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A splicing-dependent regulatory mechanism that detects translation signals

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Premature termination codons (PTCs) can cause the decay of mRNAs in the nuclear fraction of mammalian cells. This enigmatic nuclear response is of interest because it suggests that translation signals do not restrict their effect to the cytoplasm, where fully assembled ribosomes reside. Here we examined the molecular mechanism for this putative nuclear response by using the T-cell receptor-β (TCR-β) gene, which acquires PTCs as a result of programmed rearrangements that occur during normal thymic ontogeny. We found that PTCs had little or no measurable effect on TCR-β pre-mRNA levels, but they sharply depressed TCR-β mature mRNA levels in the nuclear fraction of stably transfected cells. A PTC split by an intron was able to trigger the down-regulatory response, implying that PTC recognition occurs after an mRNA is at least partially spliced. However, intron deletion and addition studies demonstrated that a PTC must be followed by at least one functional (spliceable) intron to depress mRNA levels. One explanation for this downstream intron-dependence is that cytoplasmic ribosomes adjacent to nuclear pores scan mRNAs still undergoing splicing as they emerge from the nucleus. We found this explanation to be unlikely because PTCs only 8 or 10 nt upstream of a terminal intron down-regulated mRNA levels, even though this distance is too short to permit PTC recognition in the cytoplasm prior to the splicing of the downstream intron in the nucleus. Collectively, the results suggest that nonsense codon recognition may occur in the nucleus.

Keywords: nonsense codons/premature termination codons/RNA stability/T-cell receptor

Introduction

Premature termination codons (PTCs) decrease the levels of most, but not all, known mRNAs (for reviews, see Peltz *et al.*, 1994; Maquat, 1995). Because termination codon recognition requires cytoplasmic ribosomes, it was anticipated that PTCs would cause mRNA decay in the cytoplasm. In agreement with this hypothesis, studies of *Saccharomyces cerevisiae* have shown that PTC-bearing mRNAs are present in ribosomal complexes (He *et al.*, 1993). Furthermore, the *S.cerevisiae* Upf-1 gene product, which participates in the decay of PTC-bearing mRNAs, associates with ribosomes (Atkin *et al.*, 1995). A dominant negative form of a Upf-1-interacting protein, Upf-2, has been shown to interfere with nonsense-mediated mRNA decay when directed to the cytoplasm, not the nucleus (He and Jacobson, 1995). Thus, there is evidence that PTCs mediate the decay of yeast mRNAs in the cytoplasm. However, because it is not technically possible to separate nuclear and cytoplasmic fractions from *S.cerevisiae*, it has not been possible to show directly that nonsense codon-mediated mRNA decay occurs in the cytoplasmic compartment of this organism.

In mammalian cells, in which the nuclear and cytoplasmic compartments can be physically separated, some PTC-bearing transcripts have been shown to exhibit shortened cytoplasmic half-lives (Shyu *et al.*, 1989; Lim *et al.*, 1992). However, the cytoplasmic half-lives of many PTC-bearing transcripts are essentially identical to those of their wild-type counterparts (Urlaub *et al.*, 1989; Cheng *et al.*, 1990; Baserga and Benz, 1992; Cheng and Maquat, 1993; Lozano *et al.*, 1994; Simpson and Stoltzfus, 1994), implying that the low levels of these transcripts are due to a nuclear event. In support of this latter hypothesis, studies have shown that PTCs decrease the levels of many mRNAs in the nuclear fraction of mammalian cells (Baserga and Benz, 1992; Cheng and Maquat, 1993; Belgrader and Maquat, 1994; Lozano *et al.*, 1994; Simpson and Stoltzfus, 1994). This decrease in nuclear mRNA levels imposed by PTCs is not due to a decrease in the rate of gene transcription, as shown by using the nuclear run-on assay (Jäck *et al.*, 1989; Urlaub *et al.*, 1989; Cheng and Maquat, 1993). Thus, the available evidence suggests that PTCs can cause a decrease in mRNA abundance by a nuclear post-transcriptional mechanism. The ability of PTCs to reduce the abundance of transcripts in the nucleus is a paradox since the only known entity that can scan codons is a cytoplasmic ribosome.

The immunoglobulin (Ig) and T-cell receptor (TCR) genes are attractive gene systems for studying the underlying molecular mechanism for this enigmatic nonsense-mediated nuclear regulation. Before their functional expression, these genes undergo programmed DNA rearrangements that engender random addition and deletion of nucleotides at the junctions of the rearranged gene segments (Kronenberg *et al.*, 1986; Fang *et al.*, 1996). Approximately two-thirds of the rearranged segments are not in the proper translational reading frame, resulting in the generation of PTCs. It has been shown that PTC-bearing Ig heavy and light chain genes are underexpressed (Baumann *et al.*, 1985; Jäck *et al.*, 1989; Conner *et al.*, 1994; Lozano *et al.*, 1994). This down-regulation has been reported to occur in the nucleus for the Ig light chain

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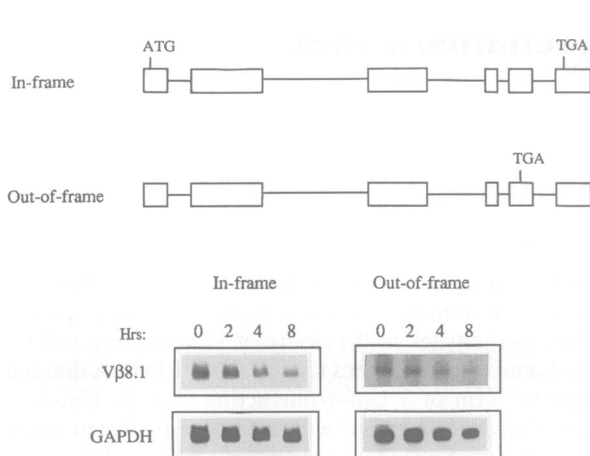

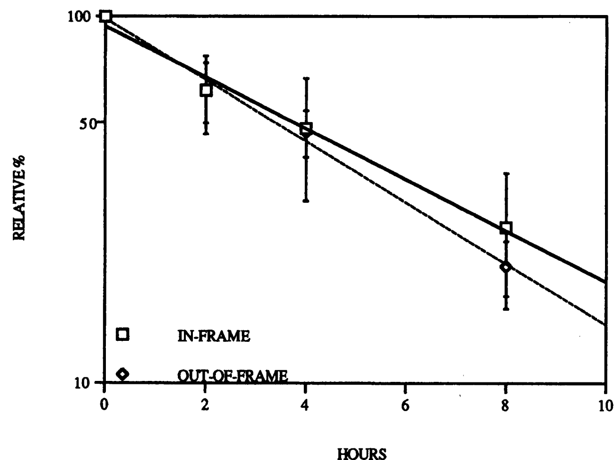

Fig. 1. The down-regulation of PTC-bearing TCR-β transcripts is not the result of a decrease in cytoplasmic decay rate. Northern blot analysis of cytoplasmic RNA (10 μg) isolated at the time points indicated after the addition of DRB from SL12.4 cells stably transfected with an in-frame (pIF) or an out-of-frame (pFS3) construct. The constructs were driven by a TCR-β promoter upstream of the ATG initiation codon in exon 1. The sizes of the exons are: exon 2, 354 nt; exon 3, 375 nt; exon 4, 18 nt; exon 5, 107 nt; and exon 6, 190 nt (this length does not include 3′ untranslated sequences from the vector). The site of transcriptional initiation from the Vβ8.1 promoter has not been precisely determined and thus the length of exon 1 is not known (the initiator ATG is 46 nt upstream of the 3′ end of exon 1). The exposure times for Northern analysis of the in-frame and out-of-frame constructs were 5 h and 2 days, respectively. Hybridization with an exon 2 (Vβ8.1) probe shows the expression of the transfected constructs. Similar loading of RNA at all time points was demonstrated by methylene blue staining of rRNA as described by Wilkinson *et al.* (1990) (data not shown) and by hybridization for stable glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts. The graph shows the average cytoplasmic decay rate for transcripts of the in-frame construct (for five experiments) and the out-of-frame construct (for four experiments). Error bars indicate the standard deviation between the experiments.

mRNA (Lozano *et al.*, 1994). In a recent report we showed that the generation of PTCs in the TCR-β gene, either by frameshift or nonsense mutation, leads to a 10- to 20-fold decrease in the level of cytoplasmic mRNA (Carter *et al.*, 1995). This down-regulation was observed for transcripts of endogenous or stably transfected TCR-β genes in T-cell lines, and from stably or transiently transfected TCR-β genes in the HeLa epithelial cell line. The down-regulation of PTC-containing transcripts could be reversed by a number of different protein synthesis inhibitors with different mechanisms of action: cycloheximide, puromycin, anisomycin, emetine, pactomycin and poliovirus. We interpreted this as showing either that the codon scanner is a ribosome or that an unstable protein is involved in the down-regulatory mechanism. These studies in transfected cell lines were corroborated by studies showing that the nonsense codon-mediated pathway also acts in intact animals: we found that mature (fully spliced) TCR-β transcripts containing PTCs were rarely detected in the normal thymus, even though PTC-bearing TCR-β genes appeared to be transcribed at a normal rate as shown by the accumulation of PTC-bearing pre-mRNAs (Carter *et al.*, 1995).

In the present study, we demonstrate that PTCs cause the down-regulation of TCR-β transcripts in the nuclear fraction of mammalian cells. We then address models that have been proposed to explain how translation signals regulate nuclear mRNA metabolism. A critical issue is whether the template for nonsense codon-mediated events is a spliced, partially spliced or an unspliced transcript. Studies on triosephosphate isomerase (TPI) transcripts from Maquat's laboratory suggest that the template for degradation is fully spliced mRNA (Cheng *et al.*, 1993; Belgrader *et al.*, 1994), while the apparent ability of PTCs to regulate RNA splicing (Naeger *et al.*, 1992; Dietz and Kendzior, 1994; Lozano *et al.*, 1994; Aoufouchi *et al.*, 1996) suggests that the template is partially spliced or unspliced mRNA. Here, we address the intron-dependence of nonsense codon-mediated regulation using the TCR-β gene as a model system. Our results, combined with previous observations from other laboratories, permit us to refine existing models and propose new models to explain the enigmatic effect of PTCs on nuclear events.

Results

Nonsense codons decrease steady-state mRNA levels in the nucleus

To determine if nonsense-mediated degradation of TCR-β transcripts occurs in the cytoplasm, the cytoplasmic decay rates of both wild-type (in-frame) and PTC-bearing (out-of-frame) TCR-β transcripts (Figure 1) were determined by using the transcriptional inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). This experiment and all other experiments in this study were performed with stably transfected cell lines because we were uncertain whether mRNAs derived from transiently transfected DNA, not incorporated into the host genome, would be normally regulated. As shown in Figure 1, the decay rate for PTC-bearing transcripts in the SL12.4 T-cell line was not significantly different from that of wild-type transcripts. This suggested that PTCs do not destabilize cytoplasmic mRNA but rather affect the levels of nuclear mRNA. However, this conclusion must be tempered by the caveat that metabolic inhibitors such as DRB cause a general decrease in cellular transcription which may influence the half-life values determined.

To assess directly whether PTCs cause a decline in nuclear TCR mRNA levels, we examined the nuclear compartment. Transfected HeLa cells were used for this analysis since we were able to obtain more purified nuclei from these epithelial cells than from the SL12.4 T-cell

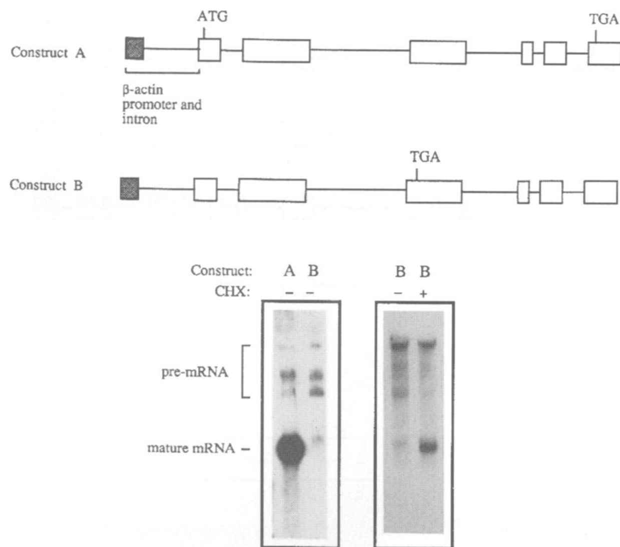

Fig. 2. A PTC dramatically reduces the level of mature mRNA but not pre-mRNA in the nucleus. Northern blot analysis of nuclear RNA (4 μg) isolated from HeLa cells stably transfected with construct A (pAc/IF) or construct B (pAc/FS2) and incubated in the presence or absence of CHX. The constructs were driven by the β-actin promoter (in the black exon); the first exon of the TCR-β gene contains the ATG initiator codon shown (69 nt of TCR-β exon 1 is present in this exon). Hybridization with an exon 2 (Vβ8.1) probe shows the expression of the transfected constructs. Methylene blue staining (Wilkinson *et al.*, 1990) of the blots showed that 32S and 45S rRNA precursors were present at high levels in the nuclear RNA and were absent in cytoplasmic RNA prepared at the same time [data not shown; see Wilkinson and MacLeod (1988) and Wilkinson (1991) for examples of gel analysis of 32S and 45S rRNA precursors in nuclear RNA]. Hybridization with a CHO-A housekeeping gene (Harpold *et al.*, 1979) probe showed that 40-fold less CHO-A mature mRNA was present in the nuclear fraction than in the cytoplasmic fraction. This indicates that no more than 3% of the cytoplasmic RNA is contaminating the nuclear RNA. The bands labeled as 'pre-mRNA' do not represent cross-hybridization with rRNA since nuclear RNA from non-transfected HeLa cells displayed no hybridization with the exon 2 probe (data not shown). The difference in the distribution of pre-mRNAs between the left and right panels is due to a difference in the transfer efficiency of larger transcripts in the two blots.

line (T-cells tend to possess fragile nuclear membranes which can easily be disrupted under conditions required to break open the outer cellular membrane). HeLa cells were deemed appropriate for these studies since we previously showed that PTC-bearing TCR-β transcripts are down-regulated in transfected HeLa cells (Carter *et al.*, 1995). Nuclei were isolated from HeLa cells by a stringent method that involved NP-40 lysis, followed by nuclear membrane stripping with deoxycholate (see Materials and methods). Figure 2 shows that nuclear RNA prepared in this way had dramatically lower levels (>50-fold) of mature mRNA containing a PTC (from construct B) than wild-type mRNA (from construct A). This decrease in the level of mature TCR-β mRNA in the nucleus was not due to a decreased rate of gene transcription, as suggested by the similar levels of pre-mRNAs with and without PTCs (Figure 2) and our previous nuclear run-on studies (Wilkinson and MacLeod, 1988). Instead, the results suggest that PTCs exert a post-transcriptional effect by blocking mRNA splicing or destabilizing mature mRNA in the nucleus. If PTCs act by inhibiting mRNA splicing, then the resulting excess pre-mRNAs must be rapidly degraded, since no appreciable build-up of pre-mRNAs was observed.

Our previous study demonstrated that cycloheximide (CHX) and other protein synthesis inhibitors with different mechanisms of action increased the cytoplasmic levels of TCR-β transcripts with PTCs (Carter *et al.*, 1995). In contrast, CHX had little or no effect on the cytoplasmic levels of TCR-β transcripts lacking PTCs (≤2-fold augmentation). Here, we examined whether CHX reverses the down-regulation of PTC-containing TCR-β mRNAs in the nucleus. Northern analysis of nuclear RNA isolated from cells treated with CHX showed an increase in the level of 1.5 kb fully spliced mRNA from a PTC-bearing gene (Figure 2). This result further supports our contention that PTCs affect nuclear TCR-β mRNA metabolism.

Rescue of mRNA expression by deletion of introns downstream of a PTC

A survey of genomic sequences shows that the vast majority of normal termination codons reside in the final exon (Hawkins, 1988). This observation suggests the hypothesis that termination codons in an internal exon (upstream of an intron) may define that termination codon as premature. To determine whether downstream introns are necessary for the down-regulation of PTC-bearing TCR-β transcripts, we created constructs in which introns downstream of a PTC were deleted. In these studies, the response to CHX treatment was our assay for 'down-regulation', i.e. if the levels of a transcript increased after CHX treatment, then that transcript was considered to be subject to down-regulation.

Figure 3 shows that a construct with introns downstream of a PTC (construct A) gave rise to mature mRNA that was down-regulated (>50-fold), whereas a construct lacking introns downstream of a PTC in the same position (construct B) expressed mature mRNA that was not down-regulated (the 2-fold increase in expression of construct B after CHX treatment is within the range exhibited by non-PTC-bearing transcripts; Carter *et al.*, 1995). The loss of regulation from construct B was unlikely to be due to the removal of specific *cis*-acting elements within the deleted introns because construct C, which lacks the same introns but has a PTC in an exon upstream of another intron, gave rise to a transcript that was down-regulated (8-fold). Collectively, the results show that the presence of one intron downstream of a PTC is sufficient to trigger down-regulation.

To further test the notion that nonsense-mediated mRNA down-regulation requires a downstream intron, we created constructs with a nonsense codon that spans the intron between exons 2 and 3. In construct D, the 'PTC-split-by-an-intron' is followed by three introns, whereas in construct E it is not (Figure 3). The significance of these two constructs is that the nonsense codon is only created after the removal of the intron it spans (i.e. after mRNA splicing) and therefore presumably cannot be recognized as a nonsense codon until the intron is spliced out. We found that PTC-containing transcripts derived from construct D (with downstream introns) were down-regulated whereas transcripts from construct E (lacking downstream introns) were not (Figure 3). This result confirmed that introns downstream of a PTC are required to trigger the down-regulatory response. More importantly, the results

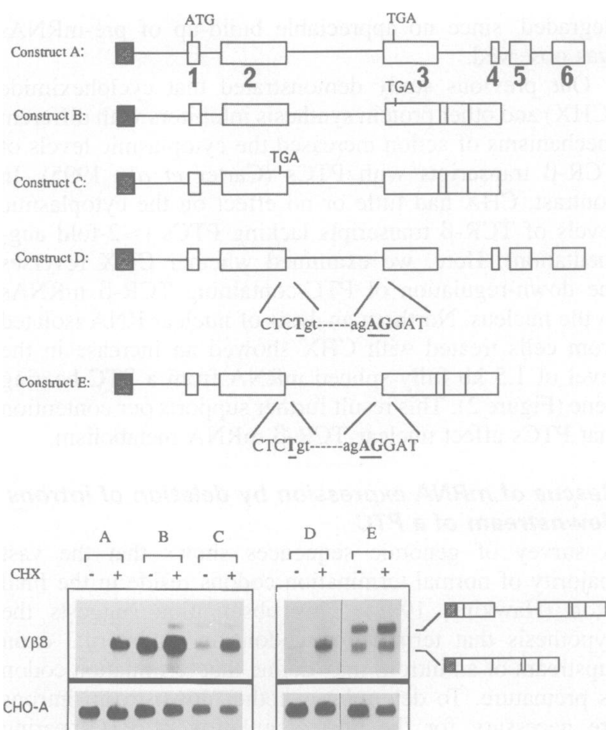

Fig. 3. An intron downstream of a nonsense codon is necessary to trigger the down-regulatory mechanism. Northern blot analysis of cytoplasmic RNA (2 μg) isolated from HeLa cells stably transfected with construct A (pAc/FS2), construct B (pAc/FS2CIVS⁻), construct C (pAc/FS4CIVS⁻), construct D (pAc/NS1) or construct E (pAc/NS1CIVS⁻), and incubated in the presence or absence of CHX. The constructs were driven by a β-actin promoter (in the black exon); the first exon of the TCR-β gene contains the ATG initiator codon shown. Hybridization with an exon 2 (Vβ8.1) probe shows the expression of the transfected constructs, whereas hybridization with the CHO-A housekeeping gene (Harpold *et al.*, 1979) probe reflects the amount of RNA loaded.

suggested that partially spliced transcripts are the target of recognition, since the nonsense codon in construct D is only likely to be revealed after RNA splicing.

Construct E also gave rise to a larger transcript that hybridized with the exon 2 probe. This transcript contained TCR-β intron 2 as assessed by Northern analysis with an intron 2-specific probe (data not shown). This 'intron-included' transcript was probably generated as a result of an inadvertent weakening of the 5′ splice site by the nonsense mutation: the consensus G normally present at the 3′ terminus of exon 2 was replaced with a T in the nonsense-codon version of the construct. Inclusion of intron 2 in construct E transcripts would generate a large terminal exon, which is typical for many vertebrate transcripts. In contrast, construct D gave rise to normally spliced mRNA that lacked intron 2 (Figure 3), perhaps because inclusion of intron 2 would have generated a large internal exon (>1 kb) that would have greatly exceeded the typical upper size limit (0.3 kb) for spliceable exons (Berget, 1995).

A 'normal' termination codon engages the down-regulatory response if introns are introduced downstream

Our results suggested that at least two signals are required to trigger down-regulation: (i) a nonsense codon and (ii) an intron downstream of the nonsense codon. One test of this two-signal hypothesis is to determine whether it is possible to convert a 'normal' nonsense codon into a 'premature' nonsense codon by simply introducing downstream introns. To test this hypothesis, we generated a construct that contained introns (and the adjacent exons, designated in gray in Figure 4) downstream of the termination codon that defines the end of the normal TCR-β reading frame (construct A). In agreement with the two-signal hypothesis, construct A expressed a normally spliced 1.9 kb transcript that exhibited increased levels after CHX treatment (Figure 4), suggesting it was subject to nonsense-mediated down-regulation. The smaller (1.5 kb) mRNA that also accumulated in these cells is an alternatively spliced transcript that is the subject of another report (see Figure 4 legend).

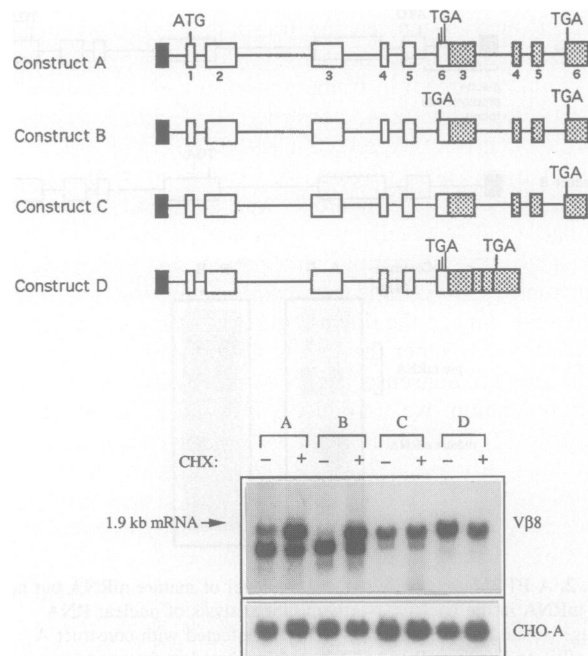

Fig. 4. A 'normal' termination codon can trigger down-regulation if introns are introduced downstream. Northern blot analysis of cytoplasmic RNA (10 μg) isolated from HeLa cells stably transfected with construct A (pS3), construct B (pS1), construct C (pS0) or construct D (pS), and incubated in the presence or absence of CHX. All of the constructs contained the β-actin promoter (in the exon depicted in black) followed by the six exons of the TCR-β gene. The sixth exon was truncated to delete the polyadenylation signal and joined to another copy of exons 3–6 (shaded in gray) that either contained the adjacent introns (constructs A–C) or lacked them (construct D). The three adjacent in-frame stop codons in constructs A and D are designated by the three ticks under the 'TGA' (the first stop codon is a TGA 19 nt downstream of the intron/exon junction). Hybridization with an exon 2 (Vβ8.1) probe shows the expression of the transfected constructs, whereas hybridization with the CHO-A housekeeping gene (Harpold *et al.*, 1979) probe reflects the amount of RNA loaded. In the figure shown, CHX increased the levels of the 1.9 kb transcripts from constructs A and B by 5- and 20-fold, respectively; experiments performed in transiently transfected cells showed that construct A and B are equally subject to down-regulation (M.S.Carter, S.Li and M.F.Wilkinson, manuscript submitted). The 1.9 kb transcript was spliced to include all exons, whereas the smaller (1.5 kb) transcript is an alternatively spliced transcript, based on size and sequence analysis of RT–PCR products (M.S.Carter, S.Li and M.F.Wilkinson, manuscript submitted). The 1.5 kb transcript is generated by triple exon skipping: it lacks the PTC-bearing internal exon as well as the next two exons and thus it contains an open reading frame to the final exon.

To create an open reading frame that extended to the final exon, the natural nonsense codon was mutated and the two downstream in-frame nonsense codons in exon 7 were deleted (Figure 4, construct C). Cells transfected with this construct expressed a 1.9 kb transcript that was not down-regulated (Figure 4). To assess whether the natural nonsense codon alone was sufficient to down-regulate mRNA levels, we generated a construct that contained only the normal termination codon in exon 7 (construct B). This single termination codon was sufficient to strongly engage the down-regulatory response. Finally, to determine whether the introns introduced downstream of the normal nonsense codon were responsible for the down-regulation, we introduced only the adjacent exons (construct D). Cells transfected with construct D expressed high levels of the 1.9 kb mRNA, demonstrating that the down-regulatory effect required the presence of the downstream introns. Collectively, the results provide further support for the notion that a downstream intron is a second signal for nonsense-mediated down-regulation. The results also suggest that a 'normal' termination codon is distinguished from a 'premature' termination codon by the presence of downstream introns, not by the local exonic environment. Finally, since we showed that constructs with a complete TCR-β reading frame can trigger down-regulation, this demonstrates that a truncated TCR-β protein is not the agent that activates the down-regulatory response to nonsense codons.

Cell type specificity of nonsense-mediated mRNA down-regulation

In agreement with the two-signal hypothesis presented in this paper, we previously showed that a nonsense codon in the penultimate TCR-β exon triggered down-regulation in the SL12.4 T-cell line (Carter *et al.*, 1995). However, when we tested a construct with a nonsense codon in the same position in transfected HeLa cells, we found little or no down-regulation (Figure 5). Thus, even though an intron was present downstream of the nonsense codon in this construct, the down-regulatory response was not engaged in this cell line. We considered the possibility that a promoter-specific effect was responsible, as the two constructs used for transfecting these two cell lines differed: the SL12.4 cells had been transfected with a construct driven by the T-cell-specific Vβ8.1 promoter (Carter *et al.*, 1995), whereas the HeLa cells were transfected with a β-actin promoter-driven construct (Figure 5). Since Enssle *et al.* (1993) demonstrated that the nature of the promoter can dictate whether nonsense-mediated mRNA down-regulation occurs, we considered the possibility that the TCR-β and β-actin promoters might differ in their ability to transcribe mRNAs permissive to down-regulation. To test this, we stably transfected the β-actin promoter-driven construct into SL12.4 cells and found that the transcript expressed from this construct was as strongly down-regulated (Figure 5) as when driven by the Vβ8.1 promoter (Carter *et al.*, 1995). We conclude that there is not a promoter-specific effect in SL12.4 T-cells and, most importantly, that the inability of HeLa epithelial cells to down-regulate the construct with a PTC in the penultimate exon is due to differences in cell type-specific factors in HeLa and T-cells.

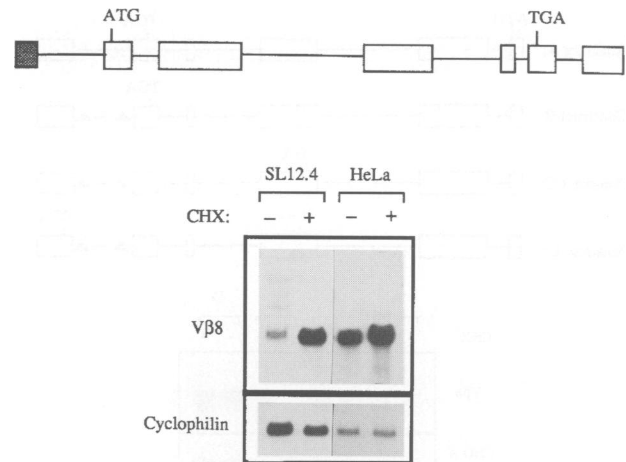

Fig. 5. Cell-type specificity of the down-regulatory response to a PTC. Northern blot analysis of cytoplasmic RNA (2 μg) from SL12.4 and HeLa cells stably transfected with pAc/pFS3 and incubated in the presence or absence of CHX. The constructs were driven by the β-actin promoter (in the black exon); the ATG initiator codon is in the first exon of the TCR-β gene and the TGA codon in the penultimate exon was present at the –65 position relative to the final intron. Hybridization with an exon 2 (Vβ8.1) probe shows the expression of the transfected gene, whereas hybridization with the housekeeping genes cyclophilin (left panel) and CHO-A (right panel) reflects the amount of RNA loaded. CHX increased Vβ8 mRNA levels by >10-fold and <2-fold in SL12.4 and HeLa cells, respectively.

Splice site deletion abolishes the ability of an intron downstream of a premature termination codon to engage the down-regulatory mechanism

Explanations for why a downstream intron is required for a nonsense codon to trigger a decrease in mRNA levels include: (1) spliceosome components must be assembled at the splice sites of the intron to trigger down-regulation; or (2) a *cis*-acting element present internally within the intron targets the mRNA for down-regulation. To distinguish between these possibilities, we deleted the 5′ and 3′ splice sites (including the polypyrimidine tract and the putative branch point) from the intron downstream of a nonsense codon in the penultimate exon (Figure 6, construct B). We found that in contrast to a control gene (construct A), which harbors an intact intron downstream of the nonsense codon, construct B gave rise to an mRNA expressed at high levels. Construct C, which was identical to construct B except that the nonsense codon was in an exon two introns upstream of the mutated intron, was efficiently down-regulated. Therefore, the inactivation of the final intron rescued only those transcripts that harbored a nonsense codon in the immediate upstream penultimate exon, and therefore did not cause a generalized inability to down-regulate all PTC-bearing transcripts. Lastly, we found that construct D, which lacks termination codons upstream of the inactivated intron, expressed high levels of transcripts not subject to down-regulation, as expected. Collectively, our results suggest that at least one functional (spliceable) intron downstream of a nonsense codon, rather than an internal *cis*-acting element within an intron, is necessary to trigger the down-regulatory response.

To extend our study of the relationship between RNA splicing and nonsense-mediated mRNA down-regulation, we ascertained whether the use of cryptic splice sites would rescue PTC-containing transcripts. To address this

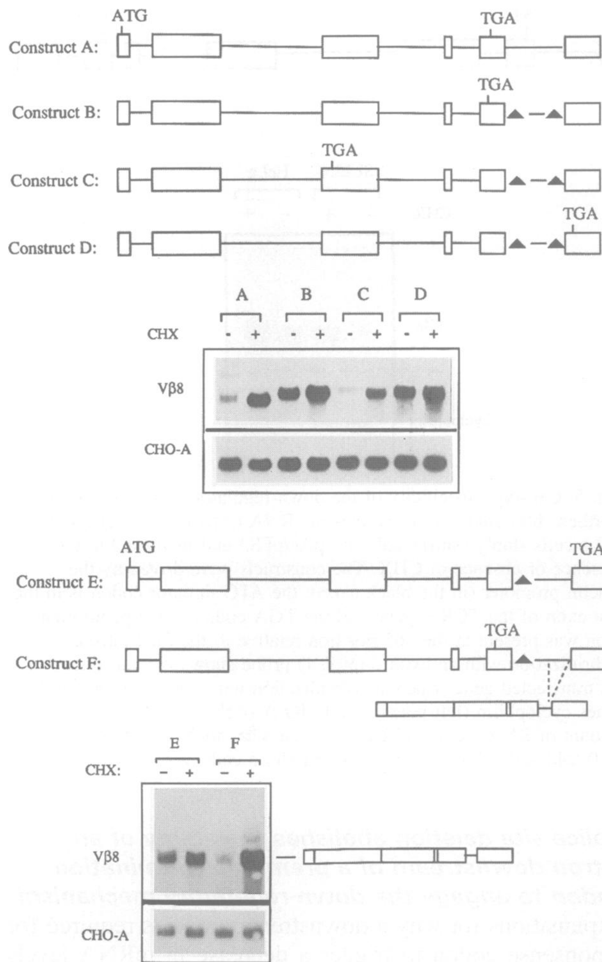

Fig. 6. A functional intron downstream of a nonsense codon is necessary to engage the down-regulatory mechanism. Northern blot analysis of cytoplasmic RNA (2 μg) from SL12.4 cells stably transfected with construct A (pAc/FS3), construct B (pAc/FS3ss⁻), construct C (pAc/FS2ss⁻), construct D (pAc/IFss⁻), construct E (pAc/IFss5⁻) or construct F (pAc/FS3ss5⁻), and incubated in the presence or absence of CHX. The constructs were driven by a TCR-β promoter upstream of the ATG initiation codon in exon 1. Retention of the non-functional intron (IVS5) was verified by Northern analysis with the intron as a probe and by RT–PCR analysis with oligos F and G. Hybridization with an exon 2 (Vβ8.1) probe shows the expression of the transfected gene, whereas hybridization with the CHO-A housekeeping gene (Harpold *et al.*, 1979) probe reflects the amount of RNA loaded. Constructs A, C and F expressed mRNAs that were up-regulated >10-fold by CHX, constructs D and E exhibited <2-fold up-regulation, and construct B showed a 3-fold increase in response to CHX.

question, we deleted the 5′ splice site from an intron that possessed an intact 3′ splice site. This deletion activated a cryptic 5′ splice site 22 nt downstream of the mutated splice site, as determined by cycle-sequencing analysis of RT–PCR products generated from cells transfected with constructs bearing this mutant intron. Northern analysis (Figure 6) showed that the use of this cryptic 5′ splice site permitted down-regulation of transcripts that possessed an upstream (construct F) but not a downstream (construct E) nonsense codon.

A test of the translational–translocation model

The translational–translocation model (Urlaub *et al.*, 1989) proposes that cytoplasmic ribosomes translate mRNAs as

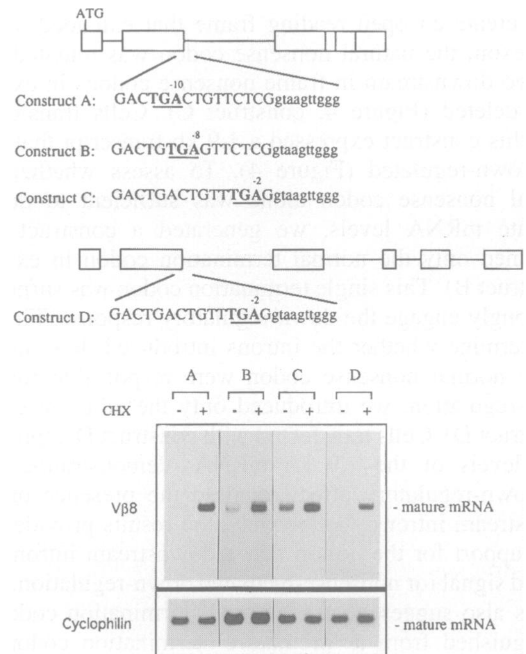

Fig. 7. A nonsense codon as close as 8 nt from the downstream intron still triggers down-regulation. Northern blot analysis of cytoplasmic RNA (2 μg) from SL12.4 cells stably transfected with construct A (pAc/FS4CIVS⁻), construct B (pAc/NS2CIVS⁻), construct C (pAc/NS3CIVS⁻) or construct D (pAc/NS3), and incubated in the presence or absence of CHX. For all constructs, the underlined TGA is in the same frame as the initiator ATG in exon 1 (after intron excision). Note that construct A has a frameshift in exon 2 which renders its reading frame different from constructs B–D. All constructs were driven by a TCR-β promoter upstream of the ATG initiation codon in exon 1. Hybridization with an exon 2 (Vβ8.1) probe shows the expression of the transfected gene, whereas hybridization with the cyclophilin housekeeping gene probe reflects the amount of RNA loaded.

they pass through the nuclear pores, while the 3′ ends of the mRNAs are still in the nucleus undergoing RNA splicing. According to this model, PTC-bearing transcripts are recognized and degraded at the nuclear pore. The model predicts that a nonsense codon located too close to the last intron would fail to permit cytoplasmic recognition prior to splicing of the intron in the nucleus, and thus the down-regulatory mechanism would fail to be engaged. Hence, this model requires that a nonsense codon be further from the last intron than the length of the nuclear pore, which has been estimated to be 15 nm (Alberts *et al.*, 1989) or ~45 nt of linear mRNA. An additional 15–18 nt would be required for the termination codon to be positioned at the P site within the translating ribosome (Beyer *et al.*, 1994). Hence, we estimate that a nonsense codon must be at least 60 nt from a downstream intron to be recognized in the cytoplasm before the intron is spliced out in the nucleus.

To test the translational–translocation model, we used a construct harboring a nonsense codon 10 nt upstream of an intron (Figure 7, construct A). In contrast to the prediction of the translational–translocation model, TCR-β transcripts from construct A were strongly down-regulated (Figure 7). To further test the model, a second construct with a nonsense codon 8 nt upstream of the intron (construct B) was also tested. Transcripts from construct B were also down-regulated. However, a third construct harboring a nonsense codon only 2 nt upstream of the

intron (construct C) gave rise to transcripts that exhibited weak down-regulation, at best (CHX increased mRNA levels by an average of 3-fold). A control construct in which three additional introns were added downstream of the –2 nt PTC (construct D) displayed strong down-regulation, indicating that a nonsense codon at this position is capable of engaging the down-regulatory mechanism. Collectively, these data indicate that the border for nonsense-mediated mRNA down-regulation lies between 2 to 8 nt from the exon/intron junction. These results are inconsistent with the translational–translocation model.

Discussion

Two signals trigger the down-regulatory mechanism

In this report, we investigated the molecular basis for how premature nonsense codons decrease nuclear mRNA levels. We showed that at least one functional (spliceable) intron downstream of a nonsense codon was necessary to down-regulate mRNA expression, based on the following evidence: (1) removal of the introns downstream of PTCs abolished the down-regulatory effect of these nonsense codons (Figure 3); (2) addition of introns downstream of a 'normal' termination codon depressed mRNA levels (Figure 4); and (3) removal of the 5′ and 3′ splice sites from an intron downstream of a PTC prevented down-regulation (Figure 6). Thus, our data support the notion that at least two signals are required to trigger mRNA down-regulation in mammalian cells: a termination codon and a spliceable downstream intron. Further evidence for this notion comes from a study on the dihydrofolate reductase gene that showed that only nonsense codons in internal exons depressed mRNA levels (Urlaub *et al.*, 1989). The possibility that this two-signal mechanism is a general one is supported by the observation that the vast majority of 'normal' termination codons in vertebrate cells are in terminal exons and thus would not trigger down-regulation (Hawkins, 1988). In contrast to vertebrate genes, most *S.cerevisiae* genes lack introns, and thus it is an inappropriate strategy for yeast to use introns as a second signal to trigger the down-regulatory response. Studies have indicated that *S.cerevisiae* uses a *cis*-acting element within the coding region as a second signal to engage nonsense-mediated mRNA decay (Peltz *et al.*, 1993; Zhang *et al.*, 1995).

Studies on TPI transcripts provided the first experimental evidence that introns play a role in nonsense-mediated down-regulation in mammalian cells (Cheng and Maquat, 1993; Cheng *et al.*, 1994). Deletion of the last intron (intron 6) in the TPI gene partially reversed the down-regulation of TPI transcripts harboring a nonsense codon in the upstream exon. Internal deletions within intron 6 partially reversed mRNA down-regulation, thus suggesting that internal *cis*-acting sequences within this intron contribute to nonsense-mediated mRNA decay. In contrast, deletion of the 5′ and 3′ splice sites in intron 6 had little or no effect on nonsense-mediated mRNA decay, implying that a spliceable intron is not the downstream signal that triggers the down-regulatory response. Our results from the TCR-β gene differ in that we found that deletion of the 5′ and 3′ splices did abrogate the ability of the downstream intron to act as a second signal (Figure 6). The basis for the difference in our results is not clear. One possible explanation is that the method of transfection may influence the results obtained. The TPI studies were performed in transiently transfected cells, whereas the TCR-β studies described here were carried out in stably transfected cells. Another possible explanation is that the regulation of TCR-β and TPI transcripts may differ. PTCs decrease TCR-β transcript levels more (⩾10 fold) than TPI transcript levels (3- to 5-fold), and thus it is possible that an additional level of regulation involving a downstream spliceable intron is exerted on TCR-β mRNAs. Lastly, the cell type may also influence nonsense-mediated mRNA down-regulation, as we demonstrated in Figure 5.

The translational–translocation model

Our results permit us to evaluate the validity of models that have been proposed to explain how nonsense codons affect nuclear events. Our results do not support the translational–translocation model (Urlaub *et al.*, 1989). The translational–translocation model proposes that during mRNA export from the nucleus to the cytoplasm, the 5′ end of a transcript is 'read' by the cytoplasmic translational machinery while the 3′ end is still undergoing mRNA splicing. According to this model, if the cytoplasmic ribosome encounters a PTC in the nuclear pore-associated mRNA this ultimately results in the decay of the mRNA. Since we showed that there is a requirement for an intron downstream of a PTC to cause down-regulation (Figures 3 and 4), we predicted that, if the translational–translocation model was correct, then a nonsense codon very close to the final intron should fail to engage the down-regulatory mechanism because the intron would have been spliced-out in the nucleus prior to recognition of the nonsense codon by the translational machinery in the cytoplasm. Contrary to this prediction, we found that a nonsense codon only 8 or 10 nt upstream of the last intron in a TCR-β construct still efficiently down-regulated mRNA expression (Figure 7). Our data complements another line of evidence against the translation–translocation model provided by Kessler *et al.* (1993) who determined that the last intron to be excised from dihydrofolate reductase and adenine phosphoribosyltransferase transcripts tends to be the most 5′ intron.

Our results differ from those obtained with the TPI gene. Cheng *et al.* (1994) determined that nonsense codons 53 nt or more upstream of the 3′ terminal TPI intron, but not 44 nt or closer, triggered down-regulation. One possible reason for the difference in the 'PTC recognition boundaries' upstream of the TCR-β and TPI introns is that the exact site of the border depends on various parameters specific to each intron. For example, the rate at which the downstream intron splices could dictate how close the nonsense codon can be to that intron and still induce down-regulation. TCR-β transcripts appear to be inefficiently spliced in cultured cells based on the observation that unspliced and partially spliced TCR-β pre-mRNAs accumulate in T-cell nuclei, as shown by the relatively insensitive Northern blot technique (Qian *et al.*, 1993a,b). TPI transcripts may splice more efficiently than TCR-β transcripts since TPI pre-mRNAs are apparently only detectable by the more sensitive RT–PCR technique (Cheng and Maquat, 1993).

The marker model

We propose a 'marker model' in which a downstream intron could act as a second signal to trigger down-regulation after the intron has already been spliced-out. The key feature of this marker model is that after removal of introns from a pre-mRNA, the previous location of the introns is 'marked' on the fully spliced mature mRNA. A similar model was proposed by Maquat's laboratory (Cheng *et al.*, 1994) in which they proposed that internal *cis*-elements in introns direct a modification (a 'mark') on adjacent exon sequences. In the marker model that we propose, the 'mark' is independent of internal intronic *cis*-acting elements, but instead requires only that the intron be spliceable (based on the data in Figure 6). Recently, it was shown that specific SR proteins remain preferentially bound to exons after RNA splicing (Blencowe *et al.*, 1995), and therefore, these proteins could serve as 'markers'. Such a nuclear marker protein could remain bound after the RNA is translocated to the cytoplasm, by analogy with known 'shuttling proteins' (Piñol-Roma and Dreyfuss, 1992; Visa *et al.*, 1996). Alternatively, the 'marker' may be a modification in the RNA itself. Many RNA molecules, including tRNAs, are known to undergo extensive post-transcriptional modification.

The notion of a marker that identifies the previous location of an intron permits consideration of a modified version of the translocational–translocation model in which a fully spliced mRNA traversing the nuclear pore is scanned by a cytoplasmic ribosome. According to this modified model, recognition of a nonsense codon and the downstream 'marker' on RNA on the cytoplasmic side of the nuclear pore would trigger mRNA decay before it has completely exited in the nucleus. By this means, an intron only 8 or 10 nt downstream of a nonsense codon (Figure 7) could still act as a second signal to engage the down-regulatory mechanism. The modified model that we propose is similar to the 'co-translational export' model proposed by Maquat (1995) except that in our model we specify a need for a downstream marker to trigger nonsense-mediated down-regulation. A consideration in evaluating such models is that the TCR is a cell surface protein and therefore it would be expected to be translated in the rough endoplasmic reticulum (ER). Previous studies have shown that mRNAs encoding proteins with hydrophobic N-terminal peptides interact with a signal recognition particle that docks the complex to the ER (Verner and Schatz, 1988). Therefore, by analogy with known cell surface and secreted proteins, TCR-β transcripts would have been transferred to the ER before recognition of nonsense codons after codon 70. Although the ER appears to be an extension of the nuclear membrane and therefore may provide an opportunity for translation to resume on TCR transcripts near the nuclear pore before they are fully released from the nucleus, it seems unlikely that all TCR transcripts would be translated near the nuclear pore. Similarly, transcripts encoding the secreted murine urinary protein, which have also been shown to be subject to nuclear nonsense-mediated mRNA decay (Belgrader and Maquat, 1994), would presumably be translated in the rough ER. An important issue for future research is to determine whether a significant proportion of the transcripts encoding cell-surface and secreted proteins undergo a first round of translation on ribosomes bound to ER associated with the nucleus.

Nuclear scanning models

An alternative model that explains our results, and those of others in the field, is the nuclear scanning model (Urlaub *et al.*, 1989). According to this model, mRNAs are scanned for PTCs in the nucleus by an entity that can read codon triplets. Although there is no direct evidence for a nuclear scanner, this model accounts for all of the important features of nonsense-mediated down-regulation of TCR-β transcripts: the decline in mRNA levels occurs in the nucleus, a spliceable intron acts as a second signal to trigger mRNA down-regulation, and a nonsense codon only 8 or 10 nt upstream of the terminal intron is able to elicit mRNA down-regulation. We offer two versions of the nuclear scanning model that explain how a downstream intron could serve as a second signal to trigger down-regulation in the nucleus proper: (i) The downstream intron leaves a 'mark' on spliced mRNA that triggers mRNA decay if the nuclear scanner senses a nonsense codon upstream. This model is the marking model proposed above. Its essential feature is that nonsense codon recognition occurs *after* intron removal. (ii) The second model proposes that the downstream intron recruits spliceosomal components that interact directly with a nuclear scanner paused at a nonsense codon and that this interaction promotes mRNA decay. According to this model, the nuclear scanner translocates across an mRNA much like a ribosome does, reading codon triplets defined by an initiator AUG. We term this the 'splice-and-scan' model, since we propose that the scanner would pause upstream of introns because of the presence of the spliceosomal complexes at these sites, but once the introns are spliced, it would continue scanning. The primary difference between the splice-and-scan model and the marker model is that the former model proposes that nonsense codon recognition occurs *before* intron removal. Consistent with the splice-and-scan model is our evidence that the template for scanning is partially spliced: nonsense codons split by an intron-triggered down-regulation only if followed by other introns (Figure 3). Similarly, Conner *et al.* (1994) showed that Ig heavy chain transcripts bearing a nonsense codon split by an intron are down-regulated only if introns are present downstream.

The splice-and-scan model does not require the excision of introns in a strict 5′ to 3′ direction, since any intron downstream of a nonsense codon, not necessarily the immediate downstream intron, can serve as a second signal for nonsense-mediated down-regulation. The order of intron removal from most pre-mRNAs has not been definitively established since most studies have depended on assaying steady-state levels of intron-bearing transcripts; the relative levels of these RNAs do not necessarily reflect a precursor–product relationship: for example, many intron-bearing mRNAs that accumulate in the nucleus may be dead-end products. Thus, it difficult to assess whether the order of intron excision in mammalian transcripts is consistent with the splice-and-scan model. It should be noted that in *Drosophila* embryos, where nascent transcripts have been directly visualized by electron microscopy, that they splice in a 5′ to 3′ order (Beyer and Osheim, 1988).

It has been reported that nonsense-mediated mRNA decay can be prevented by a hairpin loop that blocks translation or a suppressor tRNA that inhibits recognition of nonsense codons by translation release factors (Belgrader *et al.*, 1993). Although these results may be due to effects on a cytoplasmic ribosome, an equally viable interpretation is that the hairpin loop and the suppressor tRNA block the function of a ribosome or ribosome-like entity in the nucleus. As scanning for an open reading frame is similar to the process of translation, the nuclear scanner may consist, at least in part, of the same components as the translating ribosome. Factors required for translation, including eIF-2a and eIF-4E, accumulate in the nucleus (Lejbkowicz *et al.*, 1992; N.Sonnenberg, personal communication). One potential site for nuclear mRNA scanning by a ribosome-like entity is the nucleolus. Complete or nearly complete 40S and 60S ribosomal subunits, which could serve as nuclear scanners, are assembled in the nucleolus (Scheer and Weisenberger, 1994). Poly(A)$^+$ RNA accumulates in the nucleolus of heat-shocked *Schizosaccharomyces pombe* and mutant *S.cerevisiae* that lack the nucleolar protein Mtr3p and (Kadowaki *et al.*, 1995; Tani *et al.*, 1995), consistent with the notion that the nucleolus is a site for mRNA trafficking (Schneiter *et al.*, 1995). Furthermore, c-*myc*, N-*myc* and *myoD* transcripts have been shown to accumulate in the nucleolus of mammalian cells (Bond and Wold, 1993). However, if all mRNAs were scanned for open reading frames in the nucleolus, one might expect a large accumulation of mRNAs there, and this is not the case (Visa *et al.*, 1993). Furthermore, the nucleolus is not considered to be a site for RNA splicing, yet our evidence suggests that the mechanism that down-regulates mRNAs containing PTCs depends on RNA splicing.

The site we believe is the most likely location for nuclear scanning is the RNA processing arena in the nucleoplasm proper. This localization most easily explains how a downstream intron could act as a second signal to promote the down-regulatory response. It is also consistent with reports suggesting that nonsense codons can induce quantitative or qualitative alterations in RNA splicing: an internal exon in the fibrillin gene has been demonstrated to be skipped when it contains a nonsense codon (Dietz and Kendzior, 1994). Ig and minute virus of mice transcripts have been suggested to exhibit inhibited splicing in response to PTCs (Naeger *et al.*, 1992; Lozano *et al.*, 1994; Aoufouchi *et al.*, 1996). We have observed that introduction of nonsense codons in internal exons of the TCR-β gene cause the accumulation of alternatively spliced transcripts that have excised these nonsense codons (M.S.Carter, S.Li and M.F.Wilkinson, manuscript submitted). A paradox is that, in most of these examples, the splicing of an upstream splice site appears to be regulated by a downstream nonsense codon. This is inconsistent with the splice-and-scan model that we proposed to explain nonsense-mediated mRNA decay because such a model would require splicing of the upstream intron to recognize the nonsense codon. One explanation is that nonsense codons do not directly regulate splicing: instead the increased accumulation of alternatively spliced or unspliced transcripts caused by PTCs reflects alterations in mRNA stability (e.g. increased stability of alternatively spliced mRNAs that lack PTCs). Another explanation is that the splice-and-scan model only accounts for regulation of mRNA half-life by nonsense codons: another mechanism is responsible for nonsense-mediated modulation of RNA splicing. Models that explain how the use of upstream splice site could be regulated by a downstream nonsense codon are presented elsewhere (M.S.Carter, S.Li and M.F.Wilkinson, manuscript submitted).

It is of interest to determine the putative entity in the nucleus that is responsible for codon recognition. Beyer and Osheim (1988) observed ~25 nm ribonucleoprotein particles on *Drosophila* embryo transcripts that indirect evidence suggests are spliceosomes, but it is possible that some or all of them possess codon-reading capabilities. The Sperlings' group have identified a 200S ribonucleoprotein particle in mammalian cells that contains pre-mRNA and splicing factors, but it is larger than the 60S spliceosome identified in extracts used for *in vitro* RNA splicing reactions and thus may contain additional components for scanning and codon recognition (Miriami *et al.*, 1994).

In conclusion, the study of nonsense codon-induced nuclear events challenges our current basic understanding of gene expression. The observed link between RNA processing and translation-like processes suggests that the concept of nuclear and cytoplasmic compartmentalization of some events in eukaryotic cells should be re-evaluated. The nuclear down-regulation of transcripts bearing premature nonsense codons is also of interest because it may be triggered by a surveillance mechanism for inhibiting the expression of aberrant transcripts that could encode deleterious truncated proteins (Pulak and Anderson, 1993). An inherent advantage of a nuclear surveillance mechanism over a cytoplasmic-based mechanism is that cytoplasmic recognition of PTCs requires at least one round of translation for each PTC-bearing mRNA, whereas nuclear recognition does not. Therefore, toxic truncated proteins would accumulate at a lower level with a nuclear surveillance mechanism as compared with a cytoplasmic mechanism. PTCs are generated by a variety of means, including programmed gene rearrangements (such as in TCR and Ig genes), somatic mutation and errors in transcription or RNA splicing. Thus, PTCs are a significant problem, and may require a regulatory mechanism to reduce their negative consequences.

Materials and methods

Cell culture and transfections

The SL12.4 murine T-lymphoma cell clone and HeLa cell line were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Wilkinson and MacLeod, 1988; Carter *et al.*, 1995). SL12.4 and HeLa cells were stably transfected by electroporation as described previously (Carter *et al.*, 1995). Where indicated in the Results, cells were treated with 100 μg/ml CHX for 2 h. For any given construct, at least two independent cell lines were shown to display the regulatory response to CHX shown in the figures. To measure the cytoplasmic mRNA decay rate, stably transfected SL12.4 cells were treated with 67 mM DRB.

Isolation and analysis of RNA

Cytoplasmic RNA was harvested from SL12.4 and HeLa cells as described previously (Wilkinson and MacLeod, 1988; Carter *et al.*, 1995). Nuclear RNA was harvested from HeLa cells by lysis in 0.6% Nonidet P-40, 0.15 M NaCl, 10 mM Tris, pH 8.0, 0.1 mM EDTA; washed in 0.5% deoxycholate, 0.6% Nonidet P-40, 0.15 M NaCl, 10 mM Tris, pH 8.0; lysed in guanidinium isothiocyanate buffer and purified by ultracentrifugation through a 5.7 M cesium chloride cushion (Wilkinson,

1991). RNA was electrophoresed, blotted and hybridized as previously described (Qian *et al.*, 1993a). The relative amount of RNA loaded per lane was assessed by hybridization with the housekeeping genes cyclophilin or CHO-A (Harpold *et al.*, 1979; Qian *et al.*, 1993a).

Oligonucleotides and RT–PCR analysis

The following oligonucleotides corresponding to the mouse TCR-β gene (Gascoigne *et al.*, 1984; Chou *et al.*, 1987) were used: Oligo A (antisense, begins 8 nt downstream from the intron 2 splice donor site), CAACTTACAGAGAACAG; oligo B (antisense, begins 26 nt downstream from the intron 5 splice donor site), CCATTTGTCCATCCT-d11CATGGCCATCAGCACC; oligo C (antisense, begins 20 nt downstream from the intron 5 splice acceptor site), CAGGAATTTTTTT-TCTTGACd37ATGGAGCAGAGGGAAGGG; oligo D (antisense, begins 9 nt downstream from the intron 2 splice donor site), CCAACTTA-CCGAGAACTCACAGTCTGGTTCCTG; oligo E (antisense, begins 13 nt downstream from intron 2 splice donor site), GCTCCCAACTTACCT-CAAACAGTCAGTCTGG; oligo F (sense, exon 5, nucleotides 1–17), CATCCTATCATCAGGGG; oligo G (antisense, exon 6, nucleotides 167–149) GTCTGTTTCAGAGTCAAGG; and oligo H (antisense, exon 6, nucleotides 15–33) GCATAAAATATTGTCGCAGG.

The underlined letters are changes in nucleotide sequence, d denotes deleted nucleotides, and the number after each indicates the number of nucleotides deleted. All mutations were verified by DNA sequence analysis. RT–PCR was performed with Ampli Taq polymerase (Perkin Elmer, Foster City, CA), the appropriate oligonucleotides and Superscript II, according to the manufacturer's instructions (Life Technologies, Inc., Gaithersburg, MD).

Construction of plasmids

pIF, pFS3, pAc/IF, and pAc/FS2: these plasmids were prepared as described previously (Carter *et al.*, 1995).

pAc/FS2CIVS$^-$: the Cβ2 genomic segment in pAc/FS2 was replaced with Cβ cDNA from the clone 86T5 (Hedrick *et al.*, 1984) by first replacing the *Eco*RI genomic fragment with the 86T5 *Eco*RI fragment and then reintroducing part of intron 2 with the *Eco*RV–*Nco*I fragment from Cβ2.

pAc/FS4CIVS$^-$: the 1.2 kb *Sal*I–*Cla*I fragment from pAc/FS2CIVS$^-$ was replaced with a 1.2 kb *Sal*I–*Cla*I fragment from pAc/IF. A frameshift mutation was created by removing 19 nt between the *Eco*RV and *Stu*I sites in exon 2, resulting in a PTC 10 nt upstream of the exon 2 splice donor site.

pAc/NS1: a nonsense mutation was created in the codon that spans intron 2 in pAc/IF by site-directed mutagenesis with oligo A.

pAc/NS1CIVS$^-$: the 1.2 kb *Sal*I–*Cla*I segment from pAc/FS2CIVS$^-$ was replaced with a 1.2 kb *Sal*I–*Cla*I fragment from pAc/NS1.

pAc/FS3: the 2.0 kb *Cla*I–*Bam*HI segment from pAc/IF was replaced with a 2.0 kb *Cla*I–*Bam*HI fragment from pFS3.

pAc/FS3ss$^-$: the 5′ and 3′ splice sites in intron 5 of pAC/FS3 were deleted by site-directed mutagenesis with oligos B and C, respectively.

pAc/FS2ss$^-$ and pAc/IFss$^-$: these plasmids were generated in the same manner as pAc/FS3ss$^-$ by using pAc/FS2 and pAc/IF, respectively, as templates.

pAc/IFss5$^-$: the 5′ splice site in intron 5 of pAc/IF was deleted by site-directed mutagenesis using oligo B.

pAc/FS3ss5$^-$: this plasmid was generated in the same manner as pAc/IFss5$^-$ by using pAc/FS3 as template.

pAc/IFCIVS$^-$: the 1.2 kb *Sal*I–*Cla*I segment from pAc/FS2CIVS$^-$ was replaced with a 1.2 kb *Sal*I–*Cla*I pAC/IF fragment.

pAc/NS2CIVS$^-$: a nonsense mutation in pAc/IFCIVS$^-$ was created at codon 131 by site-directed mutagenesis with oligo D.

pAc/NS3CIVS$^-$: a nonsense mutation in pAc/IFCIVS$^-$ was created at codon 133 by site-directed mutagenesis using oligo E.

pAc/NS3: the 1.2 kb *Sal*I–*Cla*I segment from pAc/IF was replaced with a 1.2 kb *Sal*I–*Cla*I pAc/NS3CIVS$^-$ fragment.

pS3: exons 4–7 from pAC/FS2 were inserted into the *Bsa*BI site (within exon 6) of the pAc/IF construct.

pS1: this plasmid is a derivative of pS3 obtained by deleting 109 nt 5′ of the *Bsa*BI site.

pS0: this plasmid is identical to pS1 except that the natural termination codon in exon 6 (TGA) was converted to a TGC codon using oligo H.

pS: the *Pml*I–*Bsa*BI Cβ1 fragment from the 86T5 cDNA clone (Hedrick *et al.*, 1984) was inserted into the *Bsa*BI site of pAc/IF.

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References

Alberts,B., Bray,D., Lewis,J., Raff,M., Roberts,K. and Watson,J.D. (1989) In Robertson,M. (ed.), *Molecular Biology of the Cell*. Garland Publishing Inc., New York, pp. 422–423.

Aoufouchi,S., Yélamos,J. and Milstein,C. (1996) Nonsense mutations inhibit RNA splicing in a cell-free system: recognition of mutant codon is independent of protein synthesis. *Cell*, **85**, 415–422.

Atkin,A.L., Altamura,N., Leeds,P. and Culbertson,M.R. (1995) The majority of yeast UPF1 co-localizes with polyribosomes in the cytoplasm. *Mol. Biol. Cell*, **6**, 611–625.

Baserga,S.J. and Benz,E.J.,Jr (1992) β-Globin nonsense mutation: deficient accumulation of mRNA occurs despite normal cytoplasmic stability. *Proc. Natl Acad. Sci. USA*, **89**, 2935–2939.

Baumann,B., Potash,M.J. and Köhler,G. (1985) Consequences of frameshift mutations at the immunoglobulin heavy chain locus of the mouse. *EMBO J.*, **4**, 351–359.

Belgrader,P. and Maquat,L.E. (1994) Nonsense but not missense mutations can decrease the abundance of nuclear mRNA for the mouse major urinary protein, while both types of mutations can facilitate exon skipping. *Mol. Cell. Biol.*, **14**, 6326–6336.

Belgrader,P., Cheng,J. and Maquat,L.E. (1993) Evidence to implicate translation by ribosomes in the mechanism by which nonsense codons reduce the nuclear level of human triosephosphate isomerase mRNA. *Proc. Natl Acad. Sci. USA*, **90**, 482–486.

Belgrader,P., Cheng,J., Zhou,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.

Berget,S.M. (1995) Exon recognition in vertebrate splicing. *J. Biol. Chem.*, **270**, 2411–2414.

Beyer,A.L. and Osheim,Y.N. (1988) Splice site selection, rate of splicing, and alternative splicing on nascent transcripts. *Genes Dev.*, **2**, 754–765.

Beyer,D., Skripkin,E., Wadzack,J. and Nierhaus,K.H. (1994) How the ribosome moves along the mRNA during protein synthesis. *J. Biol. Chem.*, **269**, 30713–30717.

Blencowe,B.J., Issner,R., Kim,J., McCaw,P. and Sharp,P.A. (1995) New proteins related to the Ser-Arg family of splicing factors. *RNA*, **1**, 852–865.

Bond,V.C. and Wold,B. (1993) Nucleolar localization of *myc* transcripts. *Mol. Cell. Biol.*, **13**, 3221–3230.

Carter,M.S., Doskow,J., Morris,P., Li,S., Nihm,R.P., Sandstedt,S. and Wilkinson,M.F. (1995) A regulatory mechanism that detects premature nonsense codons in T-cell receptor transcripts *in vivo* is reversed by protein synthesis inhibitors *in vitro*. *J. Biol. Chem.*, **270**, 28995–29003.

Cheng,J. and Maquat,L.E. (1993) Nonsense codons can reduce the abundance of nuclear mRNA without affecting the abundance of pre-mRNA or the half-life of cytoplasmic mRNA. *Mol. Cell. Biol.*, **13**, 1892–1902.

Cheng,J., Fogel-Petrovic,M. and Maquat,L.E. (1990) Translation to near the distal end of the penultimate exon is required for normal levels of spliced triosephosphate isomerase mRNA. *Mol. Cell. Biol.*, **10**, 5215–5225.

Cheng,J., Belgrader,P., Zhou,X. and Maquat,L.E. (1994) Introns are *cis* effectors of the nonsense-codon-mediated reduction in nuclear mRNA abundance. *Mol. Cell. Biol.*, **14**, 6317–6325.

Chou,H.S., Anderson,S.J., Louie,M.C., Godambe,S.A., Pozzi,M.R., Behlke,M.A., Huppi,K. and Loh,D.Y. (1987) Tandem linkage and unusual RNA splicing of the T-cell receptor β-chain variable-region genes. *Proc. Natl Acad. Sci. USA*, **84**, 1992–1996.

Conner,A., Wiersma,E. and Shulman,M.J. (1994) On the linkage between RNA processing and RNA translatability. *J. Biol. Chem.*, **269**, 25178–25184.

Dietz,H.C. and Kendzior,R.J.,Jr (1994) Maintenance of an open reading frame as an additional level of scrutiny during splice site selection. *Nature Genet.*, **8**, 183–188.

Enssle,J., Kugler,W., Hentze,M.W. and Kulozik,A.E. (1993) Determination of mRNA fate by different RNA polymerase II promoters. *Proc. Natl Acad. Sci. USA*, **90**, 10091–10095.

Fang,W., Mueller,D.L., Pennell,C.A., Rivard,J.J., Li,Y.-S., Hardy,R.R., Schlissel,M.S. and Behrens,T.W. (1996) Frequent aberrant immunoglobulin gene rearrangements in Pro-B cells revealed by a bcl-x_L transgene. *Immunity*, **4**, 291–299.

Gascoigne,N.R.J., Chien,Y., Becker,D.M., Kavaler,J. and Davis,M.M. (1984) Genomic organization and sequence of T-cell receptor β-chain constant- and joining-region genes. *Nature*, **310**, 387–391.

Harpold,M.M., Evans,R.M., Salditt-Georgleff,M. and Darnell,J.E.,Jr (1979) Production of mRNA in Chinese hamster cells: relationship of the rate of synthesis to the cytoplasmic concentration of nine specific mRNA sequences. *Cell*, **17**, 1025–1035.

Hawkins,J.D. (1988) A survey of intron and exon lengths. *Nucleic Acids Res.*, **16**, 9893–9905.

He,F. and Jacobson,A. (1995) Identification of a novel component of the nonsense-mediated mRNA decay pathway by use of an interacting protein screen. *Genes Dev.*, **9**, 437–454.

He,F., Peltz,S.W., Donahue,J.L., Rosbash,M. and Jacobson,A. (1993) Stabilization and ribosome association of unspliced pre-mRNAs in a yeast *upf1* mutant. *Proc. Natl Acad. Sci. USA*, **90**, 7034–7038.

Hedrick,S.M., Nielsen,E.A., Kavaler,J., Cohen,D.I. and Davis,M.M. (1984) Sequence relationships between putative T-cell receptor polypeptides and immunoglobulins. *Nature*, **308**, 153–158.

Jäck,H.-M., Berg,J. and Wabl,M. (1989) Translation affects immunoglobulin mRNA stability. *Eur. J. Immunol.*, **19**, 843–847.

Kadowaki,T., Schneiter,R., Hitomi,M. and Tartakoff,A.M. (1995) Mutations in nucleolar proteins lead to nucleolar accumulation of Poly A^+ RNA in *Saccharomyces cerevisiae*. *Mol. Biol. Cell*, **6**, 1103–1110.

Kessler,O., Jiang,Y. and Chasin,L.A. (1993) Order of intron removal during splicing of endogenous adenine phosphoribosyltransferase and dihydrofolate reductase pre-mRNA. *Mol. Cell. Biol.*, **13**, 6211–6222.

Kronenberg,M., Siu,G., Hood,L.E. and Shastri,N. (1986) The molecular genetics of the T-cell antigen receptor and T-cell antigen recognition. *Annu. Rev. Immunol.*, **4**, 529–591.

Lejbkowicz,F., Goyer,C., Darveau,A., Neron,S., Lemieux,R. and Sonenberg,N. (1992) A fraction of the mRNA 5′ cap-binding protein, eukaryotic initiation factor 4E, localizes to the nucleus. *Proc. Natl Acad. Sci. USA*, **89**, 9612–9616.

Lim,S.-K., Sigmund,C.D., Gross,K.W. and Maquat,L.E. (1992) Nonsense codons in human β-globin mRNA result in the production of mRNA degradation products. *Mol. Cell. Biol.*, **12**, 1149–1161.

Lozano,F., Maertzdorf,B., Pannell,R. and Milstein,C. (1994) Low cytoplasmic mRNA levels of immunoglobulin κ light chain genes containing nonsense codons correlate with inefficient splicing. *EMBO J.*, **13**, 4617–4622.

Maquat,L.E. (1995) When cells stop making sense: effects of nonsense codons on RNA metabolism in vertebrate cells. *RNA*, **1**, 453–465.

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.

Naeger,L.K., Schoborg,R.V., Zhao,Q., Tullis,G.E. and Pintel,D.J. (1992) Nonsense mutations inhibit splicing of MVM RNA in *cis* when they interrupt the reading frame of either exon of the final spliced product. *Genes Dev.*, **6**, 1107–1119.

Peltz,S.W., Brown,A.H. and Jacobson,A. (1993) mRNA destabilization triggered by premature translational termination depends on at least three *cis*-acting sequence elements and one *trans*-acting factor. *Genes Dev.*, **7**, 1737–1754.

Peltz,S.W., He,F, Welch,E. and Jacobson,A. (1994) Nonsense-mediated decay in yeast. In *Progress in Nucleic Acids Research and Molecular Biology*. Vol. 47, Academic Press, New York, pp. 271–298.

Piñol-Roma,S. and Dreyfuss,G. (1992) Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. *Nature*, **355**, 730–732.

Pulak,R. and Anderson,P. (1993) mRNA surveillance by the *Caenorhabditis elegans* smg genes. *Genes Dev.*, **7**, 1885–1897.

Qian,L., Theodor,L., Carter,M., Vu,M.N., Sasaki,A.W. and Wilkinson,M.F. (1993a) T Cell receptor-β mRNA splicing: regulation of unusual splicing intermediates. *Mol. Cell. Biol.*, **13**, 1686–1696.

Qian,L., Vu,M.N., Carter,M.S., Doskow,J. and Wilkinson,M.F. (1993b) T cell receptor-β mRNA splicing during thymic maturation *in vivo* and in an inducible T cell clone *in vitro*. *J. Immunol.*, **151**, 6801–6814.

Scheer,U. and Weisenberger,D. (1994) The nucleolus. *Curr. Opin. Cell Biol.*, **6**, 354–359.

Schneiter,R., Kadowaki,T. and Tartakoff,A.M. (1995) mRNA transport in yeast: time to reinvestigate the functions of the nucleolus. *Mol. Biol. Cell*, **6**, 357–370.

Shyu,A.-B., Greenberg,M.E. and Belasco,J.G. (1989) The c-*fos* transcript is targeted for rapid decay by two distinct mRNA degradation pathways. *Genes Dev.*, **3**, 60–72.

Simpson,S.B. and Stoltzfus,C.M. (1994) Frameshift mutations in the v-src gene of avian sarcoma virus act in *cis* to specifically reduce v-src mRNA levels. *Mol. Cell. Biol.*, **14**, 1835–1844.

Tani,T., Derby,R.J., Hiraoka,Y. and Spector,D.L. (1995) Nucleolar accumulation of Poly $(A)^+$ RNA in heat-shocked yeast cells: implication of nucleolar involvement in mRNA transport. *Mol. Biol. Cell*, **6**, 1515–1534.

Urlaub,G., Mitchell,P.J., Ciudad,C.J. and Chasin,L.A. (1989) Nonsense mutations in the dihydrofolate reductase gene affect RNA processing. *Mol. Cell. Biol.*, **9**, 2868–2880.

Verner,K. and Schatz,G. (1988) Protein translocation across membranes. *Science*, **241**, 1307–1313.

Visa,N., Puvion-Dutilleul,F., Harper,F., Bachellerie,J. and Puvion,E. (1993) Intranuclear distribution of Poly(A) RNA determined by electron microscope *in situ* hybridization. *Exp. Cell Res.*, **208**, 19–34.

Visa,N., Alzhanova-Ericsson,A.T., Sun,X., Kiseleva,E., Björkroth,B., Wurtz,T. and Daneholt,B. (1996) A pre-mRNA-binding protein accompanies the RNA from the gene through the nuclear pores and into polysomes. *Cell*, **84**, 253–264.

Wilkinson,M.F. (1991) Purification of RNA. In Brown,T.J. (ed.), *Essential Molecular Biology: A Practical Approach*. IRL Press, Oxford, pp. 69–87.

Wilkinson,M.F. and MacLeod,C.L. (1988) Induction of T-cell receptor-α and -β mRNA in SL 12 cells can occur by transcriptional and post-transcriptional mechanisms. *EMBO J.*, **7**, 101–109.

Wilkinson,M.F., Doskow,J. and Lindsey,S. (1990) RNA blots: staining procedures and optimization of conditions. *Nucleic Acids Res.*, **19**, 679.

Zhang,S., Ruiz-Echevarria,M.J., Quan,Y. and Peltz,S.W. (1995) Identification and characterization of a sequence motif involved in nonsense-mediated mRNA decay. *Mol. Cell. Biol.*, **15**, 2231–2244.

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