-skipped transcripts. Admittedly, Dietz and Kendzior (1994) had presented data countering this argument. First, exon 51-skipped RNA was not detected in control individuals using a sense primer to this RNA but not to exon 51-containing RNA. Second, exon 51-skipped RNA was detectable even after RNA from a Marfan patient was supplemented with an eightfold excess of RNA from a control individual so that the relative level of non-skipped to skipped RNA should exceed that of patients by eightfold.

Undeterred, Caputi and co-workers (2002) utilized a FBN1 minigene already known to undergo exon 51 skipping when position 26 of the exon harbors either a G or A nonsense mutation (Fig. 2A; Dietz and Kendzior 1994). They found that a nonsense mutation generated within exon 51 either upstream or downstream from position 26 failed to elicit exon 51 skipping while three missense mutations generated in *cis* within the exon downstream from position 26 resulted in complete exon 51 skipping. Providing additional evidence that mutations causing exon 51 skipping disrupted an ESE, wild-type exon 51 sequences were shown to functionally substitute for a *Drosophila melanogaster doublesex* ESE. In contrast, the corresponding sequences that were derived from a Marfan patient or that harbored the three missense mutations did not. The FBN1 ESE was shown to depend on the SR protein SC35. All told, NAS in the case of FBN1 exon 51 is independent of translational reading frame and attributable to disruption of an SC35-dependent ESE.

Can the translational reading frame ever affect splice site choice?

Despite the clarity with which nonsense effects on FBN1 exon 51 can now be viewed, claims that translational reading frame can influence splicing still stand. For example, a nonsense mutation but not a missense mutation within the shared NS 1/2 exon of the minute virus of mice (MVM) has been shown to cause retention of the downstream intron in a way that was dependent on the upstream initiation codon (Fig. 2B; Gersappe et al. 1999). Similarly, a nonsense but not a missense mutation within the NS2-specific exon located downstream from the NS 1/2 exon also caused retention of this intron in a way that was suppressed by mutations that moved the nonsense mutation out of frame (Fig. 2B; Gersappe and Pintel 1999). A priori, these results are consistent with the possibility of reading frame-dependent NAS. How-

ever, it is difficult to imagine how the process of nonsense codon recognition, which is currently known to occur only via translationally active ribosomes, could influence the splicing of an upstream intron. This difficulty can be used to argue for the possibility that splice site usage may be influenced by ESEs that are disrupted by the nonsense mutations but not the missense mutation, as was found for FBN1 transcripts. Also in support of the influence of ESEs, intron retention mediated by either nonsense mutation was abrogated when the adjacent splice site was strengthened, and intron retention mediated by the downstream nonsense mutation within the NS2-specific exon was unaffected when the initiation codon was mutated. Despite arguments that can be made for or against involvement of the translational reading frame or ESEs, the actual mechanism of intron retention within MVM transcripts remains to be resolved.

Other evidence that NAS can be dependent on the translational reading frame derives from studies of the immunoglobulin (*Ig*) μ and T-cell receptor (*TCR*)- β genes (Mühlemann et al. 2001). Nonsense mutations within three different exons of transcripts that derived from the endogenous *Ig* μ gene of B cells resulted in an approximately fivefold increase in the level of the two introns that were analyzed by RNase mapping (Fig. 2C). This increase was evident at sites of DNA transcription as determined by fluorescent in situ hybridization (FISH) in a way that was not attributable to increased rates of transcription as determined by nuclear run-on assays. Notably, the increase was mechanistically distinct from NMD, which typified nonsense mutations in only two of the three exons and reduced the level of mRNA as much as 15- to 45-fold.

Similarly, a nonsense mutation within the VDJ exon of *TCR*- β transcripts derived from minigenes stably introduced into three independently generated HeLa-cell lines resulted in a twofold to fivefold increase in the level of the flanking introns as determined by RNase mapping, consistent with the increase in transcript level at the site of transcription detected by FISH (Fig. 2D). There was an accompanying 25-fold to 60-fold decrease in mRNA abundance as measured by either Northern blotting or RNase mapping. *TCR*- β transcripts harboring either a different nonsense mutation in the VDJ exon or a 10-nucleotide insertion in this exon, which generated a nonsense codon within the following exon, were also characterized by intron accumulation or NMD. In contrast, transcripts harboring either a missense mutation at the place of the nonsense mutation or a nine-nucleotide insertion at the place of the 10-nucleotide insertion were not characterized by intron accumulation and NMD. While, in theory, it could be argued that the two nonsense mutations and the 10-nucleotide insertion disrupted an ESE but the missense and the nine-nucleotide insertion did not, the finding that each of two one-nucleotide deletions generated upstream of the 10-nucleotide insertion reversed the intron accumulation can be used to argue that NAS is due to nonsense-mediated effects on the translational reading frame.

Mühlemann and co-workers (2001) offered two hypo-

thetical explanations of their data. In the first, nonsense codon recognition was proposed to involve cytoplasmic ribosomes, possibly at the nuclear envelope. Recognition would feed back specifically to the splicing machinery that is involved in processing nonsense-containing pre-mRNA either via an undefined *trans*-acting mechanism or an equally undefined physical connection between sites of cytoplasmic translation and nuclear transcription. In the second, nonsense codon recognition was proposed to involve nucleoplasmic ribosomes. Considering that nonsense codons were found to influence splicing of an upstream intron, Mühlemann and co-workers (2001) envisioned a scenario in which the accumulation of nonsense-containing mRNAs near their sites of synthesis might sequester factors required for either pre-mRNA splicing or pre-mRNA decay. For either explanation to be correct, nonsense-mediated effects on splicing must be allele specific since a nonproductively rearranged allele (i.e., one harboring a premature termination codon as a consequence of rearrangement) does not preclude proper expression of a productively rearranged allele. In the end, the authors deferred to future studies for a deeper understanding of their data.

Other data interpreted to indicate that NAS can be dependent on the translational reading frame evolved from a search to explain the observation that more than 90% of pre-mRNAs harbor at least one intronic sequence that conforms to a canonical 5' splice site but, if used, would generate mRNA harboring a nonsense codon (Li et al. 2002). For example, pre-mRNA that encodes carbamoylphosphate synthetase, aspartate transcarbamylase, and dihydroorotase (CAD) harbors an intronic 5' splice site (AG/GTGGGT) that is a better match to a canonical 5' splice site (AG/GTpuAGT) than the upstream 5' splice site (AG/GTGACA) that is generally used (Mirami et al. 1994). The finding that the intronic 5' splice site is activate when cells are heat shocked demonstrates that it can be functional, albeit under special circumstances. Li and co-workers (2002) expressed two CAD minigenes in cultured cells, and using RT-PCR, demonstrated that mutating all in-frame nonsense codons residing between the two 5' splice sites (four in the case of CAD1, and two in the case of CAD2) leads to a form of NAS in which the intronic but not usual 5' splice site is used (Fig. 2E). The authors went on to demonstrate that the failure to detect use of the intronic 5' splice site in the case of minigenes containing the in-frame nonsense codons was not attributable to competition by normally spliced transcripts. First, transcripts that were derived from the use of the intronic 5' splice site were not evident in S1 nuclease transcript mappings or in RT-PCR assays that employed primer pairs specific for these transcripts. Second, these transcripts were not evident when translation was inhibited using cycloheximide, prompting the authors to argue that NMD did not preclude detection of these transcripts. The authors also argued that mutating all in-frame nonsense codons did not activate a splicing enhancer required for use of the intronic 5' splice site, or inhibit a splicing silencer that eliminates use of the usual 5' splice site since, for example, placing

all nonsense codons out of frame activated use of the intronic splice site whereas placing only one of two nonsense codons out of frame did not. Similar results were obtained using an α -L-iduronidase (IDUA) minigene having a single in-frame nonsense codon between the usual 5' splice site and an intronic 5' splice site, including the failure of either of the translation inhibitors cycloheximide or G418 to induce intronic 5' splice site usage. Results obtained using CAD and IDUA transcripts were interpreted as evidence for the coupling of splicing and translational reading frame assessment. The authors proposed that many disease-associated exonic nonsense mutations do not elicit NAS since they reside between a 3' splice site and 5' splice site rather between two 5' splice sites, the latter of which would be somehow be subject to a different process of splice site definition.

The studies of CAD and IDUA RNAs raise a number of perplexing issues, not the least of which is how a nonsense codon downstream from a splice site can influence use of that splice site. Furthermore, if the authors are correct in claiming that effects on translational reading frame are responsible for disuse of the intronic 5' splice site, then the finding that this site was inactive in the case of CAD and IDUA minigene transcripts when translation was inhibited using cycloheximide or G418 leads one to wonder about how the intronic nonsense codons can be recognized in a way that does not involve ribosomes sensitive to cycloheximide and G418. While the authors stop short of proposing that nonsense codon recognition does not involve translation as we know it, such a proposal contorts the imagination.

The newest report of NAS derives from studies of TCR- β transcripts (Wang et al. 2002). Using RNase protections assays, nonsense mutations within the VDJ exon at all four positions tested were found to increase the level of an alternatively spliced transcript (Fig. 2D), whereas missense and silent mutations at each position elicited no increase. The alternatively spliced transcript, which is normally produced at a low level, was generated by the coupled use of a minor 5' splice site and a minor 3' splice site that skipped all four nonsense mutations and restored the site of translation termination to normal. In order to rule out the possibility of effects on ESEs or ESSs, the same frameshift mutations used by Mühlemann et al. (2001) were tested. The 10-nucleotide insertion, unlike the nine-nucleotide insertion or the 10-nucleotide insertion with the compensating one-nucleotide deletion, was also found to increase the level of NAS. Illustrating the dependence of NAS on nonsense codon position, while the 10-nucleotide insertion created a nonsense codon in the downstream exon, a nonsense mutation in this downstream exon did not up-regulate the alternatively spliced transcript. NAS was inhibited by blocks in translation brought about by cycloheximide, a stem-loop structure in the 5'-untranslated region, and a suppressor tRNA that recognizes the nonsense codon as a coding codon. Nonsense codon recognition was shown to take place after splicing since an intron-split nonsense codon (where the first nucleotide resided within the VDJ exon and last two nucleotides

resided in the downstream exons) also elicited NAS. Like Mühlemann et al. (2001), Wang et al. (2002) proposed a mechanism whereby nonsense codon recognition within normally spliced TCR- β mRNA feeds back to increase NAS. As discussed above, such feedback would need to be allele-specific, and it implies entirely new mechanistic connections either between the cytoplasm and nucleus, or within the nucleus, itself. If nonsense codon recognition within mRNA is involved, new studies of TCR- β transcripts indicate that NAS is independent of Upf2 protein and, therefore, NMD (J.T. Mendell, C. ap Rhys, and H.C. Dietz, pers. comm.). Additional studies are required to understand the molecular basis of NAS and to determine the cellular site of nonsense codon recognition if, in fact, nonsense codon recognition plays a role.

There has also been a report that nonsense mutations affect another pre-mRNA processing event: 3' end formation. In the sole example found to date, nonsense codons within transcripts encoding alcohol dehydrogenase in *Drosophila melanogaster* have been associated with the generation of alcohol dehydrogenase pre-mRNA having an abnormally long poly(A) tail (Brognia 1999). Given the mechanistic connections between transcript splicing, 3' end formation and transport (Maniatis and Reed 2002), it will be important to determine if the nonsense mutations alter 3'-end formation indirectly, possibly through effects on splicing or transport, or directly. Other issues to be resolved include determining the generality of nonsense-associated effects on 3'-end formation and if effects are sensitive to the translational reading frame.

In support of nuclear translation?

Proposals that NAS is sensitive to translational reading frame together with numerous examples of nucleus-associated NMD have resurrected an issue that began to intrigue scientists in the 1960s—can reading frame be assessed in the nucleus, or is it exclusively a cytoplasmic event? As noted earlier, if reading frame can be assessed in the nucleus, it is difficult to conceptualize a mechanism distinct from one that more or less involves translation as we know it. In fact, there are currently no mechanistic distinctions between nucleus-associated and cytoplasmic NMD, the latter of which has been shown to involve cytoplasmic translation. Therefore, this discussion will only entertain the possibility of codon recognition in the nucleus by ribosomes. Furthermore, the possibility of nuclear translation in *Saccharomyces cerevisiae* will not be considered given data indicating that the last step of 18S rRNA maturation takes place in the cytoplasm and is required for 60S subunit entry into polysomes (Udem and Warner 1973).

Whether nuclear ribosomes are translationally competent is debatable. In support of translational competency, the nuclei of *Xenopus laevis* oocytes and mammalian cells contain at least some charged tRNAs and aminoacyl-tRNA synthetases (Lund and Dahlberg 1998; Nathanson and Deutscher 2000), and the nuclei of mam-

malian cells and *Drosophila* salivary glands contain at least some translation initiation, elongation and release factors (Strudwick and Borden 2002; Brognia et al. 2002). However, studies of *Xenopus* oocyte nuclei raise issue with the possibility of translational competency; while newly synthesized but mature 40S and 60S ribosomal subunits are detectable in nuclei, 80S ribosomes are not, and mature (i.e., exportable) 60S subunits are associated the export adapter NMD3, which is removed prior to 80S ribosome formation in the cytoplasm (E. Lund and J.E. Dahlberg, pers. comm.). Furthermore, the work that jump-started recent contemplation about the possibility of nuclear translation by demonstrating amino acid incorporation at HeLa-cell nuclear foci in a way that depends on concurrent transcription (Fig. 3; Iborra et al. 2001) is not beyond reproach. The use of permeabilized cells and isolated nuclei in order to allow for the entry of exogenous charged tRNA and tRNA synthetases undoubtedly also allowed for the entry and leakage of other material, raising concern that the nuclei under study did not contain their natural constituents.

Nevertheless, there is now additional support for nuclear translation. Analyses of cultured *Drosophila melanogaster* salivary glands have demonstrated the global presence of components of the translation complex as well as amino acid incorporation at sites of transcription (Fig. 3; Brognia 2002). In situ immunostaining of squashed chromosomes in fixed glands detected 20 ribosomal proteins that comprise either the large or small ribosomal subunit, eukaryotic initiation factor 5B (which is required for subunit joining) and eRF 3 (which is required for translation termination), and in situ hybridization detected 28S rRNA. The ribosomal proteins appeared to be associated with RNA since RNase treatment eliminated or decreased staining. Furthermore, the recruitment of ribosomes and translation factors appeared to be cotranscriptional: Recruitment to either heat shock loci or ecdysone-responsive loci was induced, respectively, by heat shock-induced transcription or ecdysone-induced transcription at a rate that suggests association with nascent transcripts before splicing is completed. Finally, autoradiography detected the incorporation of ^{35}S -labeled amino acids preferentially at sites of transcription in a manner insensitive to thapsigargin, which should inhibit the import of protein synthesized in the cytoplasm, but sensitive to cycloheximide, which freezes translating ribosomes on mRNA. Notably, cycloheximide sensitivity typifies the pioneer round of translation in mammalian cells (Ishigaki et al. 2001). However, puromycin, which releases ribosomes from mRNA, did not affect ribosome recruitment or retention at transcription sites, leading the authors to propose that some nuclear ribosomal subunits may not be active in translation. Additional biochemical and microscopic work will be required in order to fully appreciate the function of chromosome-associated ribosomes.

Another pull for nuclear translation derives from the recent demonstration that a block in mRNA export by either the vesicular stomatitis virus matrix (VSV M) protein or over-expression of the UAP56 pre-mRNA splicing

Figure 3. Model for nuclear translation. Evidence exists for 80S ribosome-mediated protein synthesis in the nucleus at sites of transcription. One or more ribosomes together with initiation and termination factors are thought to associate with nascent transcripts before the completion of splicing. The degree to which ribosomes that localize to sites of transcription are translationally active remains to be determined.

and mRNA export factor fails to inhibit the nucleus-associated NMD of TCR- β mRNA (Bühler et al. 2002). Furthermore, a cycloheximide-induced block in translation after a VSV M protein-induced block in mRNA export abrogated NMD. While all of these data are consistent with the possibility of nuclear translation, the possibility that nonsense codon recognition during cytoplasmic translation feeds back to degrade nuclear mRNA was also raised. However, feedback is difficult to imagine since, in the case of NMD, it would have to be not only allele-specific but also splicing isoform-specific (e.g., see Belgrader et al. 1994). All told, the provocative possibility of nuclear translation is being received with tempered although increasing enthusiasm despite an absence of mechanistic clarity.

Fast forward to the future

At this point, there are a number of examples where NAS has been shown to be due to nonsense-mediated disruptions of exonic sequences that regulate splicing. However, there are also examples put forth where NAS is proposed to be the consequence of nonsense-mediated alterations of the translational reading frame. Future studies of NAS may weave existing threads into an indisputable story that translation termination at specific regions within certain transcripts can influence splice site choice. Whatever the mechanism, data indicate that NAS (i) typifies only certain transcripts, (ii) depends on nonsense codon position as well as splice site strength, (iii) can be provoked by a nonsense codon that does or a nonsense codon that does not elicit NMD, and (iv) must be allele-specific. Nonsense codon recognition is unlikely to take place in the context of pre-mRNA since it is difficult to imagine how a nonsense codon that influ-

ences an upstream intron can be read when residing in *cis* to the intron. It has been proposed that nonsense codon recognition takes place after splicing and subsequently transmits information about the status of termination to the splicing machinery. Such a transmission pathway, while not impossible, is currently without precedent.

If data strengthen the view that translational reading frame can influence splicing, then major issues to be resolved will include the cellular location of reading frame assessment and the mechanism by which reading frame wields influence on splicing. While it is easy to rationalize NMD as a quality control mechanism, it is less easy to find a general purpose for NAS: even when NAS restores the reading frame to normal, which is not always the case, the encoded protein is unlikely to manifest normal function. It has been proposed that NAS has evolutionary significance by generating proteins of diverse function (Dietz and Kendzior 1994). It follows that the NAS of TCR- β and Ig μ transcripts might be viewed as a constructive means to augment the level of immune diversity achieved by somatic gene rearrangement and hypermutation. However, the alternatively spliced TCR- β transcript that is up-regulated by four different nonsense mutations (Wang et al. 2002) is not thought to be functional. For transcripts in general, it may be that NAS, whether sensitive to the translational reading frame or not, is more opportunistic than purposeful.

Acknowledgments

I thank Chris Burge, Jim Dahlberg, Hal Dietz, Jens Lykke-Andersen, Elsebet Lund, Josh Mendell, Oliver Mühlemann, Mike Rosbash, Ann-Bin Shyu, and Jun Wang for sharing results that are unpublished or in press; Karen Beemon, Tom Blumenthal, Arlen Johnson, and Fabrice Lejeune for helpful conversa-

Maquat

tions; and Fabrice Lejeune for generating figures. I also apologize to those whose papers could not be cited because of page limitations. The Maquat lab is supported by Public Health Service Grants DK 33938 and GM 59614 from the National Institutes of Health.

References

- Belgrader, P., Cheng, J., Zou, X., Stephenson, L.S., and Maquat, L.E. 1994. Mammalian nonsense codons can be *cis* effectors of nuclear mRNA half-life. *Mol. Cell Biol.* **14**: 8219–8228.
- Blencowe, B.J. 2000. Exonic splicing enhancers: mechanism of action, diversity and role in human genetic diseases. *Trends Biochem. Sci.* **25**: 106–110.
- Brett, D., Hanke, J., Lehmann, G., Haase, S., Delbruck, S., Krueger, S., Reich, J., and Bork, P. 2000. EST comparison indicates 38% of human mRNAs contain possible alternative splice forms. *FEBS Lett.* **474**: 83–86.
- Brogna, S. 1999. Nonsense mutations in the alcohol dehydrogenase gene of *Drosophila melanogaster* correlate with an abnormal 3' end processing of the corresponding pre-mRNA. *RNA* **5**: 562–573.
- Brogna, S., Sato, T.-A., and Rosbash, M. 2002. Ribosomes are associated with sites of transcription. *Mol. Cell* (In press).
- Bühler, M., Wilkinson, M.F., and Mühlemann, O. 2002. Intracellular degradation of nonsense codon-containing mRNA. *EMBO Rep.* **3**: 646–651.
- Caputi, M., Kendzior, R.J., and Beemon, K.L. 2002. A nonsense mutation in the fibrillin-1 gene of a Marfan syndrome patient induces NMD and disrupts an SC35-dependent splicing enhancer. *Genes & Dev.* (this issue).
- Cartegni, L., Chew, S.L., and Krainer, A.R. 2002. Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nat. Rev. Genet.* **3**: 285–298.
- Cooper, T.A. and Mattox, W. 1997. The regulation of splice-site selection, and its role in human disease. *Am. J. Hum. Genet.* **61**: 259–266.
- Dietz, H.C. and Kendzior, Jr., R.J. 1994. Maintenance of an open reading frame as an additional level of scrutiny during splice site selection. *Nat. Genet.* **8**: 183–188.
- Dietz, H.C., Valle, D., Francomano, C.A., Kendzior, Jr., R.J., Pyeritz, R.E., and Cutting, G.R. 1993. The skipping of constitutive exons in vivo induced by nonsense mutations. *Science* **259**: 680–683.
- Fairbrother, W.G. and Chasin, L.A. 2000. Human genomic sequences that inhibit splicing. *Mol. Cell Biol.* **20**: 6816–6825.
- Fairbrother, W.G., Yeh, R.-F., Sharp, P.A., and Burge, C.B. 2002. Predictive identification of exonic splicing enhancers in human genes. *Science* (In press).
- Fong, Y.W. and Zhou, Q. 2001. Stimulatory effect of splicing factors on transcriptional elongation. *Nature* **414**: 929–933.
- Gersappe, A. and Pintel, D.J. 1999. A premature termination codon interferes with the nuclear function of an exon splicing enhancer in an open reading frame-dependent manner. *Mol. Cell Biol.* **19**: 1640–1650.
- Gersappe, A., Burger, L., and Pintel, D.J. 1999. A premature termination codon in either exon of minute virus of mice P4 promoter-generated pre-mRNA can inhibit nuclear splicing of the intervening intron in an open reading frame-dependent manner. *J. Biol. Chem.* **274**: 22452–22458.
- Graveley, B.R. 2000. Sorting out the complexity of SR protein functions. *RNA* **6**: 1197–1211.
- Hentze, M.W. and Kulozik, A.E. 1999. A perfect message: RNA surveillance and nonsense-mediated decay. *Cell* **96**: 307–310.
- Iborra, F.J., Jackson, D.A., and Cook, P.R. 2001. Coupled transcription and translation within nuclei of mammalian cells. *Science* **293**: 1139–1142.
- Ishigaki, Y., Li, X., Serin, G., and Maquat, L.E. 2001. Evidence for a pioneer round of mRNA translation: mRNAs subject to nonsense-mediated decay in mammalian cells are bound by CBP80 and CBP20. *Cell* **106**: 607–617.
- Krawczak, M., Reiss, J., and Cooper, D.N. 1992. The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. *Hum. Genet.* **90**: 41–54.
- Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W. et al. 2001. Initial sequencing and analysis of the human genome. *Nature* **409**: 860–921.
- Lejeune, F., Ishigaki, Y., Li, X., and Maquat, L.E. 2002. The exon junction complex is detected on CBP80-bound but not eIF4E-bound mRNA in mammalian cells: Dynamics of mRNP remodeling. *EMBO J.* **21**: 3536–3545.
- Li, B., Wachtel, C., Miriami, E., Yahalom, G., Friedlander, G., Sharon, G., Sperling, R., and Sperling, J. 2002. Stop codons affect 5' splice site selection by surveillance of splicing. *Proc. Natl. Acad. Sci.* **99**: 5277–5282.
- Liu, H.X., Cartegni, L., Zhang, M.Q., and Krainer, A.R. 2001. A mechanism for exon skipping caused by nonsense or missense mutations in BRCA1 and other genes. *Nat. Genet.* **27**: 55–58.
- Liu, W., Qian, C., and Francke, U. 1997. Silent mutation induces exon skipping of fibrillin-1 gene in Marfan syndrome. *Nat. Genet.* **16**: 328–329.
- Lund, E. and Dahlberg, J.E. 1998. Proofreading and aminoacylation of tRNAs before export from the nucleus. *Science* **282**: 2082–2085.
- Maniatis, T. and Reed, R. 2002. An extensive network of coupling among gene expression machines. *Nature* **416**: 499–506.
- Maquat, L.E. 2001. The power of point mutations. *Nat. Genet.* **27**: 5–6.
- . 2002. Nonsense-mediated mRNA decay in mammalian cells: from pre-mRNA processing to mRNA translation and degradation. In *Translation Mechanisms*. (eds. J. Lapointe et al.) Landes Bioscience, Georgetown, Texas, (in press). <http://www.eurekah.com/chapter.php?chapid=660&bookid=59&catid=54>
- Maquat, L.E. and Carmichael, G.G. 2001. Quality control of mRNA function. *Cell* **104**: 173–176.
- Mendell, J.T. and Dietz, H.C. 2001. When the message goes awry: Disease-producing mutations that influence mRNA content and performance. *Cell* **107**: 411–414.
- Miriami E., Sperling, J., and Sperling, R. 1994. Heat shock affects 5' splice site selection, cleavage and ligation of CAD pre-mRNA in hamster cells, but not its packaging in InRNP particles. *Nucleic Acids Res.* **22**: 3084–3091.
- Modrek, B., Resch, A., Grasso, C., and Lee, C. 2001. Genome-wide detection of alternative splicing in expressed sequences of human genes. *Nucleic Acids Res.* **29**: 2850–2959.
- Mühlemann, O., Mock-Casagrande, C.S., Wang, J., Li, S., Custodio, N., Carmo-Fonseca, M., Wilkinson, M.F., and Moore, M.J. 2001. Precursor RNAs harboring nonsense codons accumulate near the site of transcription. *Mol. Cell* **8**: 33–43.
- Nathanson, L. and Deutscher, M.P. 2000. Active aminoacyl-tRNA synthetases are present in nuclei as a high molecular weight multienzyme complex. *J. Biol. Chem.* **275**: 31559–31562.
- Rajavel, K.S. and Neufeld, E.F. 2001. Nonsense-mediated decay of human HEXA mRNA. *Mol. Cell Biol.* **21**: 5512–5519.
- Reed, R. and Hurt, E. 2002. A conserved mRNA export machin-

- ery coupled to pre-mRNA splicing. *Cell* **108**: 523–531.
- Strasser, K., Masuda, S., Mason, P., Pfannstiel, J., Oppizzi, M., Rodriguez-Navarro, S., Rondon, A.G., Aguilera, A., Struhl, K., Reed, R., et al. 2002. TREX is a conserved complex coupling transcription with messenger RNA export. *Nature* **417**: 304–308.
- Strudwick, S. and Borden, K.L. 2002. The emerging roles of translation factor eIF4E in the nucleus. *Differentiation* **70**: 10–22.
- Udem, S.A. and Warner, J.R. 1973. The cytoplasmic maturation of a ribosomal precursor ribonucleic acid in yeast. *J. Biol. Chem.* **248**: 1412–1416.
- Valentine, C.R. 1998. The association of nonsense codons with exon skipping. *Mutat. Res.* **411**: 87–117.
- Wang, J., Hamilton, J.I., Carter, M.S., Li, S., and Wilkinson, M.F. 2002. Nonsense codons induce alternatively spliced mRNA by a translation-like mechanism. *Science* **297**: 108–110.
- Wilkinson, M.F. and Shyu, A.-B. 2002. RNA surveillance by nuclear scanning? *Nat. Cell Biol.* **4**: E144–E147.
- Zeng, Y. and Cullen, B.R. 2002. RNA interference in human cells is restricted to the cytoplasm. *RNA* **8**: 855–860.

NASy effects on fibrillin pre-mRNA splicing: another case of ESE does it, but proposals for translation-dependent splice site choice live on

Lynne E. Maquat

Genes Dev. 2002, **16**:

Access the most recent version at doi:[10.1101/gad.1014502](https://doi.org/10.1101/gad.1014502)

References

This article cites 41 articles, 16 of which can be accessed free at:
<http://genesdev.cshlp.org/content/16/14/1743.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).