A Regulatory Mechanism That Detects Premature Nonsense Codons in T-cell Receptor Transcripts in Vivo Is Reversed by Protein Synthesis Inhibitors in Vitro*

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Gene rearrangement during the ontogeny of T- and B-cells generates an enormous repertoire of T-cell receptor (TCR)1 and immunoglobulin (Ig) genes. Because of the error-prone nature of this rearrangement process, two-thirds of rearranged TCR and Ig genes are expected to be out-of-frame and thus contain premature termination codons (ptcs). We performed sequence analysis of reverse transcriptase-polymerase chain reaction products from fetal and adult thymus and found that newly transcribed TCR-β pre-mRNAs (intron-bearing) are frequently derived from ptc-bearing genes but such transcripts rarely accumulate as mature (fully spliced) TCR-β transcripts. Transfection studies in the SL12.4 T-cell line showed that the presence of a ptc in any of several TCR-β exons triggered a decrease in mRNA levels. Ptc-bearing TCR-β transcripts were selectively depressed in levels in a cell clone that contained both an in-frame and an out-of-frame gene, thus demonstrating the allelic specificity of this down-regulatory response. Protein synthesis inhibitors with different mechanism of action (anisomycin, cycloheximide, emetine, pactamycin, puromycin, and poliovirus) all reversed the down-regulatory response. Ptc-bearing transcripts were induced within 0.5 h after cycloheximide treatment. The reversal by protein synthesis inhibitors was not restricted to lymphoid cells, as shown with TCR-β and β-globin constructs transfected in HeLa cells. Collectively, the data suggest that the ptc-mediated mRNA decay pathway requires an unstable protein, a ribosome, or a ribosome-like entity. Protein synthesis inhibitors may be useful tools toward elucidating the molecular mechanism of ptc-mediated mRNA decay, an enigmatic response that can occur in the nuclear fraction of mammalian cells.

In some TCR and Ig genes, diversity (D) elements are also included in this rearrangement process. The tremendous combinatorial possibilities afforded by this rearrangement mechanism permit the generation of a wide variety of antigen receptors. Additional variability is provided by the enzyme terminal transferase which introduces random nucleotides at the junctions between V, D, and, J elements (1, 2). Variability is also engendered by the low fidelity of the rearrangement event itself; the borders of each element are not fixed, sometimes leading to small deletions at the junctions between the V, D, and J elements. The collective result of these insertional and deletional events is that a large fraction of rearrangement events will generate out-of-frame (nonproductive) genes that contain premature termination codons (ptcs).

Since out-of-frame TCR and Ig genes are commonly generated during normal lymphocyte development, there may exist a mechanism that diminishes the expression of these nonfunctional ptc-containing genes. Consistent with this hypothesis, most Ig and TCR cDNA clones obtained from cDNA libraries have been in-frame (3–6). Studies with cultured cells have provided evidence that out-of-frame Ig transcripts are down-regulated compared with in-frame transcripts (7–10). In addition, several other genes exhibit depressed mRNA levels when mutated to contain ptc (Refs. 11–26; reviewed in Ref. 27). One approach toward analyzing mechanisms responsible for ptc-mediated decay is to study this event in simpler eukaryotic organisms that are more amenable to genetic analysis. Toward this end, it has been shown that Saccharomyces cerevisiae and Caenorhabditis elegans down-regulate ptc-bearing mRNAs (28–32). In S. cerevisiae, the decay of ptc-bearing mRNAs appears to be a cytoplasmic event. Several genes have been identified (the upf and smg series) that participate in ptc-mediated mRNA decay in S. cerevisiae and C. elegans (28–32). Determination of the precise functional role of the upf and smg proteins may contribute greatly to our understanding of this novel regulatory process.

Vertebrates may use a ptc-mediated decay mechanism that differs from the down-regulatory mechanism in lower eukaryotic cells. In vertebrates it has been shown that many ptc-bearing mRNAs are degraded in the nuclear fraction, rather than the cytoplasmic fraction. Preferential nuclear degradation has been demonstrated for ptc-bearing triosephosphate isomerase, dihydrofolate reductase, β-globin, v-src, major urinary protein (MUP), and Ig transcripts (9, 12, 14–17, 20, 23, 24, 26). This is an unexpected observation, since termination codons are generally considered to be recognized only by ribosomes in the cytoplasm. Several models have been put forward to explain how ptc could affect nuclear events (see “Discussion”). In order to elucidate the novel mechanism that is responsible for the recognition and decay of ptc-bearing mRNAs in verte-
brates, it is necessary to identify a system that efficiently down-regulates such aberrant mRNAs. In addition, the identification of an approach to reverse the down-regulatory response would be a useful tool toward understanding the underlying mechanism and its associated components.

In our previous work (33, 34) we showed that mature mRNA derived from a transcriptionally active TCR-β gene in the SL12.4 T-lymphoma cell clone failed to accumulate in the cytoplasm, although TCR splicing intermediates were easily detectable in the nucleus. We showed that incubation of SL12.4 cells with several different protein synthesis inhibitors dramatically induced the accumulation of mature TCR transcripts in the cytoplasm (35). In the present study, we show that the rearranged TCR-β gene in the SL12.4 T cell clone is out-of-frame and therefore contains ptc. We then demonstrate in transfection experiments that the inducibility by protein synthesis inhibitors is exclusively restricted to ptc-bearing TCR-β transcripts. Protein synthesis inhibitors efficiently reversed the down-regulatory effect of ptc in any of a number of TCR exons. The reversal of ptc-mediated mRNA decay by protein synthesis inhibitors was a general response that was not restricted to particular stages of T-cells, nor was it restricted to lymphoid cells. The ability to reverse this response may be useful toward studying the molecular mechanism of nonsense codon-mediated decay in the nucleus of vertebrate cells.

Since nonfunctional ptc-bearing TCR genes are generated as a part of normal thymic ontogeny, we then asked if this down-regulatory mechanism operates in normal T-cells in vivo. We show that although ptc-bearing TCR-β genes are transcribed normally and give rise to normal levels of pre-mRNA in the thymus, the levels of mature mRNA derived from these nonfunctional genes is selectively depressed. This constitutes the first in vivo evidence in higher eukaryotes that the ptc-mediated mRNA decay pathway selectively depletes mature mRNA levels, not pre-mRNA levels. Our findings reveal new insights into the ptc-induced down-regulatory mechanism and serve to further generalize the importance of this mechanism.

MATERIALS AND METHODS

Cell Culture, Transfections, and Northern blot Analysis

The SL12.4, R54.2, and AKR1 murine T-lymphoma cell clones were cultured as described previously (33, 36). Cells were treated with cycloheximide (CHX) for 2 h at a concentration (100 μg/ml) that we have previously shown is sufficient to inhibit protein synthesis (as assessed by [35S]methionine incorporation) by >95% (33, 36). Stable transfections of the T-lymphoma cell clones were performed by electroporating 5–20 μg of DNA in 1 × HBS buffer (280 mM NaCl, 1.5 mM Na2HPO4, 50 mM Hepes, pH 7.05) with a Moonlight Cat Door electroporator (Seattle, WA) at 2900–3200 V. After transfection, the cells were split at different dilutions (1:5 to 1:100) among the wells in a 24-well plate. The antibiotic G418 (800 μg/ml) was added the following day, and after 12–20 days of selection in the presence of the antibiotic, all wells with viable cells were tested for expression of the constructs by Northern blot analysis. For any given construct, at least two independent wells were shown to express the construct and display the regulatory response to CHX shown in the figures. Cytoplasmic RNA from the T-lymphoma cell lines was prepared as described (33).

HeLa cells were stably transfected with 5–20 μg of DNA by calcium phosphate precipitation (37), and individual stably transfected clones were isolated for Northern blot analysis. Transient transfections were performed by electroporation with an Invitrogen Electroporator II according to the manufacturer’s instructions (Invitrogen Corporation, San Diego, CA). Total cellular RNA was harvested 2 days after transfection. For polio virus infection, near confluent HeLa cells were infected with polio virus type I (Mahoney strain) at a concentration of 75 plaque-forming unit/cell. The virus was incubated with the HeLa cells for 30 min at room temperature, followed by addition of Dulbecco’s modified Eagle’s medium without serum (designated time point 0 of the infection). Total RNA from HeLa cells was prepared as described (33). Cytoplasmic RNA was prepared by lysis in 0.6% Nonidet P-40, 0.15 mM NaCl, 10 mM Tris (pH 8), 0.1 mM EDTA. RNA was electrophoresed, blotted, and probed as described (34). The relative amount of RNA loaded per lane was assessed by methylene blue staining of the RNA content (38) as well as hybridization with the housekeeping genes cyclin D1 and CHF-A (39).

Oligonucleotides

The following oligonucleotides were used: Vβ5, sense orientation (ends at the 9 position with respect to the initiator ATG of Vβ3.1, CAAAGACGATCCTGA; Vβ5, antisense, Vβ5 exon 1, nucleotides 1–17, 2 nucleotide mismatch to generate Sod1 site), GAGAGCTCAAACAAGGAGAC- TCTAGAT; E1-A, antisense (Gγ1 exon 1, nucleotides 2–23, induces Sod1 site at 5′ end), ACACCGGCGTGGTAGCTCATT- TCTCAGAT; E1-B, antisense (Gγ1 exon 2, nucleotides 25–47, 2 nucleotide mismatch to generate Sod1 site), GAGGAAGATTACCAACAGAGGACCTT; E2, antisense (Gγ1 exon 1, nucleotides 1–17), TGAGGTAAT- CCCACAGT; glob, antisense (corresponds to amino acids F42–P36 of human β-globin), AAGAACCTTACAGTTCAGAA. The sequence information used to design the following oligonucleotides was obtained from the following sources: Vβ1-Vβ2, Vβ8, and Vβ8.4 (40), 41, and 42 (43, 44). The Vβ8 oligo- nucleotide was designed on the basis of our own sequence analysis of cloned Vβ8.1 genomic DNA.

Reverse Transcriptase-PCR Analysis and Sequencing

Total cellular RNA (1 μg) from fetal (day 16 post-coitum) or adult thymus from BALB/c mice was used to prepare cDNA. To analyze Vβ8 mRNAs, reverse transcriptase-PCR was performed as described (33) using the oligo(dT)18 primer for cDNA synthesis. The following oligonucleotides for the PCR reaction (40 cycles). Mature Vβ8.1-Vβ8.2, and Vβ8.3 amplifications were performed using oligonucleotides Vβ8.1-A + E1-A. Pre-mRNA transcripts were amplified using either oligonucleotide Vβ8.2-A + I-Jβ-A oligonucleotide Vβ8.3-A + I-Jβ-B, or by nested PCR using Vβ8.2-A + I-Jβ-B (10 cycles), followed with Vβ8.3-A + I-Jβ-B (30 cycles). Anchored PCR was performed according to the manufacturer’s instructions (Life Technologies, Inc., Gaithersburg, MD) for 5′ rapid amplification of cDNA ends. In brief, cDNA was generated using a primer complementary to TCR-β sequences, the cDNA was dC-tailed with terminal transferase, then PCR was performed using an “anchor primer” (complementary to the C-tail) and an oligonucleotide complementary with TCR-β mRNA. For TCR-β mature mRNA, the E1-A oligonucleotide was used for cDNA synthesis and the E1-B oligonucleotide was used for PCR. For TCR-β pre-mRNA, the I-Jβ oligonucleotide was used to generate cDNA, and the I-Jβ-A was used for PCR. The PCR products were subcloned into Bluescript KS (Strategene Inc.), followed by dideoxy sequencing analysis. The sequences obtained were compared with known Vβ, Dβ, Jβ, and Cγ1 sequences (40, 43) to determine whether the V(D)Jβ exon was in-frame with respect to the Cγ1 first exon. N-region diversity was observed at the V, D, and J junctions of most PCR products. The sequence of the V, D, and J elements was as reported in the literature, except that polymorphisms were sometimes noted for Jβ1 (nucleotide 14 (C) was deleted) and Jβ1 (a G was inserted after the Vβ sequence at position 9). When several identical sequences were obtained from a given PCR reaction, only one sequence was considered in the results shown in Table I.

To obtain the sequence of the Vβ5.1-Jγ5.1 transcript in the SL12.4 cell clone, cDNA was prepared using 1 μg of RNA from SL12.4 cells treated with 6 μg with CHX (100 μg/ml). Oligo(dT) was used as a primer for cDNA synthesis, and the oligonucleotides Vβ8.1 and E2 were used for PCR.

Plasmid Constructions

pVδ8Bc2Jβ—A productively rearranged Vδ8.1-Dδ2-Jβ-Cγ2 genomic fragment was subcloned into pUC13 (obtained from M. Blackman, P. Mar- rak, and J. Kappler).

pNED—the β-actin promoter region of pHAPr-1 (43) was removed by EcoRI/BamHI digestion and replaced with the multiple cloning site of Bluescript KS present on a 0.5 kb PvuII fragment.

pF—An 18-kb KpnI/Sall fragment, derived from pVδ8Bc2, that contains Vδ8.1-Dδ2-Jβ-Cγ2 and the downstream enhancer, was subcloned as.
into pNEO.

pVDJ—A 3.2-kb KpnI/ClaI fragment, containing the V<sub>8.1</sub>D<sub>3</sub> region, subcloned into a modified version of Bluescript KS that lacked sequences between the EcoRV and Smal sites.

pRV—10-nucleotide XhoI linkers were introduced into the unique EcoRV site within the V<sub>β</sub>1 exon of pVDJ. The number of XhoI linkers inserted into individual recombinant clones was determined by DNA sequence analysis.

pFS1—A 3.2-kb KpnI/ClaI fragment that contains the V<sub>8.1</sub>D<sub>3</sub> region was released from pRV and used to replace the analogous region of pV<sub>β</sub>B<sub>3</sub>I. An 18-kb KpnI/Sall fragment that contains the entire gene was then excised and subcloned into pNEO.

pS<sub>1</sub>Stu was generated analogously to pRV except that a single pStu—KpnI linker was introduced into the unique Stul site.

pCD2—A 2-kb PstI C<sub>β</sub>2 fragment subcloned into the PstI site of pKS.

pF52—The V<sub>8.1</sub>D<sub>3</sub>C<sub>β</sub>2 region of pStu was subcloned into pCD2 cut with KpnI and ClaI. A 0.8-kb BamHI fragment containing the TCR-β enhancer was then subcloned into the unique BamHI site downstream of C<sub>β</sub>2. The V<sub>8.1</sub>D<sub>3</sub> region was then excised with Xbal and KpnI, and this 6-kb fragment was subcloned into pNEO.

pFS5—A fragment containing C<sub>β</sub>2 exons 1 and 2 was released from pCD2 by linearizing with BglII, followed by S1 nuclease treatment and digestion with XhoI. This 1.25-kb blunt-end/XhoI fragment was subcloned into Bluescript KS at the XhoI and EcoRV sites. To introduce a frameshift, the plasmid was then linearized with Ncol, made blunt with Klenow enzyme, and religated to create a four nucleotide insertion at the Ncol site (confirmed by sequence analysis).

pPrV<sub>β</sub>J8—A promoterless V<sub>β</sub>8.1 fragment was generated by PCR using pVDJ as a template and oligonucleotides V<sub>β</sub>C and -D. The PCR fragment was blunt-ended and inserted into Bluescript KS at the Stul and EcoRV sites.

pPrV<sub>β</sub>D<sub>1</sub>—pVDJ was digested with NdeI and BamHI and the 2.8-kb fragment was inserted into the NdeI and BamHI sites of pPrV<sub>β</sub>J8.

pPrStu—pPrStu was created analogous to pPrV<sub>β</sub>D<sub>1</sub> except that a pStu NdeI/BamHI fragment was used.

pAc1F—pPrV<sub>β</sub>D<sub>1</sub> was digested with Sall and BamHI, and the 3.2-kb fragment was inserted into the Sall and BamHI sites of pH<sub>Ac</sub>Pror-1.

pAcF52—pAcF52 was created analogous to pAc1F except that a pPrStu Sall/BamHI fragment was used.

pAc1D—pAcG<sub>β</sub>1α is identical to pAc1F except that it contains a deletion between the PmlI site in C<sub>β</sub>2 exon 1 and the BsaBI site in C<sub>β</sub>2 exon 4.

pGLOB—The human β-globin gene was isolated from pIGG339/pSP64 (Promega, Madison, WI), then released with HindIII and PstI, subcloned into pSP72, and expressed in day 16 fetal thymus. At day 16 of gestation, the thymus is dominated by immature double-negative (CD4<sup>-</sup>CD8<sup>-</sup>) and double-positive (CD4<sup>+</sup>CD8<sup>-</sup>) thymocytes that might be subject to different regulation than more mature thymocytes. Our analysis revealed that TCR-β pre-mRNAs were commonly out-of-frame (38%), but mature mRNAs rarely were (4%; Table I). Collectively, the results show that both fetal and adult thymus actively transcribe in-frame and out-of-frame TCR-β genes but selectively depress the levels of mature out-of-frame transcripts.

pGLOB39—A β-globin fragment containing exons 1 and 2 was released from pIGG339/pSP64 with HindIII and Dral, the ends were filled in with Klenow enzyme, and the 1.1-kb fragment was subcloned into Bluescript KS at the EcoRV site. A nonsense mutation (UAG) was created at codon 39 using the Bio-Rad mutagenesis kit (Hercules, CA) with the oligonucleotide glob, the 1.1-kb insert was released with HindIII and BamHI and subcloned into pNEO. This construct was then linearized with BamHI and a 1.6-kb fragment containing the 3′ end of β-globin was subcloned into the BamHI site of pCD2.

pUA<sub>E</sub>—Codon 50 in pVDJ was mutated using the oligonucleotide V<sub>β</sub>E<sub>E</sub> by the approach described for pGLOB39. A 3.2-kb KpnI/ClaI fragment containing this mutation was used to replace the equivalent region in pFS2.

pUAC—pUAC was generated in the same manner as pUA<sub>E</sub>.

**RESULTS**

**Down-regulation of Out-of-frame TCR mRNA Levels by Premature Termination Codons**—To study the mechanism responsible for the decreased abundance of out-of-frame TCR-β mRNAs, we assessed the expression of TCR-β constructs in stably transfected T-cell lines. First, the expression of several frameshifted versions of a V<sub>β</sub>8.1D<sub>3</sub>C<sub>β</sub>2 (V<sub>β</sub>8.1) gene were compared with the in-frame version of this gene. These frameshifted genes possessed ptc in either the V<sub>β</sub>D<sub>1</sub> exon, the C<sub>β</sub>2 exon, or the C<sub>β</sub>2.3 exon (the second, third, or fifth exons of the TCR-β gene, respectively; see Fig. 1). Northern blot analysis revealed that the in-frame version of the V<sub>β</sub>8.1 gene was expressed at high levels in the SL12.4 T-lymphoma cell clone (Fig. 1). In contrast, all out-of-frame V<sub>β</sub>8.1 constructs were expressed at very low levels in SL12.4 cells (Fig. 1). Transcript levels for the three frameshifted constructs were all at least 10-fold lower than for the in-frame construct, as assessed by densitometry.

The basis for the decrease in mRNA levels may be due to recognition of the premature nonsense codon by a ribosome,
we tested whether the addition of protein synthesis inhibitors could reverse the down-regulation. Incubation with the protein synthesis inhibitor CHX dramatically induced mRNAs derived from the three constructs that possessed ptc's (Fig. 1). In contrast, CHX had little or no effect on mRNA expression from the in-frame construct (Fig. 1). Thus, incubation with CHX specifically reversed the down-regulation of out-of-frame TCR-β transcripts.

Two of the out-of-frame VDJβ constructs shown in Fig. 1 were generated by adding a 10-nucleotide Xho linker at different sites within the exon. To test if the reading frameshift caused by the Xho linker was depressing expression, we tested the effect of adding three Xho linkers to the normal gene (construct A); this would maintain the correct frame by introducing 30 nucleotides. The construct containing three Xho linkers at the EcoRV site of the VDJβ exon was expressed at high levels, regardless of the presence of CHX (data not shown). In contrast, when four Xho linkers were introduced at the EcoRV site so that the construct was now out-of-frame, the expression pattern was the same as the construct with a single Xho linker (e.g. CHX-inducible).

Next, a nonsense codon was introduced into an in-frame TCR-β gene to determine if a ptc could trigger the down-regulatory response without a frameshift. A UAA nonsense codon was introduced in place of a UAU codon within the VDJβ exon. This single nucleotide mutation was sufficient to efficiently down-regulate mRNA levels (Fig. 2). Treatment with CHX reversed the down-regulation (Fig. 2). As a control, the UAU codon was converted to a synonymous UAC codon. This construct expressed high levels of TCR-β mRNA regardless of whether CHX was present or absent (Fig. 2).

To determine whether the down-regulation mechanism displayed stage specificity in its effects, an out-of-frame Vβ8.1 gene was transfected into two T-lymphoma cell clones (RS4.2 and AKR1) that display a more mature phenotype than the SL12.4 cell clone used in our study. Although the RS4.2 cell clone expresses a double negative phenotype (CD4-CD8-) like SL12.4 cells, it constitutively expresses TCR-β transcripts and displays a pattern of cell surface markers indicative of a more mature phenotype (35, 46). The AKR1 clone expresses an even more mature phenotype: it is a double-positive cell clone that constitutively expresses both TCR-α and -β mRNA (33). We observed that the transfected out-of-frame Vβ8.1 gene was expressed at very low levels in stably transfected lines of either RS4.2 or AKR1 cells (Fig. 3). CHX treatment induced the out-of-frame Vβ8.1 transcript in both cell lines (Fig. 3). Thus, the RS4.2 and AKR1 T-cell clones exhibit the same down-regulatory response as the less mature SL12.4 cell clone.

Allelic Specificity of the Down-regulatory Mechanism—In past studies, we showed that although the SL12.4 T-lymphoma cell clone possesses a fully rearranged endogenous TCR-β gene that is transcriptionally active (as assessed by nuclear run-on analysis) and gives rise to abundant nuclear pre-mRNAs (detected by Northern blot analysis), it expresses very low levels of mature mRNAs in the cytoplasm (33, 34). Since we found that CHX induces TCR-β transcripts in this cell clone by a post-transcriptional mechanism (33), we considered the possibility that the rearranged TCR-β gene in the SL12.4 cell clone is out-of-frame. Sequence analysis of reverse transcriptase-PCR products generated from these cells showed that indeed SL12.4 cells expressed an out-of-frame Vβ8.1 gene possessing a ptc in the first Cβ exon (Fig. 4). Northern blot analysis showed that SL12.4 cells expressed transcripts from this nonproductively rearranged Vβ8.1 gene only after CHX treatment (Fig. 4). In contrast, an in-frame Vβ8.1 gene stably transfected into the same cell clone generates high levels of transcripts whether CHX was present or not (Fig. 4). Since the in-frame Vβ8.1 and out-of-frame Vβ8.1 mRNAs are differentially regulated in the same cell clone, this demonstrates that the down-regulatory mecha-
nism is allelic-specific.

To further confirm allelic specificity, TCR-β mRNA expression was examined in the BW5147 cell clone, which possesses a productively rearranged Vβ1 gene and a nonproductively rearranged Vβ5.2 gene (46). Northern blot analysis showed that levels of the mature 1.3-kb Vβ1 transcript were high, while the 1.3-kb Vβ5.2 transcript was barely detectable (at least a 30-fold difference in expression; data not shown). This further supports the notion that the down-regulatory mechanism discriminates between in-frame and out-of-frame transcripts within a single cell.

To assess the cell type specificity of the ptc-mediated regulatory mechanism, in-frame and out-of-frame TCR-β constructs were transfected into HeLa (epithelial) cells. For these experiments, the TCR-β promoter was replaced with the ubiquitously transcribed β-actin promoter (Fig. 5A). Fig. 5B shows that the presence of a ptc strongly down-regulated TCR-β mRNA levels in stably transfected HeLa cells. Addition of CHX selectively reversed the down-regulation of the out-of-frame transcripts and had no discernible effect on in-frame transcript levels (Fig. 5B). The kinetics of induction triggered by CHX was rapid. An increase in out-of-frame TCR-β mRNA levels was evident as early as 0.5 h following CHX treatment (Fig. 5B).

We next assessed whether transiently transfected TCR-β genes are subject to the same down-regulatory response as stably integrated TCR-β genes. To our knowledge, a comparative study of the ptc-mediated regulatory mechanism in transiently and stably transfected cells has not been reported previously. HeLa cells were chosen for the transient transfection experiments, since they are more efficiently transfected than SL12.4 cells. Fig. 5D (left panel) shows that an out-of-frame TCR-β construct was expressed at dramatically reduced levels compared with the in-frame construct in transiently trans-
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HeLa cells. We observed that the wild type TCR-β-globin construct was expressed at low levels relative to a co-transfected in-frame mutant TCR-β-globin gene that contained a deletion (construct C, Fig. 5A) to permit independent analysis of its expression (Fig. 5D, right panel). CHX only weakly induced the out-of-frame transcripts after either 2 h or 4 h (Fig. 5D) incubation in these transiently transfected cells. We conclude that although the down-regulatory mechanism is able to act efficiently on ptc-bearing transcripts derived from "free DNA" in transiently transfected cells, CHX is not able to efficiently reverse this down-regulatory response.

Nonsense Codon-mediated mRNA Decay Is Reversed by Protein Synthesis Inhibitors with Different Mechanisms of Action—Since CHX may induce out-of-frame TCR-β transcripts by a nonspecific mechanism independent of its effects on protein synthesis, we tested the effect of other known protein synthesis inhibitors. Fig. 6 shows that the protein synthesis inhibitors anisomycin, emetine, pactamycin, and puromycin induced out-of-frame TCR transcripts in stably transfected HeLa cells as effectively as CHX. We also tested the effect of polio virus infection, since polio virus is known to be an efficient inhibitor of translation (47). Infection with polio virus caused a time-dependent increase in out-of-frame TCR-β transcripts in HeLa cells (Fig. 7). In contrast, polio virus infection caused a gradual decline of in-frame transcript levels, presumably due to the block in transcription known to be triggered by polio virus infection (48).

CHX Reverses the Down-regulation of β-Globin mRNA That Possesses a Premature Termination Codon—To determine whether CHX has a general capacity to reverse nonsense codon-mediated down-regulation, its effect on the expression of β-globin mRNA was assessed. A ptc was introduced at codon 39 of the human β-globin gene, since that has been previously shown to depress globin mRNA levels (16, 17, 26). The mutant and wild type β-globin constructs were stably transfected into HeLa cells. We observed that the wild type β-globin construct was expressed at high levels in HeLa cells, regardless of whether CHX was present or not, while the ptc-bearing construct was expressed at high levels only if the cells were incubated with CHX (Fig. 8). Thus, CHX is able to reverse the down-regulatory effect of ptc's in nonlymphoid transcripts expressed in nonlymphoid cells.

Fig. 6. Protein synthesis inhibitors with different mechanisms of action all reverse the down-regulatory response. Northern blot analysis of cytoplasmic RNA (2 μg) from HeLa cells stably transfected with construct B from Fig. 5. The cells were incubated with cycloheximide (100 μg/ml), pactamycin (3 μg/ml), anisomycin (100 μg/ml), emetine (300 μg/ml), and puromycin (300 μg/ml), or medium alone for 2 h. Hybridization with the Vβ8.1 probe shows the expression of the transfected genes, while hybridization with the CHO-A probe shows the amount of RNA loaded.

Fig. 7. Polio virus infection selectively induces ptc-bearing TCR transcripts. Northern blot analysis of cytoplasmic RNA (2 μg) from HeLa cells stably transfected with constructs A and B (from Fig. 5) followed by infection with polio virus. Hybridization with the Vβ8.1 probe shows the expression of the transfected genes, while hybridization with the CHO-A probe depicts the expression of a control transcript.

Fig. 8. Cycloheximide reverses the down-regulation of β-globin transcripts that possess a premature nonsense codon. Upper panel: construct A, pGLOB (wild type β-globin construct); construct B, pGLOB39 (β-globin construct that possesses a ptc at position 39). Lower panel, Northern blot analysis of cytoplasmic RNA (2 μg) obtained from HeLa cells stably transfected with pGLOB or pGLOB39. Hybridization with the β-globin probe (exon 2) shows the expression of the transfected genes, while hybridization with the CHO-A probe shows that the blots are equally loaded.

DISCUSSION

We have shown that ptcs trigger a diminution in mature TCR-β transcript levels in fetal and adult thymocytes in vivo (Table I) and in transfected T-cells cultured in vitro (Fig. 2). Out-of-frame TCR genes that bear ptcs are commonly generated by programmed rearrangement during lymphocyte development. Most T-cells possess a nonproductively rearranged TCR-β gene on one chromosome and a productively rearranged TCR-β gene on the other chromosome (2). Thus, it is critical that the down-regulatory mechanism exhibits allelic specificity; that it only depress the expression of the ptc-bearing gene, not the functionally rearranged gene. Our demonstration that the down-regulatory mechanism is indeed allelic-specific (Fig. 4) suggests that this mechanism may be biologically relevant to the immune system. Consistent with our results, Maquat's laboratory has shown that the down-regulation of ptc-bearing triosephosphate isomerase transcripts is allelic-specific (11) and that ptcs act in cis, not in trans, to down-regulate triosephosphate isomerase mRNA levels (23). A general down-regulatory mechanism may be present in all cell types, where it serves to protect cells from dominant negative mutations that result from mutant nonsense codons. Since mutant proteins of the dominant negative class can be potent inhibitors of the corresponding wild-type protein (49), it is likely that there has been selection pressure to evolve a mechanism to reduce the expression of such deleterious proteins. In the case of the TCR-β protein, it is known that a truncated amino-terminal version that has lost the ability to bind to CD3 molecules gains the
ability to be secreted (50) and thus could potentially interfere with immune reactions.

It is not clear how the down-regulatory mechanism can distinguish a premature termination codon from a bona fide termination codon. One possibility is that the termination codons which trigger down-regulation lie upstream of an intron. This model is consistent with the fact that most normal stop codons in mammalian genes lie in the terminal exon (51) and hence would not be followed by an intron. Evidence for this model is that ptcS in any of the internal TCR-β exons we tested, including the penultimate exon, all result in strongly reduced expression (Fig. 1). Furthermore, removal of the introns downstream of a ptc in the TCR-β gene reversed the down-regulatory response. Similarly, it has been shown that removal of introns downstream of ptcS in the triosephosphate isomerase gene inhibits the degradation of the encoded message (14, 21). If nonsense codon-mediated decay is intron-dependent, then it is reasonable to suppose that the down-regulatory mechanism involves the nucleus. In fact, several reports have provided evidence that the presence of ptcS in transcripts causes their decay in the nuclear fraction of mammalian cells, not in the cytoplasmic fraction (9, 12, 14–17, 20, 23, 24, 26). We have also found this to be the case for TCR-β transcripts, based on nuclear subcellular fractionation studies and cytoplasmic half-life measurements. Two models have been proposed by Urlaub et al. (12) to explain how translation signals may affect nuclear mRNA stability. The translational translocation model proposes that translation of mRNA in the cytoplasm is first initiated while mRNA is still traversing through the nuclear pore. According to this model, if the ribosome encounters a ptc, export of the mRNA is interrupted, leading to degradation of the mRNA while it is still associated with the nucleus. In contrast, the nuclear-scanning model proposes that a codon scanner exists in the nucleus that searches for ptcS in mRNAs. Recognition of a ptc by the codon-scanner leads to nuclear degradation of the mRNA. More recently, a third model was suggested by Cheng & Maquat (19) in which recognition of a ptc by a cytoplasmic ribosome causes the transmission of a signal to the nucleus, triggering the degradation of the ptc-bearing mRNA in the nucleus. The key difference between these models is the locality of ptc recognition. For the translational translocation and the signaling models, recognition occurs in the cytoplasm, whereas the nuclear scanning model proposes that recognition of nonsense codons takes place in the nucleus.

Given that the down-regulation of many mRNAs occurs in the nuclear fraction of mammalian cells, it is important to determine if the regulation is exerted on pre-mRNA or mature mRNA. In this study, we assessed this question in fetal and adult thymus and determined that the presence of ptcS dramatically down-regulated mature TCR-β mRNAs, but had little or no effect on TCR-β pre-mRNA levels (Table I). This implies that the nuclear down-regulation triggered by ptcS in vivo is not due to a decreased rate of gene transcription or decreased stability of the primary transcript. Thus, the most likely mechanisms by which ptcS depress TCR-β mature mRNA levels are by: 1) inhibiting the splicing of one or more introns present in TCR-β pre-mRNAs or 2) decreasing the stability of partially or fully spliced TCR-β transcripts. Our conclusions are consistent with several reports that show that ptcS have no effect on gene transcription in cultured cell lines (8, 12, 16, 19). In contrast, no consensus has emerged regarding the effect of ptcS on pre-mRNA levels in cell lines; ptcS exert no discernible effect on triosephosphate isomerase pre-mRNA levels and thus appear not to affect splicing (19), while ptcS apparently inhibit the splicing of Igκ chain and MVM pre-mRNAs (9, 15).

Protein synthesis inhibitors prevented the down-regulation of ptc-bearing TCR transcripts in T-cells at different stages of maturation (Fig. 3) and in nonlymphoid cells (Figs. 5–7). In addition, we showed that CHX treatment induced a reversal in ptc-mediated down-regulation of β-globin transcripts (Fig. 8). Consistent with our observations, it was reported that CHX induces ptc-bearing iduronidase transcripts in fibroblasts, as assessed by Northern blot analysis (25). In contrast, Lozano et al. (9) showed that CHX only marginally affects the levels of ptc-bearing Igκ transcripts, as judged by reverse transcriptase-PCR analysis. The mechanistic basis for the apparent difference between Ig and TCR regulation is not known. CHX may induce events in B-cells that renders them still sensitive to the effects of nonsense codons. Alternatively, B- and T-cells may utilize different regulatory mechanisms to detect nonsense codons. Although this latter hypothesis is formally possible, it seems unlikely, since we find that protein synthesis inhibitors reverse the down-regulatory effect of ptcS in HeLa cells (Figs. 5–8), as well as T-cells (35). In addition, the effect of CHX does not appear to be context-specific, since CHX induced transcripts containing ptcS in any of several different exons (Fig. 1).

We suggest that protein synthesis inhibitors debilitate the down-regulatory mechanism in one of two ways. One possibility is that protein synthesis inhibitors directly prevent a ribosome in the cytoplasm or a ribosome-like entity in the nucleus from reading mRNAs to determine if they possess mutant nonsense codons. The rapid reversal of the down-regulatory response by CHX treatment (Fig. 5C) is consistent with this possibility. This hypothesis is also supported by a study that showed that ptc-mediated mRNA decay is partially inhibited by the presence of a stable hairpin loop in the 5’ end of an mRNA or by co-transfection with a nonsense suppressor tRNA (18). Our observation that protein synthesis inhibitors that act by different mechanisms all efficiently reverse the down-regulation of ptc-bearing TCR-β transcripts (Fig. 6) suggests that either a translocating ribosome or a modified translocating ribosome is responsible. This entity is likely to contain components of the 60S ribosomal subunit, since anisomycin, CHX, and puromycin all affect the function of this ribosomal subunit. Components of the 40S subunit are also likely to be involved, since emetine, which specifically binds the 40S subunit, also reverses the down-regulatory response. Since many of the inhibitors that we used are polysome stabilizers, it is possible that they act by eliciting a build-up of ribosomes on ptc-bearing RNAs, thus shielding these mRNAs from ribonuclease attack. This is unlikely since the polysome destabilizers puromycin and pactamycin, which generate naked ribosome-free transcripts, also induced ptc-bearing TCR-β transcripts in HeLa cells (Fig. 6) and T-cells (35). Interestingly, puromycin is known to cause premature termination of protein synthesis, yet like the other metabolic inhibitors we used, it induced the accumulation of ptc-bearing mRNAs. This suggests that the act of premature translational termination is not sufficient to trigger the down-regulatory response. Instead, recognition of a nonsense codon is critical to engage the down-regulatory mechanism.

A second possible mechanism by which protein synthesis inhibitors reverse the down-regulatory response may be by preventing the translation of an unstable protein critical for the down-regulatory response. Since ptc-mediated down-regulation appears to involve the nucleus, an emerging possibility is that this response may not be mediated by a classical ribosome. An unstable protein(s) may be an essential component of this putative nonclassical ribosome. Alternatively, an unstable pro-

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tein could be involved in putative ptc-mediated signaling events (19), or it could mediate the degradation of ptc-bearing mRNAs. Our observation that ptc-bearing mRNAs are induced within 30 min after CHX treatment suggests that the half-life of such a labile protein would be relatively short. Evidence suggests that a labile protein also regulates the half-life of wild type c-fos transcripts (52), although other evidence suggests that the rate of c-fos mRNA decay is regulated by other factors (53). The nature of the labile proteins that may mediate the decay of unstable normal mRNAs (such as as c-fos) and aberrant mRNAs containing premature nonsense codons remains to be determined.

The use of various drugs to inhibit protein synthesis allowed us to determine that de novo protein synthesis is necessary for ptc-mediated degradation. However, their use does not address the question of whether a cytoplasmic ribosome or a nuclear ribosome-like entity is involved in this regulation, since these drugs can accumulate in the nucleus as well as the cytoplasm. To address this question, we chose to use the polio virus, since it replicates in the cytoplasm of infected cells and selectively inhibits cytoplasmic translation by cleaving a protein that is a component of one of the initiation factors necessary for cap-dependent translation (47). Infection with polio virus resulted in an increase in the levels of ptc-containing TCR-β transcripts (Fig. 7). This result supports the notion that cytoplasmic ribosomes are involved in this mechanism. Hence, polio virus infection could be preventing ribosomal recognition of ptcs in the cytoplasm. Alternatively, polio virus may prevent the cytoplasmic translation of an unstable protein destined for either the nucleus or the cytoplasm that is necessary for ptc-mediated mRNA decay. One caveat with interpreting this data is that since polio virus affects many host processes (48), it is possible that the reversal of the down-regulatory response elicited by polio virus may be due to one of its other activities.

Although CHX strongly induced ptc-bearing TCR-β mRNAs in stably transfected SL12.4 cells (Figs. 1–4) and HeLa cells (Figs. 5, A–C), CHX was a weak inducer in transiently transfected HeLa cells (Fig. 5D). Thus, it appears that ptc-bearing TCR-β transcripts are not efficiently rescued by protein synthesis inhibitors in transiently transfected cells. Possible explanations for this result include: (i) the declining rate of transcription from DNA templates two days after transient transfection may preclude significant increases in mRNA levels when protein synthesis inhibitors are added at this time; (ii) the high level of mRNAs transcribed from the large number of transiently transfected DNA templates (in the small percentage of cells that efficiently incorporate the exogenous DNA) may overwhelm the putative alternative pathway for nuclear mRNA transport used when the ptc-scanning pathway is blocked by protein synthesis inhibitors; (iii) the nonchromosomally integrated DNA generated by transient transfection may be localized inappropriately for the transcribed RNA to be efficiently exported from the nucleus when the ptc-scanning pathway is blocked.

The level of down-regulation triggered by ptcs appears to vary depending on the gene. For example, TCR and Ig transcripts display a decrease in mRNA levels of 10–100-fold in response to ptcs (Refs. 7–10 and herein), while fully spliced triose-phosphate isomerase, v-scr, and minute virus of mice transcripts are down-regulated by only 3–4-fold by the presence of ptcs (11, 14, 15, 24). The basis for this difference is not known. Ptc-bearing TCR and Ig genes are generated as a part of normal lymphoid ontogeny, and thus it is possible that these genes have evolved cis-acting elements that improve their ability to be regulated by the ptc-mediated mRNA decay pathway. Since the rapid decay of some ptc-bearing mammalian transcripts appears to require introns (14–21), it is conceivable that splicing efficiency could influence the level of down-regulation induced by ptcs. TCR transcripts exhibit inefficient splicing (34) and thus may be localized to the nucleus for sufficient lengths of time to permit stringent scanning for ptcs.

Chromosomal context may be important for maximal down-regulation of ptc-bearing transcripts. The out-of-frame Vβ8.1, Jβ1.1, cDNA gene in SL12.4 cells gave rise to almost undetectable levels of mature transcripts; levels were induced by ~50-fold after CHX treatment (Refs. 33–35 and herein, Fig. 4). In contrast, transfected out-of-frame TCR-β genes that presumably integrated at nonhomologous sites gave rise to mature mRNAs that were expressed at low-to-moderate levels and induced only 10–20-fold by CHX (Figs. 1–3). Similarly, it has been shown that endogenous out-of-frame Ig genes are expressed at almost undetectable levels (up to 100-fold less than in-frame genes), while transfected out-of-frame Ig genes are less dramatically down-regulated (7–10). The presence of ptcs depressed dihydrofolate reductase mRNA levels by 5–20-fold when the mRNA was derived from the endogenous gene, but not at all when the mRNA was derived from stably transfected dihydrofolate reductase genomic clones (12). More recent experiments suggest that the promoter dictates whether the down-regulatory response is engaged: the β-globin and cytomegalovirus immediate-early promoters permitted regulation, whereas the HSV tk promoter did not (17). Taken together, these results suggest that nuclear context may strongly influence the down-regulatory mechanism engaged by ptcs. The TCR-β gene is a useful model for studying the molecular mechanism underlying this down-regulatory network, since: (i) the TCR-β gene acquires mutant nonsense codons during normal development; (ii) its expression is efficiently suppressed by premature nonsense codons; and (iii) this regulation can be manipulated by treatment with protein synthesis inhibitors.

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