Alternatively Spliced TCR mRNA Induced by Disruption of Reading Frame

Jun Wang, John I. Hamilton, Mark S. Carter,* Shulin Li,† Miles F. Wilkinson‡

Nonsense codons that prematurely terminate translation generate potentially deleterious truncated proteins. Here, we show that the T cell receptor–β (TCRβ) gene, which acquires in-frame nonsense codons at high frequency during normal lymphocyte development, gives rise to an alternatively spliced transcript [alternative messenger RNA (alt-mRNA)] that skips the offending mutations that generate such nonsense codons. This alt-mRNA is up-regulated by a transfer RNA–dependent scanning mechanism that responds specifically to mutations that disrupt the reading frame. The finding that translation signals regulate the levels of alternatively spliced mRNAs generated in the nucleus may alter the current view of how gene expression is controlled in eukaryotic cells.

Approximately 15% of all point mutations responsible for human genetic diseases cause alternative RNA splicing (*1*). Surprisingly, mutations that generate premature termination (nonsense) codons (PTCs) sometimes increase the levels of alternatively spliced transcripts that skip the offending mutations, thereby potentially saving protein function (*2–7*). This putative RNA correction response is paradoxical, because translation and RNA splicing are thought to occur in different cellular compartments. A solution to this paradox has been that this response is not specific to nonsense mutations but rather results merely from any mutation that disrupts RNA splicing enhancers critical for exon inclusion (*6–8*). Although this is one mechanism by which nonsense mutations can act, we demonstrate here unambiguously the existence of a nonsense codon–specific up-regulatory mechanism that acts independently of splicing enhancer disruption.

The gene we used for our studies is the TCRβ gene, because it commonly acquires PTCs as a result of programmed rearrangements during T cell development (*3*). Although such nonproductively rearranged TCR genes transcribe high levels of precursor messenger RNAs (mRNAs), little or no mature mRNA accumulates as a result of the nonsense-mediated decay (NMD) pathway (*3*, *9*). In our earlier transfection studies, we used a rearranged mouse $V_{\beta 8.1}$ $D_{\beta 2}$ $J_{\beta 2.3}$ $C_{\beta 2}$ TCR gene to demonstrate that this NMD response is indistinguishable in lymphoid and nonlymphoid cells, it occurs in the nuclear fraction, it depends on an intron downstream of the PTC, and it requires a translation-like process (*9–12*). Here, we report the identification and characterization of a complementary response in which transcript levels are increased, rather than decreased, in response to nonsense codons. In particular, we identified an induced alternatively spliced transcript (alt-mRNA) that skips offending mutations that generate PTCs. This alt-mRNA is generated by simultaneous use of both an alternative splice acceptor and donor (Fig. 1A). Use of both is critical for maintaining an open-reading frame, as use of either alternative site alone generates in-frame PTCs as a result of frameshifts (fig. S1). The alt-mRNA was up-regulated severalfold in response to a PTC in the VDJ exon (Fig. 1B, left panel). The spliced intron liberated as a result of alternative splicing also showed increased levels (fig. S3), indicating that nonsense codons act by increasing the amount of alt-mRNA that is spliced.

There have been several other reports of genes harboring PTCs that express elevated levels of alternatively spliced transcripts that have skipped the PTC, as judged by semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis (*5–7*). The prevailing explanation for this phenomenon is that nonsense mutations disrupt the function of splicing enhancers, *cis* elements that bind to RNA-splicing factors that promote exon inclusion (*6–8*). To test this enhancer-disruption model, we introduced nonsense mutations at various sites in the VDJ exon of the TCR gene. Because splicing enhancers occur only at specific sites, we postulated that if the enhancer-disruption model is correct, PTCs at most positions would fail to increase alt-mRNA levels. We deliberately mutated

Department of Immunology, The University of Texas M. D. Anderson Cancer Center, Box 180, 1515 Holcombe Boulevard, Houston, TX 77030, USA.

*Present address: Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305–5124, USA.

†Present address: Department of Surgery/Otolaryngology, 2799 West Grand Boulevard, Henry Ford Health System, Detroit, MI 48202, USA.

‡To whom correspondence should be addressed. E-mail: mwilkins@mail.mdanderson.org

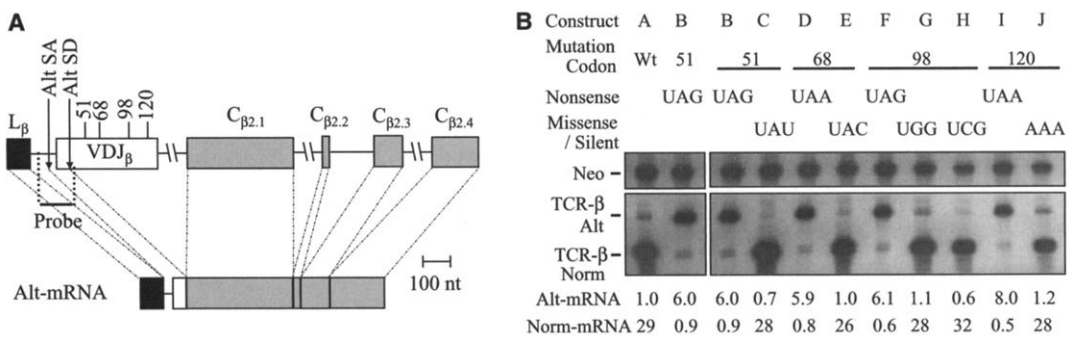

Fig. 1. Up-regulation of an alternatively spliced mRNA specifically in response to nonsense codons. (**A**) Structure of the TCRβ gene (*9*) and the alternatively spliced transcript (alt-mRNA) that we identified. Alt SA, alternative splice acceptor; Alt SD, alternative splice donor. The codons we mutated in the VDJ_β exon are indicated. (**B**) Ribonuclease protection assay (RPA) of total cellular RNA (5 μg) from HeLa cells transiently transfected with the constructs shown. Construct A [pAc/IF (*9*)] contains a TCRβ gene with a full-length open reading frame. Constructs B through J are derivatives of construct A with mutations in the VDJ exon generated by site-specific mutagenesis (*30*). The sizes of the alt- and norm-mRNA bands protected by the TCRβ probe were ~84 and ~72 nt, respectively, which are the sizes expected on the basis of the position of the splice sites in the alt- and norm-mRNAs. Steady-state levels of alt- and norm-mRNA were normalized against the level of neomycin (neo) mRNA, which was expressed from the same plasmids as was TCRβ mRNA. Band intensities were determined with a direct radioactivity scanner (InstantImager, Packard Instrument Co., Downers Grove, IL). A value of 1.0 was arbitrarily assigned to the level of alt-mRNA from constructs A and E in the left and right panels, respectively. Similar results were obtained for each construct in at least three independent transfections. Titration experiments demonstrated that the RPA was quantitative under the conditions we used (fig. S2). Cell transfection, RNA purification, and RPA were performed as described (*24*).

positions distant from known splicing-enhancer elements, such as purine- or AC-rich sequences (*13*). We also introduced missense and silent mutations at those same positions, because such mutations are theoretically as likely as nonsense mutations to disrupt splicing enhancers. Contrary to the predictions of the enhancer-disruption model, we found that nonsense codons at all four positions tested (codons 51, 68, 98, and 120) increased the level of the alt-mRNA, whereas missense and silent mutations at those positions did not (Fig. 1B, right panel). Even a UGG missense codon (construct G), which closely resembles a nonsense codon (because it contains a U followed by two purines), did not increase alt-mRNA levels. Normally spliced (norm) mRNA exhibited the opposite response, as it was decreased in levels in response to all the nonsense mutations.

If nonsense codons cause alt-mRNA up-regulation, we further predicted that a frameshift that generates a downstream nonsense codon should also up-regulate alt-mRNA. In agreement with this prediction, we found that a frameshift-inducing 10-nucleotide (nt) insert (Fig. 2A, construct K) increased the levels of alt-mRNA (Fig. 2B). To distinguish whether the insert induced the alt-mRNA because it generates an in-frame nonsense codon or because it interferes with a splicing enhancer, we tested two constructs with compensating downstream frameshifts so that a PTC would not be generated by the 10-nt insert (constructs L and M). These compensating mutations prevented the up-regulation of the alt-mRNA (Fig. 2B). Introduction of a 9-nt insert (construct N), which would disrupt a splicing enhancer if one were present but would not change reading frame, also did not up-regulate the alt-mRNA (Fig. 2B). We conclude that the up-regulation of the alt-mRNA is not a consequence of disruption of a splicing-regulatory element, but rather is a specific regulatory response to nonsense codons.

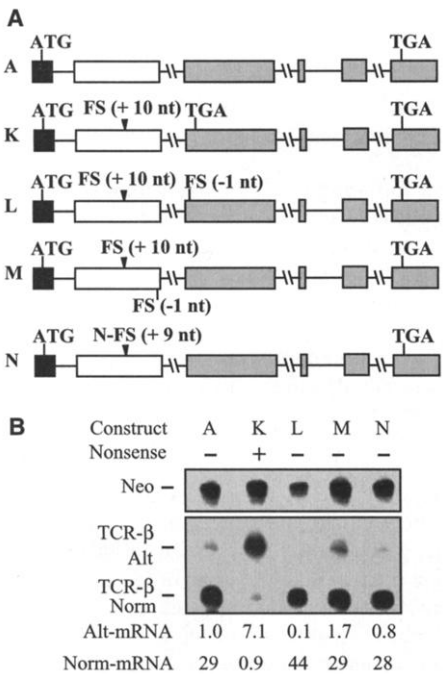

Fig. 2. Up-regulation of alt-mRNA specifically in response to disruption of the VDJ reading frame. (**A**) TCRβ gene constructs [described in (*24*)]. (**B**) RPA of total cellular RNA (5 μg) from HeLa cells transiently transfected with the constructs shown and analyzed in the same manner as in Fig. 1. Similar results were obtained in at least two independent transfection experiments.

To further examine the specificity of the up-regulatory response, we evaluated the effect of PTCs at positions that cannot be eliminated by the alternative splicing event that generates the alt-mRNA. We found that nonsense mutations in the $C_{\beta 2.1}$ and $C_{\beta 2.3}$ exons decreased, rather than increased, alt-mRNA levels unless the cells were incubated with the protein synthesis/NMD inhibitor cycloheximide, strongly suggesting that these transcripts are subject to NMD (fig. S4). This result, together with the results obtained with nonsense mutations and frameshifts at other sites (Figs. 1B and 2B), indicates that the up-regulatory response is highly specific, as it is only triggered by PTCs and frameshifts at sites skipped in the alt-mRNA (i.e., most of the VDJ exon).

We found that this alt-mRNA up-regulatory response required a mechanism with the characteristics of translation. First, it depended on a start AUG to define the reading frame, because disruption of both initiator AUGs prevented alt-mRNA up-regulation (Fig. 3A). Second, a scanning mechanism appeared to be necessary for the response, because a translation-blocking stem loop (*14*, *15*) introduced into the 5′-leader exon prevented alt-mRNA up-regulation (Fig. 3B). Third, it was a transfer RNA (tRNA)–dependent mechanism, because it was inhibited by specific suppressor tRNAs (fig. S5).

How can translation affect the amount of alt-mRNA generated? If the cytoplasmic translation apparatus was responsible, this would necessitate the existence of a signaling mechanism that travels between the cytoplasm and the nucleus to somehow direct increased alt-mRNA splicing. If instead nonsense codon recognition occurred in the nucleus, this would be a more direct mechanism to influence alt-mRNA generation. The notion of codon recognition occurring in the nucleus is controversial, but nevertheless, increasingly data support it, including the localization of charged tRNAs and translation factors in the nucleus, the observation that NMD can occur in the nuclear fraction of mammalian cells, and the ability of nonsense codons to increase the levels of unspliced mRNA in the nucleus at or near the site of transcription (*3*, *4*, *16*–*24*). Direct support for nuclear translation comes from a recent study suggesting that about 10% of amino acid incorporation in permeabilized mammalian cells occurs in the nucleus coupled with transcription (*25*).

If nuclear translation is responsible for triggering alt-mRNA up-regulation, how is this achieved? To begin to address this, we assessed whether a nonsense codon only recognizable after normal splicing (a PTC split by an intron) was capable of triggering alt-mRNA up-regulation. We found that this PTC strongly up-regulated the alt-mRNA (Fig. 4), which has important implications for models explaining the effect, because the normal splicing event that generates this PTC precludes alternative splicing. Thus, if a *cis* mechanism is responsible for alt-mRNA up-regulation, this would necessitate that the normally spliced intron be "reverse spliced" after PTC recognition to restore the pre-mRNA and allow subsequent alternative splicing. Alternatively, the normally spliced intron would require being looped out (but not spliced) to permit PTC recognition followed by alternative splicing. Because neither scanning across looped-out introns or reverse splicing are known to occur and both suffer from mechanistic implausibility, we believe that neither of these *in cis* mechanisms are likely. Instead, we suggest that an *in trans* (feedback) mechanism, in which the

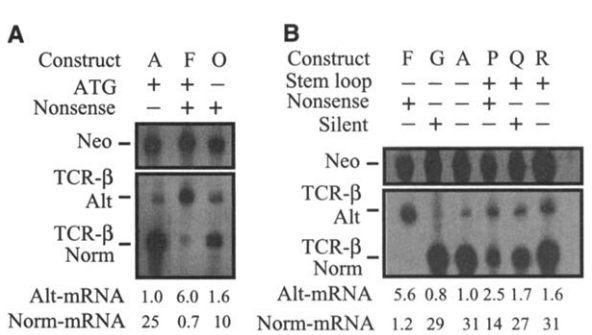

Fig. 3. Alt-mRNA up-regulation in response to nonsense codons depends on a translation-like mechanism. (**A** and **B**) RPA of total cellular RNA (5 μg) from HeLa cells transiently transfected with the constructs shown and analyzed in the same manner as in Fig. 1. TCRβ gene constructs were described in (*12*). Construct O contains a PTC at codon 68 and has mutated initiator ATGs rendered defective as described (*11*). Constructs P, Q, and R contain a stem-loop (ΔG –61 kcal/mol) identical to that previously shown to impede translation (*14*) at a site 42 nt upstream of the initiator AUG in constructs D, E, and A, respectively. Similar results were obtained in at least two independent transfection experiments.

decay of normally spliced PTC-bearing mRNA triggers the increase in alt-mRNA, is more plausible. This could be achieved in many ways; one possibility is that there is massive decay of the normally spliced TCR mRNA at the site of transcription (as a result of PTC recognition there), which consequently alters the concentration of splicing factors and the rate of splicing reactions specifically in that local milieu of the nucleus. Splicing factor levels could either increase or decrease, depending on whether they are prematurely released from degraded PTC-bearing transcripts or are instead degraded along with the TCR transcripts harboring nonsense codons.

Two other well-established examples of nonsense codon–associated altered splicing (*4*) are the up-regulation of exon-skipped transcripts from the fibrillin gene (*5*, *26*) and a splicing enhancer–debilitated version of minute virus of mice (MVM) (*19*). In both cases, frameshifting experiments showed that recognition of in-frame stop codons was responsible for increased levels of alternatively spliced transcript, just as we showed here for TCRβ. However, only nonsense mutations at a single codon position adjacent to exonic splicing enhancers in fibrillin and MVM have been shown to trigger this event (*5*, *19*, *27*). In contrast, we found that TCRβ alt-mRNA was up-regulated in response to nonsense mutations and frameshifts at many different positions in the VDJ exon by a mechanism apparently independent of splicing enhancers. The discovery that classic translation signals (nonsense codons) act through a translation-like pathway to up-regulate alternatively spliced mRNA in the nuclear fraction of cells suggests that a novel mode of regulating gene expression exists in eukaryotic cells. It is important to follow up on initial indications that this regulatory response reduces the severity of some genetic diseases by generating alternative proteins that retain at least partial function (*5*–*7*, *28*).

Note added in proof: Recent further evidence for a relationship between nonsense codons and RNA splicing is the finding that mutation of the stop codon between natural and latent 5′ splice sites can induce alternatively spliced transcripts derived from the latent site (*29*).

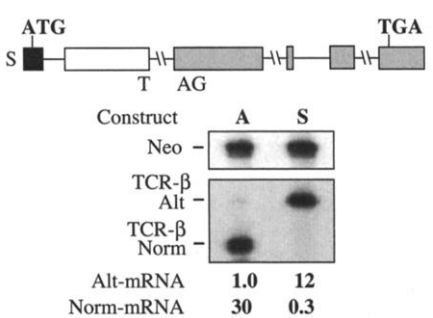

Fig. 4. Alt-mRNA up-regulation is triggered by nonsense codon recognition after normal splicing. RPA of total cellular RNA (5 μg) from HeLa cells transiently transfected with construct S [pAC/NS1 (9)] and analyzed in the same manner as in Fig. 1. Previous analysis (*10*) showed that the normally spliced mRNA from construct S contains the TAG nonsense codon. Similar results were obtained in at least two independent transfection experiments.

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Supporting Online Material

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Figs. S1 to S5
References

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Reversion of B Cell Commitment upon Loss of *Pax5* Expression

Ingvild Mikkola,*† Barry Heavey,* Markus Horcher, Meinrad Busslinger‡

The transcription factor Pax5 is essential for initiating B cell lineage commitment, but its role in maintaining commitment is unknown. Using conditional *Pax5* inactivation in committed pro-B cells, we demonstrate that Pax5 is required not only to initiate its B lymphoid transcription program, but also to maintain it in early B cell development. As a consequence of *Pax5* inactivation, previously committed pro-B cells regained the capacity to differentiate into macrophages in vitro and to reconstitute T cell development in vivo in *RAG2*$^{-/-}$ mice. Hence, Pax5 expression is continuously required to maintain B cell lineage commitment, because its loss converts committed pro-B cells into hematopoietic progenitors with multilineage potential.

The hematopoietic stem cell gives rise to all lymphoid lineages by differentiating through the intermediary stage of the common lymphoid progenitor (*1*). Entry of this progenitor into the B cell pathway depends on the transcription factors E2A, EBF, and Pax5 (BSAP), which control two separate transcriptional programs (*2*). The regulators E2A and EBF coordinately activate the expression of B cell–specific genes (*3*) and are essential for the generation of the earliest B cell progenitors (pro-B cells) (*4*, *5*). However, mere activation of the B lymphoid expression program is not sufficient for commitment to the B cell lineage, because Pax5-deficient pro-B cells still retain the broad developmental potential of early hematopoietic progenitors (*6*, *7*) despite normal expression of E2A and EBF (*8*). Instead, Pax5 restricts the multilineage potential of early progenitors to the B cell pathway by repressing lineage-inappropriate genes and further activating B cell–specific genes (*6*, *7*).

Research Institute of Molecular Pathology, Vienna Biocenter, Dr. Bohr-Gasse 7, A-1030 Vienna, Austria.

*These authors contributed equally to this work.
†Present address: Department of Biochemistry, Institute of Medical Biology, University of Tromsø, N-9037 Tromsø, Norway.
‡To whom correspondence should be addressed: E-mail: busslinger@nt.imp.univie.ac.at