Nonsense mutations create premature termination codons (PTCs), leading to the generation of truncated proteins, some of which have deleterious gain-of-function or dominant-negative activity. Protecting cells from such aberrant proteins is nonsense-mediated decay (NMD), an RNA surveillance pathway that degrades transcripts harboring PTCs. A second response to nonsense mutations is the up-regulation of alternatively spliced transcripts that skip the PTC. This nonsense-associated altered splicing (NAS) response has the potential to rescue protein function, but the mechanism by which it is triggered has been controversial. Some studies suggest that, like NMD, NAS is triggered as a result of nonsense mutations disrupting reading frame, whereas other studies suggest that NAS is triggered when nonsense mutations disrupt exonic splicing enhancers (ESEs). Using T-cell receptor-β (TCRβ), which naturally acquires PTCs at high frequency, we provide evidence that both mechanisms act on a single type of mRNA. Mutations that disrupt consensus ESE sites up-regulated an alternatively spliced TCRβ transcript that skipped the mutations independently of reading frame disruption and the NMD factor UPF1. In contrast, reading frame-disrupting mutations that did not disrupt consensus ESE sites elicited UPF1-dependent up-regulation of the alternatively spliced TCRβ transcript. Restoration of reading frame prevented this up-regulation. Our results suggest that the response of an mRNA to a nonsense mutation depends on its context.

Nonsense mutations lead to the generation of truncated proteins with the potential to have dominant-negative or deleterious gain-of-function activities (1). To reduce these harmful effects, the nonsense-mediated decay (NMD)2 pathway recognizes and rapidly degrades transcripts harboring nonsense mutations (2–5). Although generally regarded as a beneficial quality control mechanism, NMD can sometimes be detrimental. For example, if the degraded transcript encodes a truncated protein that retains full or partial function, a reduction in its level by NMD has the potential to worsen clinical symptoms (1, 6, 7). A solution to this problem is provided by a second mechanism induced by nonsense mutations: nonsense-associated altered splicing (NAS). In NAS, alternative splicing produces a transcript that no longer contains the premature termination codon (PTC) generated by the nonsense mutations (8–10). If the resulting transcript is in-frame and the skipped region does not encode a vital portion of the protein, NAS can produce a truncated protein that retains the function of the original, full-length protein. An example of beneficial NAS occurs in the dystrophin gene. Nonsense mutations induce an alternatively spliced dystrophin transcript encoding a truncated but active protein that produces a mild (Becker’s) muscular dystrophy phenotype (11–13). A second example of beneficial NAS occurs in the LAMA2 gene; a nonsense mutation at position 2230 induces an alternatively spliced transcript encoding a truncated laminin α2 protein associated with a mild form of congenital muscular dystrophy (14).

Two distinct mechanisms have been proposed for NAS. In class-I NAS, alternative splicing is triggered by mutations that disrupt exonic splicing enhancers (ESEs), short degenerate sequence elements that are bound by serine arginine (SR)-rich proteins (15, 16). Although the actual mechanism is not completely understood, ESEs are thought to improve splicing by recruiting SR proteins, which in turn recruit spliceosome components (15, 17, 18). When an ESE is disrupted by mutation, SR protein binding is reduced or prevented, which in turn lowers the inclusion frequency of the exon harboring that ESE. NAS is triggered if alternative splice sites are available to compete successfully with the normal splice sites for the spliceosomal apparatus. Because ESEs can be disrupted not only by nonsense, but also by missense and silent mutations, a hallmark of an exonic site susceptible to class-I NAS is that all three types of mutations have the potential to up-regulate the alternatively spliced transcript (15). Another feature of class-I NAS is that it is independent of reading frame. Documented examples of class-I NAS come from the BRCA1, fibrillin, and immunoglobulin μ (Igμ) genes (19–22).

In class-II NAS, the level of alternatively spliced mRNA is up-regulated by virtue of the disruption of the reading frame by...
the nonsense and frameshift mutations. Three lines of evidence support the existence of class-II NAS. First, nonsense mutations, but not missense mutations at corresponding positions, trigger an increase in the level of alternatively spliced transcripts from several genes, including minute virus of mice, fibrillin, and T-cell receptor β (TCRβ) (8–10, 23–26). Second, the up-regulation of the alternatively spliced transcripts in response to nonsense mutations is inhibited by blocking translation; i.e. by introducing a stem loop before the translation initiation start site or by mutating the start AUG or the Kozak consensus sequence (9, 10, 25). Third, suppressor tRNAs repress the up-regulation of nonsense codon-bearing TCRβ transcripts (9).

A similar phenomenon called the suppression of splicing response has been documented for the carbamoyl-phosphate synthetase, aspartate transcarbamylase, dihydroorotase, and human α-t-iduronidase transcripts. In this mechanism, in-frame PTCs within the intron appear to prevent the splicing machinery from using cryptic 5’-splice sites in the intron. This mechanism is likely to be distinct from NAS, because it is not affected by the protein synthesis inhibitor G418, suppressor tRNAs, or depletion of UPF1 by RNAi (27).

Recently, evidence challenging the existence of reading frame-dependent (class-II) NAS was reported (28). Mohn et al. (28) found that up-regulation of the alternatively spliced transcript from a TCRβ minigene did not always correlate with disruption of reading frame, leading the authors to conclude that the up-regulation was not caused by disruption of reading frame. Here we reinvestigate this issue by introducing a series of nonsense, missense, and frameshift mutations at well defined positions in the TCRβ gene. Transfection experiments with these mutant TCRβ constructs indicated that mutations at consensus ESE-rich sites increased the level of alternatively spliced transcripts regardless of whether they disrupted reading frame, whereas mutations at sites devoid of consensus ESEs up-regulated alternatively spliced transcripts only when the mutations also disrupted reading frame. Our results provide evidence that TCRβ transcripts are subject to both classes of NAS and that the consequences of reading frame-disrupting mutations depend on context.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Construct A (β-290) is a wild-type TCRβ gene, as described previously (pAc/IF) (29). All other TCRβ constructs were derived from construct A by site-directed mutagenesis PCR (30). Construct B (β-1136) contains a single-nucleotide nonsense mutation at codon 123. Construct C (β-1135) contains a single-nucleotide missense mutation at codon 123. Constructs D (β-1011) and E (β-1012) contain one- or three-nucleotide insertions, respectively, at codon 91. Construct F (β-1013) contains a one-nucleotide deletion at codon 123. Construct G (β-1041) combines the mutations in constructs D and F. Construct H (β-1148) contains a single-nucleotide nonsense mutation at codon 126. Construct I (β-1147) contains a single-nucleotide missense mutation at codon 126. Construct J (β-1094) contains a one-nucleotide deletion at codon 126. Construct K (β-1115) combines the mutations in constructs D and J.

**Transfections**—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, as previously described (5). They were grown to ~40% confluence in 60-mm plates for transfection. Plates were transfected with 500 ng of TCRβ reporter construct and 50 ng of a β-globin expression vector construct (G-435) using Lipofectamine (Invitrogen), according to the manufacturer’s instructions. The transfection mixture was removed after 20 h, and 3 ml of fresh serum-containing medium was added to each plate. Cells were harvested after additional 24-h incubation.

**RNA Interference**—UPF1 and SMG1 were transiently depleted using siRNA oligonucleotides (Ambion) containing sequences previously described (8, 31). The luciferase (Luc) siRNA was previously described (32). HeLa cells were grown to ~15% confluence in 60-mm plates before being transfected with siRNA oligonucleotides using Lipofectamine 2000, following the manufacturer’s standard protocol. The final concentration of siRNA oligonucleotide was 100 nM for UPF1 and 200 nM for SMG1. The transfection mixture was removed after 20 h, and the cells were incubated for ~24 h with 3 ml of fresh serum-containing medium and then transfected with 500 ng of TCRβ reporter construct and 50 ng of β-globin expression vector using Lipofectamine according to the manufacturer’s standard protocol. The transfection mixture was removed after 20 h, 3 ml of fresh serum-containing medium was added, and the cells incubated an additional 24 h before harvest.

elf4AIII was transiently depleted using an siRNA oligonucleotide containing sequences previously described (33). HeLa cells were grown to ~40% confluence in 60-mm plates before being transfected with elf4AIII and control (Luc) siRNA oligonucleotides (at a final concentration of 100 nM) using Lipofectamine 2000. After 6-h incubation, the cells were transfected with 500 ng of TCRβ reporter construct and 50 ng of β-globin expression vector using Lipofectamine. The transfection mixture was removed after 20 h, 3 ml of fresh serum-containing medium was added, and the cells incubated an additional 24 h before harvest.

UPF3a and/or UPF3b were depleted by transiently transfecting short hairpin (sh) RNA constructs against these factors using Lipofectamine in HeLa cell clones. These clones, grown to ~40% confluence on 60-mm plates, already stably expressed UPF3a and/or UPF3b shRNAs (5). In addition to the shRNA constructs, the cells were transiently transfected with 500 ng of TCRβ reporter construct and 50 ng of β-globin expression vector. After 20-h incubation, the transfection mixture was removed, 3 ml of fresh serum-containing medium was added, and the cells were incubated an additional 24 h before harvest.

**RNA Isolation and Ribonuclease Protection Analysis**—RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Ribonuclease protection analysis was performed as described previously, using 10 μg of total cellular RNA (30, 34, 35). The SMG1 riboprobe was generated from a 193-nucleotide SMG1 cDNA fragment subcloned into pGEM-T Easy (Promega) after PCR amplification from a SMG1 cDNA template (G-376, provided by Dr. Alan P. Fields (36)) using oligonucleotides MDA1935 (5’-caaagatggcacaacag-3’) and MDA1936 (5’-gacgggaaactgagg-3’). The UPF3a (G-539), UPF3b (G-540), and β-actin (G-98) riboprobes were previously
Nonsense-associated Altered Splicing

A

Alt-mRNA

NAS

Pre-mRNA

Norm-mRNA

NMD
decay

B

Alt-SA

Alt-SD

Pre-mRNA

Norm-mRNA

VDJ riboprobe

Alt-mRNA

FIGURE 1. NAS and NMD. A, TCRβ precursor mRNA (Pre-mRNA) gives rise to normally spliced transcript (Norm-mRNA) and an alternatively spliced transcript (Alt-mRNA). The presence of a PTC triggers the norm-mRNA to be degraded by NMD and the alt-mRNA to be up-regulated by NAS. White box, L exon; diagonal striped box, VDJ exon; gray boxes, C exons; white octagon, norma stop codon; black octagon, PTC. B, the alt-mRNA is produced through the use of an alternative splice acceptor (Alt-SA) in the intron upstream of the rearranging VDJ exon and an alternative splice donor (Alt-SD) in the VDJ exon. The alternative splicing maintains the reading frame but generates a truncated protein lacking most of the TCRβ variable region.

described (5, 34, 35). mRNA levels were quantified by normalization to an internal control using the Instant Imager (Packard Instrument), a direct sensor of β radiation.

Western Blot Analysis—Western blot analysis was performed as described before (34). In brief, cells were harvested and lysed on ice for 30 min using radioimmunoprecipitation assay buffer, the lysates were cleared by centrifugation at 14,000 × g for 20 min, the supernatant was collected, and the protein concentration was determined using the DC protein assay kit (Bio-Rad). 5–20 μg of protein was electrophoresed on SDS-polyacrylamide gels, transferred to Hybond ECL nitrocellulose (Amersham Biosciences), incubated with a polyclonal antiserum against human UPF1 (provided by Dr. Jens Lykke-Andersen, University of Colorado, Boulder) at 1:5,000 dilution or a monoclonal antibody against β-actin (Sigma) at 1:10,000 dilution in 5% milk solution, and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antisera (Amersham Biosciences), respectively. Signals were visualized using ECL reagent (Pierce).

RESULTS

Reading Frame-dependent NAS—As with our past studies (9, 10), we utilized a productively rearranged TCRβ gene to study NAS. This gene, composed of the Lβ8.1 exon, a rearranged Vβ8.1-DJβ2-Jβ2.3 exon, and four Cβ exons, produces the normally spliced transcript (norm-mRNA) shown in Fig. 1 (A and B). It also produces an alternatively spliced transcript (alt-mRNA) derived as a result of an alternative splice-site acceptor (alt-SA) located in the intron upstream of the VDJ exon and an alternative splice-site donor (alt-SD) in the 5′-end of the VDJ exon (Fig. 1, A and B). The resulting alt-mRNA contains a portion of the intron and only the 5′-end of the VDJ exon but remains in frame. The vast majority of transcripts expressed from this TCRβ gene are norm-mRNA, with only a trace amount of alt-mRNA (9, 10). However, introduction of nonsense and frameshift mutations leads to the degradation of most of the norm-mRNA by NMD and an increase in the level of alt-mRNA by NAS (9, 10, 29, 30, 37).

To assess whether the up-regulation of alt-mRNA occurs by a reading frame-dependent mechanism, we scanned the VDJ exon for positions at which to introduce nonsense mutations that would disrupt the reading frame but not known ESEs. Toward this end, we used the software program ESEfinder to identify consensus binding sites for the SR proteins ASF/SF2, SC35, SRp40, and SRp55 (38). ESEs above the default threshold are shown in supplemental Fig. S1. From this analysis we selected codon 123 in which to make mutations. A nonsense mutation in this codon does not disrupt any known ESEs, according to ESEfinder analysis (construct B in supplemental Fig. S2A). As a control, we generated a missense mutation in the same codon that likewise does not affect any known consensus ESEs (construct C).

To determine the effects of these mutations, we transiently transfected the constructs into HeLa cells, extracted total cellular RNA, and used ribonuclease protection analysis to quantify the levels of alt- and norm-mRNA. As we have described before (9, 10), we used a riboprobe that gives a larger band for the alt-mRNA than for norm-mRNA, because the riboprobe hybridizes with the entire length of the alternative VDJ exon in alt-mRNA but only a portion of the VDJ exon in norm-mRNA (Fig. 1B). Consistent with our past studies demonstrating that TCRβ transcripts are subject to NMD (5, 9, 10, 29, 30, 34, 37, 39, 40), we found that the norm-mRNA was specifically down-regulated by the nonsense mutation but not the missense mutation (constructs B and C, respectively, Fig. 2A). Relevant to the present study, the nonsense mutation also triggered NAS, as it increased the level of the alt-mRNA (Fig. 2A). Using β-globin as an internal control, we found that alt-mRNA levels were increased by 3-fold in response to the nonsense mutation in construct B (Fig. 2B). In contrast, the missense mutation did not significantly increase alt-mRNA level (Fig. 2B). These results supported the hypothesis that the alternatively spliced TCRβ mRNA is up-regulated in response to disruption of reading frame.

As an independent test of this hypothesis, we introduced frameshift mutations in the region of the VDJ exon skipped in the alt-mRNA. A frameshift in this region creates a PTC in the norm-mRNA but not the alt-mRNA, and thus we predicted it would trigger NAS. To test this prediction, we created construct D, which contains a one-nucleotide insertion at codon 91 that does not disrupt any consensus ESEs (supplemental Fig. S2B). As a control, we generated a three-nucleotide insertion in the same codon that likewise does not affect any known consensus ESEs (construct E). We also generated a downstream compensatory one-nucleotide deletion in codon 123 (construct F) that does not disrupt consensus ESEs (supplemental Fig. S2B). Finally, we generated a construct that
combines the one-nucleotide insertion and deletion, restoring the original reading frame (construct G in supplemental Fig. S2).

We transiently transfected these constructs into HeLa cells and determined whether the mutations present in the constructs triggered NAS and NMD. We predicted that only the constructs with disrupted reading frame would give rise to transcripts regulated by NAS and NMD. In agreement with this prediction, we found that the level of alt-mRNA was increased and that of norm-mRNA decreased when the reading frame was disrupted by the one-nucleotide insertion or deletion (constructs D and F, respectively, Fig. 3, A and B). In contrast, the levels of alt- and norm-mRNA remained unchanged when the mutation was reading frame neutral or when the original frameshift was corrected by a downstream compensatory frameshift (constructs E and G, respectively, Fig. 3, A and B).

The results from these four insertion and deletion constructs, in addition to those described earlier containing nonsense and missense mutations, support the hypothesis that TCRβ transcripts are subject to reading frame-dependent (class-II) NAS.

Reading Frame-dependent NAS Depends on UPF1—Because, like NMD, class-II NAS is elicited by an in-frame nonsense codon, we predicted it would require the NMD factor UPF1. To test this, we depleted UPF1 with a double-stranded siRNA previously shown to effectively knockdown UPF1 (8). We found that transient transfection of this siRNA into HeLa cells reduced UPF1 protein levels to <10% of its level in control cells transfected with an siRNA against firefly luciferase (Fig. 4A). To determine whether depletion of UPF1 interferes with class-II NAS, we first transfected HeLa cells with UPF1 siRNA to knock down UPF1 and then 40 h later transfected these UPF1-depleted cells with wild-type and mutant TCRβ constructs. We found that the reduced level of UPF1 partially reversed the NMD response triggered by the nonsense mutation (construct B; Fig. 4, B and C) and the frameshift mutations (constructs D and F; Fig. 4, D and E) but had no effect on norm-mRNA level in response to mutations that did not disrupt reading frame (Fig. 4, B–E). Similarly, we found that depletion of UPF1 partially reversed NAS, as alt-mRNA levels from the PTC-containing constructs (B, D, and F) were significantly decreased after transfection of the UPF1 siRNA (Fig. 4, B–E). This reversal was specific, because depletion of UPF1 did...
not significantly affect alt-mRNA levels from the in-frame constructs (A, C, E, and G, Fig. 4, B–E). We conclude that the up-regulation of the TCR/H9252 alt-mRNA in response to reading frame-disrupting mutations that do not disrupt consensus ESEs is UPF1-dependent. Although we cannot rule out the possibility that the dependence on UPF1 is the result of an indirect effect, the most straightforward interpretation of our results is that, like NMD, NAS requires UPF1 to perceive a nonsense codon as being in a premature position.

UPF1 is phosphorylated by SMG-1, a member of the phosphatidylinositol 3-kinase-related kinase family that is required for NMD in Caenorhabditis elegans, augments NMD in Drosophila melanogaster, and reduces NMD when knocked down by RNAi in mammalian and D. melanogaster cell lines (36, 41–44). Because we found that class-II NAS requires UPF1, this led to the possibility that class-II NAS also requires SMG-1. To address this, we used an siRNA that was previously shown to strongly deplete the level of SMG-1 (31). We were not able to monitor SMG-1 knockdown efficiency at the protein level, because the extremely large size of SMG-1 (~400 kDa) precluded our ability to detect it by Western blot analysis. Instead, we used ribonuclease protection analysis to assess the knockdown of SMG-1 at the mRNA level. This analysis showed that the SMG-1 siRNA knocked down SMG-1 mRNA levels by >60% compared with the control firefly luciferase siRNA (Fig. 5A). This is likely to be a conservative estimate of the knockdown of SMG1 protein levels, because siRNAs often reduce not only mRNA stability but also translation (45). Supporting the notion that we had effectively knocked down SMG-1, we found that NMD was strongly reversed after transfection of the SMG-1 siRNA (~4- to 6-fold up-regulation of norm-mRNA from construct B, Fig. 5, B and C). In contrast, knockdown of SMG-1 did not have an effect on class-II NAS, because the increased levels of alt-mRNA resulting from NAS showed no significant change when SMG-1 was depleted (construct B, Fig. 5, B and C). Although it is possible that the modest level of SMG-1 remaining after siRNA treatment was sufficient for NAS, we believe this is unlikely, because the SMG-1 siRNA reversed NMD by a magnitude similar to that elicited by the NAS-reversing UPF1 siRNA (Fig. 4, B–E).

In mammalian NMD, the exon junction complex (EJC) serves as a second signal to identify a nonsense codon as aberrant (46–49). We examined the role of the EJC in class-II NAS by using RNAi to deplete three EJC factors: eIF4AIII, UPF3a, and UPF3b. eIF4AIII is a key component of the EJC that binds directly to the mRNA after splicing and probably serves as the anchor upon which all other EJC proteins assemble (50, 51). To deplete eIF4AIII, we used an siRNA that was previously shown to reduce the level of eIF4AIII (33). We found that transient transfection of this eIF4AIII siRNA reduced the level of eIF4AIII compared with cells transfected with the control luciferase siRNA (Fig. 5D). Although the eIF4AIII mRNA level was not strongly reduced (~40% of the control), this reduction was sufficient to strongly reverse NMD, because the level of norm-mRNA from construct B was increased by 3- to 6-fold (Fig. 5, E and F). In contrast, there was no change in the level of the alt-mRNA (construct B, Fig. 5, E and F), providing evidence that eIF4AIII does not have a role in NAS. Conditions favoring greater knockdown of eIF4AIII...
Nonsense-associated Altered Splicing

FIGURE 5. Class-II NAS is not affected by depletion of SMG1, elf4AIII, UPF3a, or UPF3b. A, D, G, and J, ribonuclease protection analysis of the NMD and EJC factors shown using total cellular RNA from HeLa cells transfected with the indicated siRNAs or shRNAs. The luciferase siRNA is a negative control and β-actin is an internal loading control. B, E, H, and K, ribonuclease protection analysis (performed as in Fig. 2) of total cellular RNA from HeLa cells cotransfected with the siRNAs and shRNAs indicated, the TCRβ constructs shown in Fig. 2A, and a β-globin expression vector as an internal control. C, quantitation of four independent transfections, with error bars indicating standard error; *, p < 0.001; **, p < 0.05 using the two-tailed Student’s t test. F, I, and L, quantitation of three independent transfections, with error bars indicating standard error; *, p < 0.05, two-tailed Student’s t test.

(higher siRNA concentrations, longer incubations with siRNAs, and lower cell confluency at the time of transfection) caused considerable cell death, and thus we did not use them to investigate the role of elf4AIII in NAS.

UPF3a and UPF3b are EJC proteins that have a role in β-globin NMD (52, 53), but probably not in TCRβ NMD, because knockdown of these two factors (by ≥80%) has no effect on TCRβ NMD (5). We therefore hypothesized that their knockdown would also have no effect on TCRβ NAS. To test this, we strongly depleted UPF3a and UPF3b using our previously described short hairpin RNA (shRNA) approach (5). In brief, this approach involves stably transfecting HeLa cells with constructs expressing UPF3a and UPF3b shRNAs from the mouse U6 promoter, isolating individual cell clones that have depleted levels of UPF3a or UPF3b, and transiently transfecting these cell clones with the same shRNA constructs to further knockdown UPF3a and/or UPF3b. By using this “double RNAi” approach, we knocked down the level of UPF3a mRNA to below 10% and UPF3b to below 20% compared with the control firefly luciferase shRNA (Fig. 5, G and J). As we had previously reported (5), knockdown of UPF3a and/or UPF3b had no effect on TCRβ NMD (Fig. 5, H, I, K, and L). Knockdown of either or both of these factors also did not significantly affect TCRβ NAS (Fig. 5, H, I, K, and L): These data, together with the data from cells depleted of elf4AIII, indicate that class-II NAS either requires much lower levels of EJC factors than NMD or is completely independent of the EJC.

Reading Frame-independent NAS—The experiments just described provided several lines of evidence that the TCRβ alt-mRNA is up-regulated by a UPF1-dependent mechanism elicited in response to nonsense codons. The data, along with our published evidence that translation is required for TCRβ alt-mRNA to be up-regulated in response to nonsense mutations (9), make a strong case for the existence of reading frame-dependent (class-II) NAS. We next determined whether the TCRβ alt-mRNA could also be up-regulated in response to nonsense mutations in a reading frame-independent manner (class-I NAS). In particular, we assessed whether alt-mRNA level could be increased by nonsense mutations that disrupt splicing enhancers in the VDJ exon. Using ESEfinder we identified a nonsense mutation in codon 126 of the VDJ exon that completely eliminated consensus SC35 and SRp40 binding sites and weakened a consensus ASF/SF2 binding site (construct H). We predicted that disruption of these consensus ESEs would reduce usage of the splice sites needed for inclusion of the full-length VDJ exon found in the norm-mRNA and favor inclusion of the drastically truncated VDJ exon found in the alt-mRNA. To test this prediction, we made a construct containing the mutation and compared its expression with that of the wild-type control in transfected HeLa cells. In agreement with our predictions, we found that the nonsense mutant (construct H) expressed higher levels of alt-mRNA than did the wild type (construct A) (Fig. 6, A and B). We should note that the level of alt-mRNA up-regulation triggered by the nonsense mutation in construct H varied in different experiments (between ~2.5- to 4.5-fold), but nonetheless we found that this mutation consistently up-regulated the alt-mRNA.

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To determine whether disruption of consensus ESEs in this region was sufficient to elicit alt-mRNA up-regulation, we made a missense mutation in codon 126 that disrupted consensus ESEs (construct I in supplemental Fig. S3A). We found that this missense mutant expressed elevated levels of alt-mRNA relative to that of the control (Fig. 6, A and B). This result indicates that alt-mRNA can be up-regulated in the absence of reading frame disruption and thus provides evidence that TCRβ transcripts can undergo class-I NAS.

As an independent test of whether TCRβ transcripts can undergo class-I NAS, we disrupted consensus ESEs using frameshift mutations. Construct J contained a one-nucleotide deletion in codon 126 predicted to completely eliminate three ESEs (supplemental Fig. S3B). As with construct H, we predicted that the mutation in construct J would up-regulate the levels of the alt-mRNA as a consequence of the disruption of consensus ESEs. In agreement with this prediction, construct J had increased levels of alt-mRNA as compared with the wild-type control (Fig. 6, C and D). To further investigate this issue, we made construct K, which contained the consensus ESE-disrupting mutation in construct J together with a compensatory one-nucleotide insertion that restores the reading frame but does not disrupt known ESEs (Fig. 6D and supplemental Fig. S3B). We predicted that this double-frameshift mutant would also express elevated levels of alt-mRNA because consensus ESEs are disrupted at codon 126 even though the reading frame is restored. Our prediction was met, as we found that construct K expressed 2- to 3-fold higher levels of alt-mRNA than the control (construct A).

Reading Frame-independent NAS Is Not Impaired by Depletion of NMD or EJC Factors—We next examined whether class-I NAS requires the NMD factor UPF1. To test this, we depleted UPF1 by RNAi and examined its effect on the up-regulation of alt-mRNA in response to class-I NAS mutations. We found that none of the consensus ESE-disrupting mutants exhibited significantly altered expression of the alt-mRNA after depletion of UPF1. This included the nonsense and missense mutants (constructs H and I, respectively, Fig. 7, A and B) and the frameshift mutants (constructs J and K, Fig. 7, C and D). This lack of an effect on class-I NAS occurred under conditions in which UPF1 depletion strongly reversed NMD, as levels of norm-mRNA were increased by ~5-fold for construct H (Fig. 7, A and B) and by ~8-fold for construct J (Fig. 7, C and D). The finding that UPF1 depletion had no effect on the up-regulation of the alt-mRNA in response to either the nonsense and frameshift mutations suggests that class-I NAS is dominant over class-II NAS (because these mutations disrupt both consensus ESEs and the reading frame). Consistent with the finding that UPF1 deple-
Nonsense-associated Altered Splicing

We next examined whether class-I NAS depends on the EJC factors eIF4AIII, UPF3a, and UPF3b. We predicted that class-I NAS would not require these EJC factors, because class-I NAS results from alterations in splice-site recognition, not post-splicing events. RNAi-mediated depletion experiments demonstrated that reduction in the level of these three EJC factors had no effect on the level of alt-mRNA expressed from the class-I NAS substrate construct H (Fig. 5, E, F, H, I, K, and L). Depletion of these factors also had no effect on alt-mRNA expression from construct I, which harbors a mutation that only affected consensus ESEs, not the reading frame (Fig. 5, E, F, H, I, K, and L). Collectively, these results suggest that reductions in the level of NMD and EJC factors sufficient to strongly abrogate NMD have no effect on class-I NAS.

DISCUSSION

In this study we have provided evidence for two distinct NAS mechanisms. In class-I NAS, the up-regulation of alternatively spliced transcript is caused by the disruption of splicing elements such as ESEs. In support of this mechanism, we found that mutations that disrupted consensus ESEs, regardless of whether they disrupted the reading frame, up-regulated alternatively spliced mRNA (Fig. 6B). In class-II NAS, mutations that disrupt the reading frame trigger an increase in alternatively spliced transcript levels. Several lines of evidence support this mechanism: First, frame-disrupting mutations that do not disrupt consensus ESEs (as determined using ESEfinder) increased the level of alternatively spliced mRNA (Figs. 2 and 3) (9, 10). Second, a missense mutation at the same position or restoration of reading frame had no observable effect on alternatively spliced mRNA levels (Figs. 2 and 3) (9, 10, 40). Third, RNAi-mediated depletion of the NMD factor UPF1 decreased the level of the alternatively spliced mRNA induced by reading frame-disrupting mutations (Fig. 4, B–E) (8). Finally, we obtained several lines of evidence that class-II NAS depends on translation, because inhibiting translation by various means (mutating the start AUG or the Kozak consensus sequence or introducing a stem loop before the translation start site) specifically decreased the level of the alternatively spliced mRNA (9, 10).

If indeed two classes of NAS exist, this complicates interpreting the effects of nonsense and frameshift mutations. This may explain why some groups reported that nonsense mutations up-regulate alternatively spliced mRNAs by disrupting reading frame (8–10, 23–26), whereas others reported that up-regula-
tion of alternatively spliced transcripts occurs as a result of the disruption of ESEs (19–22). It also provides an explanation for why Mohn et al. (28) found that TCRβ alt-mRNA was up-regulated by only some frame-disrupting mutations at codon 58 in a TCRβ minigene. Although their analysis using ESE-RESCUE (54) showed that codon 58 was free of consensus ESEs, we found using ESEfinder that consensus ESEs were enhanced and/or supplemented by new consensus ESEs as a result of the codon 58 mutations. This enhancement and acquisition of ESEs would favor normal splicing and thus counteract the alt-mRNA up-regulatory response triggered by frame-disrupting mutations (class-II NAS). We should note, however, that this interpretation requires experimental verification, because ESEfinder is only a prediction program and does not detect all classes of ESEs. One way to examine the role of specific ESEs is to delete the specific SR proteins predicted to bind to the ESE in question. Although potentially powerful, this approach suffers from the difficulty of distinguishing between direct and indirect effects of SR proteins.

Although our RNAi depletion studies suggested that class-II NAS is independent of the EJC factors elf4AIII, UPF3a, and UPF3b, we cannot rule out the possibility that class-II NAS requires low levels of these factors. However, if this were the case for elf4AIII, the level of elf4AIII required for NAS would be lower than that required for NMD, because we found that TCRβ NMD was reversed by the same magnitude of elf4AIII depletion (Fig. 5, E and F). Furthermore, siRNA-mediated depletion of elf4AIII reversed TCRβ NMD to approximately the same extent as did siRNA-mediated depletion of UPF1, and yet only the latter inhibited TCRβ NAS (Fig. 4, B–E). In the case of UPF3b, whereas reduction in its level by double shRNA treatment had no effect on TCRβ NMD (Fig. 5, F and K) (5), the same treatment reversed β-globin and triosephosphate isomerase NMD (5), arguing that if UPF3b is required for NAS, lower levels are required than for NMD. Lastly, in the case of UPF3a, while its depletion did not affect the levels of standard NMD substrates (5), we have identified endogenous human transcripts with NMD features that are up-regulated in response to UPF3a depletion. Thus, the finding that UPF3a depleted in the same way did not affect TCRβ NAS suggests a differential effect of this factor on NMD and class-II NAS. Lastly, we should note that the notion that class-II NAS is EJC-independent is not entirely surprising, because an alternative branch of the NMD pathway was recently identified with this characteristic (55) and the decay of PTC+ Igμ transcripts under some circumstances appears to be elf4AIII-independent (56). Furthermore, NMD in D. melanogaster, C. elegans, and Saccharomyces cerevisiae does not depend on EJC factors (44, 57, 58).

TCRβ class-II NAS is paradoxical for two reasons. First, recognition of a PTC in the TCRβ VDJ exon requires that it be spliced with the exon upstream that harbors the start AUG (9), an event that removes one of the splice sites required to generate the alt-mRNA. If PTC recognition precludes the ability to generate the alt-mRNA, how does it up-regulate the alt-mRNA? One possibility that we believe is unlikely is that normally spliced introns are reintroduced into the mRNA after PTC recognition (by reverse splicing), allowing alternative splicing to occur. Another possibility is that the translation apparatus reads across introns (by looping), allowing the PTC to be recognized without the necessity for normal splicing (24). This possibility is unlikely as well for several reasons, including architectural constraints imposed by the ribosome and the spliceosome and our finding that a PTC generated only after RNA splicing was able to efficiently trigger TCRβ NAS (9). The second paradox is that class-II NAS is triggered by a frame-reading mechanism (only definitively known to exist in the cytoplasm), but it requires alternative RNA splicing (a nuclear event). How can an mRNA be scanned for PTCs in the cytoplasm and then be alternatively spliced in the nucleus?

The model we favor to explain these two paradoxes is that class-II NAS is an in trans mechanism, rather than an in cis mechanism. In this in trans mechanism, recognition of a PTC in a transcript does not trigger alternative splicing in that particular transcript, but rather it generates a “signal” that increases the amount of alternatively spliced transcripts derived from newly synthesized precursor mRNA molecules. The signal that increases the level of the alternatively spliced transcript could be an mRNA splicing factor that is sequestered or released as a result of NMD (59). Alternatively, it could be an mRNA decay/stabilization factor that is sequestered or released as a result of NMD, leading to stabilization of the precursor mRNA, allowing more alt-mRNA to be generated. This latter idea is consistent with the finding that nonsense and frameshift mutations can increase the level of TCRβ and Igμ precursor transcripts (35). In some cases, the alt-mRNA itself could also be stabilized, but we found that TCRβ alt-mRNA was not stabilized by PTCs, indicating that this is not the mechanism responsible for TCRβ NAS (10).

If PTC recognition occurs as a result of translation in the cytoplasm, the factor sequestered or released by NMD would necessarily need to be present in limiting concentrations within the entire cell. Alternatively, if PTC recognition and NMD occurs in the nucleus, this would require only that the factor’s level be altered in the local nuclear microenvironment where the precursor mRNA is transcribed and processed. Although it is a potentially attractive possibility, there is evidence both for and against nuclear translation (2, 59–67).

NAS may be physiologically important by virtue of its ability to partially restore gene function. For example, NAS may reduce the phenotype of muscular dystrophy caused by a subset of dystrophin mutations. Patients with this disease often harbor nonsense or frameshift mutations in the dystrophin gene that target the normally spliced dystrophin transcript for NMD. Some muscular dystrophy patients exhibiting a less severe disease phenotype harbor nonsense or frameshift mutations that cause the exon with these mutations to be skipped, producing an alternatively spliced transcript that is up-regulated by NAS (11, 12). This suggests that the alternatively spliced transcript encodes a functional dystrophin protein that partially compensates for the loss of the normal dystrophin protein. This discovery led to a therapeutic approach in which NAS is artificially induced to reduce muscular dystrophy symptoms. In this approach, van Deutekom et al. (13) introduced into muscle cells

3 W.-K. Chan and M. F. Wilkinson, unpublished observations.
a series of antisense transcripts corresponding to exon 46 to inhibit inclusion of this exon, leading to the production of truncated dystrophin protein that correctly localized to myotubes. We suggest that this approach can be used on a wide variety of genes harboring nonsense or frameshift mutations to up-regulate or induce alternatively spliced transcripts encoding functional protein products.

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