*culosis* challenge. In contrast, the unvaccinated macaques developed lethargy, anorexia, and wasting; they subsequently died and they showed evidence of miliary tuberculosis 4 to 6 weeks after *M. tuberculosis* aerosol challenge. We realize that the BCG-vaccinated, *M. tuberculosis*-infected monkeys may progress to a subclinical form of tuberculosis, because they did exhibit detectable *M. tuberculosis* and its mRNA in BAL cells (Fig. 4B). Nevertheless, this potential outcome would not negate our observation that a rapid recall response of $V\gamma2V\delta2^+$ T cells coincided with immune protection against the acutely fatal tuberculosis in monkeys.

Our studies provide strong evidence that $V\gamma2V\delta2^+$ T cells, like $\alpha\beta^+$ T cells, contribute to adaptive immune responses in mycobacterial infections. The adaptive immune responses of $V\gamma2V\delta2^+$ T cells are indeed driven by BCG nonpeptide antigens (*19*). The contribution of these cells to vaccine protection against tuberculosis was demonstrated in the juvenile rhesus model. BCG-mediated protection against the fatal form of tuberculosis has been reported in children, although its protective efficacy for chronic pulmonary tuberculosis in adults and monkeys is controversial (*22–29*). It seems that the antigen specificity, TCR diversity, and recall features of $V\gamma2V\delta2^+$ T cells separate this $\gamma\delta$ T cell subset from innate cells including peripheral mononuclear cells (PMN), monocytes, and natural killer (NK) cells as well as those $\gamma\delta$ T cells that express invariant $\gamma\delta$ TCR (*30*, *31*). The unique ability of $V\gamma2V\delta2^+$ T cells to mount rapid and large expansions in mycobacterial infections suggests that vaccine-elicited $V\gamma2V\delta2^+$ T cell immunity may be both possible and useful. Thus, $V\gamma2V\delta2^+$ T cells may broadly contribute to both innate and acquired immunity against microbial infections.

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# An mRNA Surveillance Mechanism That Eliminates Transcripts Lacking Termination Codons


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**Translation is an important mechanism to monitor the quality of messenger RNAs (mRNAs), as exemplified by the translation-dependent recognition and degradation of transcripts harboring premature termination codons (PTCs) by the nonsense-mediated mRNA decay (NMD) pathway. We demonstrate in yeast that mRNAs lacking all termination codons are as labile as nonsense transcripts. Decay of "nonstop" transcripts in yeast requires translation but is mechanistically distinguished from NMD and the major mRNA turnover pathway that requires deadenylation, decapping, and 5′-to-3′ exonucleolytic decay. These data suggest that nonstop decay is initiated when the ribosome reaches the 3′ terminus of the message. We demonstrate multiple physiologic sources of nonstop transcripts and conservation of their accelerated decay in mammalian cells. This process regulates the stability and expression of mRNAs that fail to signal translational termination.**


Eukaryotes have evolved surveillance mechanisms that are intimately linked to translation to eliminate errors in mRNA biogenesis. The decay of transcripts containing PTCs by the NMD pathway effectively prevents expression of deleterious truncated proteins. In prokaryotes, protein products encoded by transcripts lacking termination codons are marked for degradation by the addition of a COOH-terminal tag encoded by tmRNA (*1*, *2*). Thus, both the presence and context of translational termination can regulate gene expression. In order to determine whether the presence of translational termination influences mRNA stability, we assayed PGK1 transcripts in *Saccharomyces cerevisiae* derived from the following constructs (*3*): wild-type PGK1 (WT-PGK1), a nonsense form of PGK1 harboring a PTC at codon 22 [PTC(22)-PGK1], and nonstop-PGK1 that was created by removing the bona fide termination codon and all in-frame termination codons in the 3′ UTR (untranslated region) from the WT-PGK1 transcript. Nonstop-PGK1 transcripts were as labile as their nonsense-containing counterparts (Fig. 1). At least three *trans*-acting factors (Upf1p, Upf2p, and Upf3p) are essential for NMD in *S. cerevisiae* (*4*, *5*). Remarkably, nonstop transcripts were not stabilized in strains lacking Upf1p, distinguishing the pathway of decay from NMD (Fig. 1).

The turnover of normal mRNAs requires deadenylation followed by Dcp1p-mediated decapping and degradation by the major 5′-to-3′ exonuclease Xrn1p (*6*, *7*). NMD is distinguished in that these events occur without prior deadenylation (*8*, *9*). Nonstop-PGK1 transcripts showed rapid decay


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in strains lacking Xrn1p or Dcp1p activity (Fig. 1), providing further evidence that they are not subject to NMD. This result was surprising since both of these factors are also required for the turnover of normal mRNAs after deadenylation by the major deadenylase Ccr4p (*10*). Nonstop decay was also unaltered in a strain lacking Ccr4p (Fig. 1). Therefore, degradation of nonstop-PGK1 transcripts requires none of the factors involved in the pathway for degradation of wild-type or nonsense mRNAs.

Additional experiments were performed to assess the role of translation in nonstop decay. Treatment with cycloheximide (CHX) or depletion of charged tRNAs in yeast harboring the conditional *cca1-1* allele (*11*) grown at the nonpermissive temperature substantially increased the stability of nonstop-PGK1 transcripts (Fig. 1) (*12*). It has been shown that translation into the 3′ UTR of selected transcripts can displace bound *trans*-factors that are positive determinants of message stability (*13, 14*). To determine whether this mechanism is relevant to nonstop decay, we examined the performance of transcript Ter-poly(A)-PGK1 which contains a termination codon inserted one codon upstream of the site of polyadenylate [poly(A)] addition (*15*) in transcript nonstop-PGK1. The addition of a termination codon at the 3′ end of the 3′ UTR of a nonstop transcript resulted in substantial (threefold) stabilization (Fig. 1). Unlike nonstop-PGK1 transcripts, the half-life of Ter-poly(A)-PGK1 was increased in the absence of Xrn1p (Fig. 1), indicating that this transcript is degraded by the pathway for normal mRNA and not by the nonstop pathway. These data suggest that the instability of nonstop-PGK1 transcripts cannot fully be explained by ribosomal displacement of factors bound to the 3′ UTR.

There is an emerging view that the 5′ and 3′ ends of mRNAs interact to form a closed loop and that this conformation is required for efficient translation initiation and normal mRNA stability. Participants in the interaction include components of the translation initiation complex eIF4F as well as Pab1p. The absence of a termination codon in nonstop-PGK1 is predicted to allow the ribosome to continue translating through the 3′ UTR and poly(A) tail, potentially resulting in displacement of Pab1p, disruption of normal mRNP structure, and consequently accelerated degradation of the transcript. However, while mRNAs in *pab1Δ* strains do undergo accelerated decay, the rapid turnover is a result of premature decapping and can be suppressed by mutations in *XRN1* (*16*), allowing distinction from nonstop decay. Lability of nonstop transcripts might also be a consequence of ribosomal stalling at the 3′ end of the transcript. It is tempting to speculate that 3′-to-5′ exonucleolytic activity underlies the decapping- and 5′-to-3′ exonuclease–independent, translation-dependent accelerated decay of nonstop transcripts. An appealing candidate is the exosome, a collection of proteins with 3′-to-5′ exoribonuclease activity that functions in the processing of 5.8*S* RNA, rRNA, small nucleolar RNA

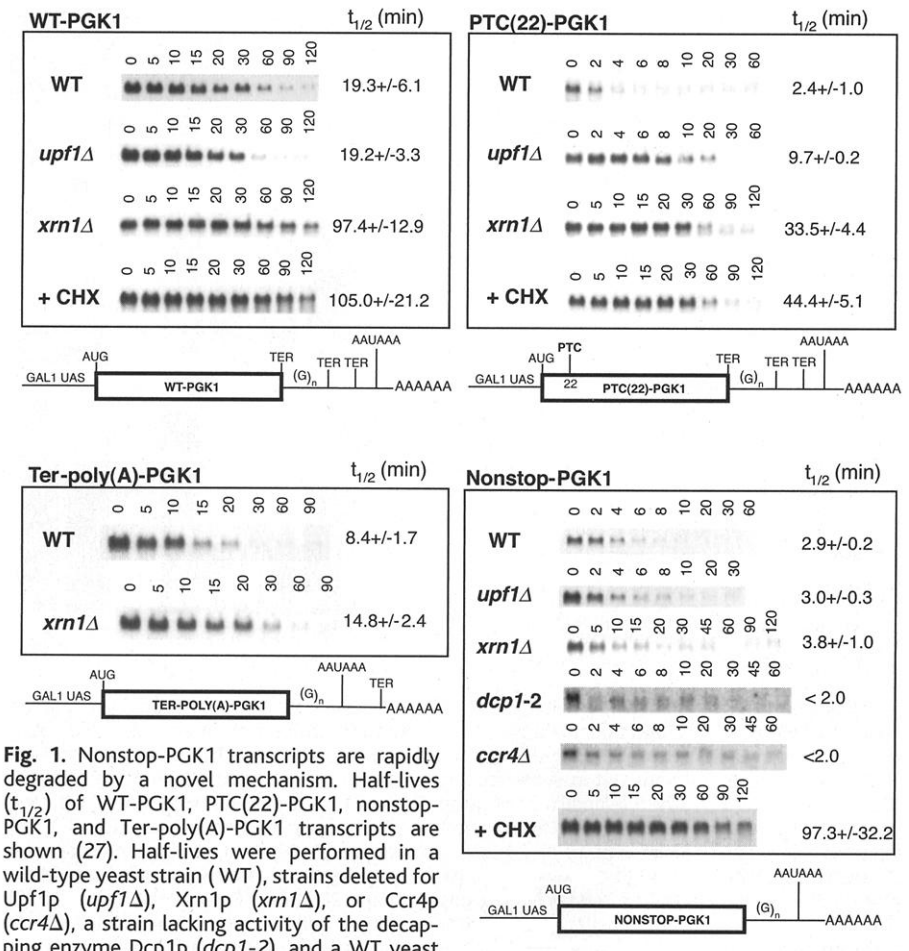


**Fig. 1.** Nonstop-PGK1 transcripts are rapidly degraded by a novel mechanism. Half-lives ($t_{1/2}$) of WT-PGK1, PTC(22)-PGK1, nonstop-PGK1, and Ter-poly(A)-PGK1 transcripts are shown (*27*). Half-lives were performed in a wild-type yeast strain (WT), strains deleted for Upf1p (*upf1Δ*), Xrn1p (*xrn1Δ*), or Ccr4p (*ccr4Δ*), a strain lacking activity of the decapping enzyme Dcp1p (*dcp1-2*), and a WT yeast strain treated for 1 hour with 100 μg/ml of cycloheximide (+CHX).

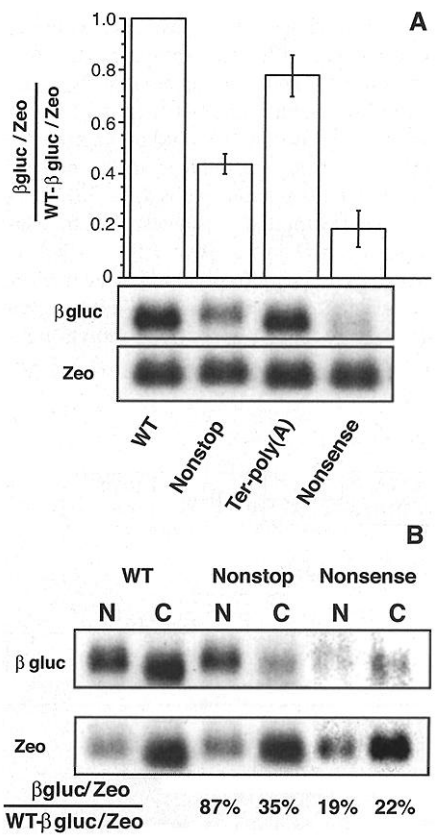


**Fig. 2.** Nonstop decay is conserved in mammalian cells. (**A**) Steady-state abundance of β-glucuronidase transcripts derived from wild type–(WT), nonsense-, nonstop-, and Ter-poly(A)-βgluc minigene constructs after transient transfection into HeLa cells (*28*). Zeocin (zeo) transcripts served as a control for loading and transfection efficiency. For each construct, the βgluc/Zeo transcript ratio is expressed relative to that observed for WT-βgluc. (**B**) Steady-state abundance of WT-βgluc, nonstop-βgluc, and nonsense-βgluc transcripts were determined in the nuclear (N) and cytoplasmic (C) fractions of transfected cells (*28*). The ratio of normalized nonstop- or nonsense-βgluc transcript levels to WT-βgluc transcript levels are shown for each cellular compartment. Reported results are the average of three independent experiments.

(snoRNA), small nuclear RNA (snRNA), and other transcripts. Data presented in van Hoof *et al.* (*17*) validate this prediction.

In order to determine whether nonstop decay functions in mammals, we assessed the performance of transcripts derived from β-glucuronidase (βgluc) minigene constructs containing exons 1, 10, 11, and 12 separated by introns derived from the endogenous gene (*18*). The abundance of transcripts derived from a nonstop-βgluc version of this minigene was significantly reduced relative to WT-βgluc, suggesting that a termination codon is also essential for normal mRNA stability in mammalian cells (Fig. 2A). Addition of a stop codon one codon upstream from the site of poly(A) addition in nonstop-βgluc [Ter-poly(A)-βgluc (*15*)] increased the abundance of this transcript to near—wild-type levels (Fig. 2A). Thus, all functional characteristics of nonstop transcripts in yeast appear to be relevant to mammalian systems.

Both the abundance and stability of most nonsense transcripts, including nonsense-βgluc (Fig. 2B), are reduced in the nuclear fraction of mammalian cells (*19, 20*). This has been interpreted to suggest that translation and NMD initiate while the mRNA is still associated with (if not within) the nuclear compartment. If translation is initiated on nucleus-associated transcripts, so might nonstop decay. Subcellular fractionation studies localized mammalian nonstop decay to the cytoplasmic compartment, providing further distinction from NMD (Fig. 2B).

The conservation of nonstop decay in yeast and mammals suggests that the pathway serves an important biologic role. There are many potential physiologic sources of nonstop transcripts that warrant consideration. Mutations in bona fide termination codons would not routinely initiate nonstop decay due to the frequent occurrence of in-frame termination codons in the 3′ UTR and could not plausibly provide the evolutionary pressure for maintenance of nonstop decay. In contrast, alternative use of 3′-end processing signals embedded in coding sequence has been documented in many genes including CBP1 in yeast and the growth hormone receptor (GHR) gene in fowl (*21–23*). Moreover, a computer search of the human mRNA and *S. cerevisiae* open reading frame (ORF) databases revealed many additional genes that contain a strict consensus sequence for 3′-end cleavage and polyadenylation within their coding region (Fig. 3A). Utilization of these premature signals would direct formation of truncated transcripts that might be substrates for the nonstop decay pathway. Indeed, analysis of 3425 random yeast cDNA clones sequenced from the 3′ end (i.e., 3′ ESTs) revealed that 40 showed apparent premature polyadenylation within the coding region (*24*).

The truncated GHR transcript in fowl is apparently translated, as evidenced by its association with polysomes (*22*). As predicted for a substrate for nonstop decay, the ratio of truncated–to–full-length GHR transcripts was dramatically increased after treatment of cultured chicken hepatocellular carcinoma cells with the translational inhibitor emetine (Fig. 3B). Other truncated transcripts, both bigger and smaller than the predicted 0.7-kb nonstop transcript, did not show a dramatic increase in steady-state abundance upon translational arrest suggesting that they are derived from other mRNA processing events, perhaps alternative splicing (Fig. 3B) (*12*). To directly test whether the nonstop mRNA pathway degrades prematurely polyadenylated mRNAs, we analyzed CBP1 transcripts in yeast. The CBP1 gene produces a 2.2-kb full-length mRNA and a 1.2-kb species with premature 3′ end processing and polyadenylation within the coding region (*25*). As expected from the observation that nonstop decay requires the exosome in yeast (*17*), deletion of the gene encoding the exosome component Ski7p stabilized the

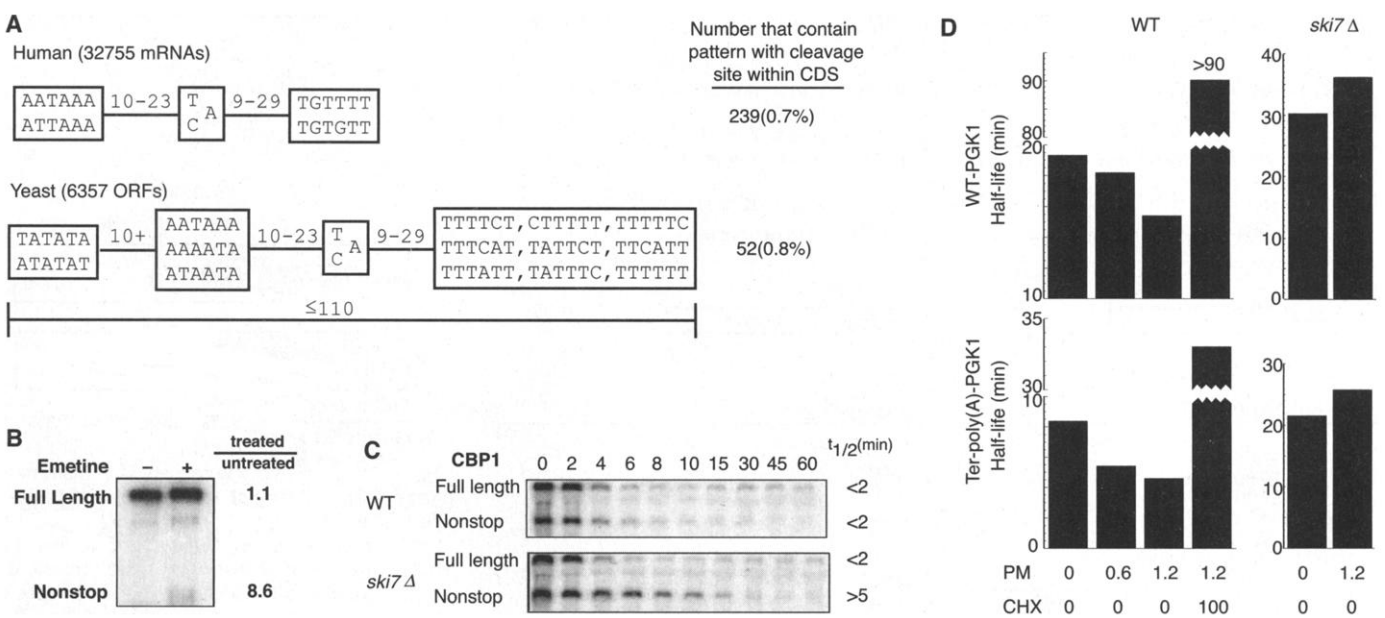


**Fig. 3.** Many physiologic transcripts are substrates for nonstop decay. (**A**) 239 human mRNAs contain a polyadenylation signal consisting of either of the two most common words for the positioning, cleavage, and downstream elements (*29*), separated by optimal distances (*30*), where the cleavage site occurs between the start and stop codons as determined from the cDNA coding sequence (CDS). Similarly 52 *S. cerevisiae* ORFs contained the yeast polyadenylation signal consisting of optimal upstream, positioning, and cleavage signals followed by any of nine common "U-rich" signals (*24*) with optimal spacing of the elements. Computer search was performed using the human mRNA database and the *S. cerevisiae* ORF database and an analysis program written in PERL that is available upon request. (**B**) Abundance of nonstop and full-length cGHR transcripts in chicken hepatocellular carcinoma cells (CRL-2117 purchased from the American Type Culture Collection, Manassas, VA) treated with 100 μg/ml of emetine for 6 hours. Northern analysis was performed as described (*22*). The relative ratio of each transcript in untreated and treated cells is reported. (**C**) Half-lives ($t_{1/2}$) of the full-length and nonstop CBP1 mRNAs measured in wild-type– (WT) and *SKI7*-deleted (*ski7*Δ) yeast strains carrying plasmid pG::-26 (*25, 31*). (**D**) Half-lives of WT-PGK1 and Ter-poly(A)-PGK1 transcripts in wild-type– (WT) and *SKI7*-deleted (*ski7*Δ) yeast strains treated with the indicated dose of paromomycin (mg/ml) (PM) for 20 hours. For cycloheximide (CHX) experiments, yeast were treated identically except 100 μg/ml of CHX was added during the last hour before half-life determination. Half-lives were determined as described (*27*).

prematurely polyadenylated mRNA but had no effect on the stability of the full-length mRNA (Fig. 3C). These data indicate that physiologic transcripts arising from premature polyadenylation are subject to nonstop mRNA decay. The cytoplasmic localization of ski7p is consistent with our observation that nonstop decay occurs within the cytoplasm (Fig. 2B).

Any event that diminishes translational fidelity and promotes readthrough of termination codons could plausibly result in the generation of substrates for nonstop decay. In view of recent attempts to treat genetic disorders resulting from PTCs with long-term and high-dose aminoglycoside regimens, this may achieve medical significance. As a proof-of-concept experiment, we examined the performance of transcripts with one [Ter-poly(A)-PGK1] or multiple (WT-PGK1) in-frame termination codons (including those in the 3′ UTR) in yeast strains after treatment with paromomycin, which induces ribosomal readthrough. Remarkably, both transcripts showed a dose-dependent decrease in stability that could be reversed by inhibiting translational elongation with CHX or prevented by deletion of the gene encoding Ski7p (Fig. 3D). These data suggest that nonstop decay can limit the efficiency of therapeutic strategies aimed at enhancing nonsense suppression and might contribute to the toxicity associated with aminoglycoside therapy.

The degradation of nonstop transcripts may be regulated. The relative expression level of truncated GHR transcripts compared to full-length transcripts varies in a tissue-, gender-, and developmental stage-specific manner (*22*) and the relative abundance of truncated CBP1 transcripts varies with growth condition (*21*). Data presented here warrant the hypothesis that regulation may occur at the level of nonstop transcript stability rather than production. The conservation of the GHR coding sequence 3′-end processing signal throughout avian phylogeny and conservation of premature polyadenylation of the yeast RNA14 transcript and its fruitfly homolog su(f) support speculation that nonstop transcripts or derived protein products serve essential developmental and/or homeostatic functions that are regulated by nonstop decay (*21, 26*).

Many processes contribute to the precise control of gene expression including transcriptional and translational control mechanisms. In recent years, mRNA stability has emerged as a major determinant of both the magnitude and fidelity of gene expression. Perhaps the most striking and comprehensively studied example is the accelerated decay of transcripts harboring PTCs by the NMD pathway. Nonstop decay now serves as an additional example of the critical role that translation plays in monitoring the fidelity of gene expression, the stability of aberrant or atypical transcripts, and hence the abundance of truncated proteins.

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28. HeLa cells [maintained in DMEM with 10% fetal bovine serum (FBS)] were grown to ~80 to 90% confluency, trypsinized, and resuspended in 300 μl of RPMI 1640 without FBS, 10 mM glucose and 0.1 mM dithiothreitol (DTT). Plasmid DNA (10 μg) was added and cells were electroporated (Bio-Rad, Gene Pulser II Electroporation System) at a voltage of 0.300 kV and a capacitance of 500 μF. Poly(A) RNA (Oligotex midi kit, Qiagen, 2 μg), isolated 36 hours after transfection, was separated on a 1.6% agarose formaldehyde gel, transferred to a nylon membrane, and hybridized with a polymerase chain reaction (PCR) product comprising exons 10 through 12 of the β-gluc gene that had been radioactively labeled by nick translation (Random Primed DNA Labeling Kit, Boehringer Mannheim). The membrane was subsequently stripped with boiling 0.5% SDS and probed with radioactively labeled zeocin cDNA. Northern blot results were quantitated using an Instant Imager (Packard). For subcellular fractionation, transiently transfected HeLa cells were trypsinized, washed in cold 1× phosphate buffered saline, and pelleted by centrifuging at 2040*g* for 5 min at 4°C. Cells were then resuspended in 200 μl of 140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.6), 0.5% NP-40, and 1 mM DTT, vortexed, and incubated on ice for 5 min. Nuclei were pelleted by centrifuging at 12,000*g* for 45 s at 4°C and subsequently separated from the aqueous (cytoplasmic) phase.
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