The EMBO Journal (2007) 26, 2327–2338 | © 2007 European Molecular Biology Organization | All Rights Reserved 0261-4189/07
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THE EMBO JOURNAL

Translation of nonSTOP mRNA is repressed post-initiation in mammalian cells

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We investigated the fate of aberrant mRNAs lacking in-frame termination codons (called nonSTOP mRNA) in mammalian cells. We found that translation of nonSTOP mRNA was considerably repressed although a corresponding reduction of mRNA was not observed. The repression appears to be post-initiation since (i) repressed nonSTOP mRNAs were associated with polysomes, (ii) translation of IRES-initiated and uncapped nonSTOP mRNA were repressed, and (iii) protein production from nonSTOP mRNA associating with polysomes was significantly reduced when used to program an *in vitro* run-off translation assay. NonSTOP mRNAs distributed into lighter polysome fractions compared to control mRNAs encoding a stop codon, and a significant amount of heterogeneous polypeptides were produced during *in vitro* translation of nonSTOP RNAs, suggesting premature termination of ribosomes translating nonSTOP mRNA. Moreover, a run-off translation assay using hippuristanol and RNAse protection assays suggested the presence of a ribosome stalled at the 3′ end of nonSTOP mRNAs. Taken together, these data indicate that ribosome stalling at the 3′ end of nonSTOP mRNAs can block translation by preventing upstream translation events.

The EMBO Journal (2007) **26,** 2327–2338. doi:10.1038/sj.emboj.7601679; Published online 19 April 2007
Subject Categories: RNA; proteins
Keywords: nonSTOP; ribosome; RNA surveillance; translational repression

Introduction

Gene expression is an inherently complex process and errors often happen in the course of transcription and RNA processing. Both prokaryotic and eukaryotic cells have evolved remarkable surveillance mechanisms to verify mRNA quality, thus ensuring their suitability for translation and avoiding the synthesis of potentially deleterious proteins from aberrant mRNAs (Wagner and Lykke-Andersen, 2002; Fasken and Corbett, 2005). The mRNA surveillance system not only contributes to the maintenance of cellular homeostasis but is also implicated in certain diseases (Holbrook *et al*, 2004; Kuzmiak and Maquat, 2006).

One of the best-studied mRNA surveillance pathways is nonsense-mediated mRNA decay (NMD), which recognizes aberrant transcripts having premature termination codons and targets these for degradation (Gonzalez *et al*, 2001; Baker and Parker, 2004; Weischenfeldt *et al*, 2005; Amrani *et al*, 2006; Behm-Ansmant and Izaurralde, 2006). Although the NMD pathway is conserved in eukaryotes, recent studies in several organisms have revealed that different mechanisms have evolved to discriminate premature stop codons from natural stop codons and to degrade the targeted mRNAs (Conti and Izaurralde, 2005). Another example of aberrant mRNAs is that lacking in-frame termination codons (referred herein as nonSTOP mRNA). NonSTOP mRNAs can be generated when 3′ end formation occurs within the coding region of cistrons as a consequence of genetic mutations (Temperley *et al*, 2003; Chatr-Aryamontri *et al*, 2004), transcriptional pausing (Cui and Denis, 2003), or usage of cryptic polyadenylation sites (Edwalds-Gilbert *et al*, 1997; Sparks and Dieckmann, 1998). It has been estimated that 40 of 3622 yeast ESTs have 3′ ends located upstream of the *bona fide* termination codon, suggesting that nonSTOP mRNAs are truly produced *in vivo* (Graber *et al*, 1999). These nonSTOP mRNAs have the potential to produce C-terminally truncated proteins, which in some cases could act as dominant-negative or gain-of-function mutants. To circumvent this danger, it is reasonable to hypothesize that organisms have evolved specialized surveillance pathways that detect nonSTOP mRNAs and prevent them from producing potentially harmful products.

In eubacteria, translation of nonSTOP mRNAs results in stalled ribosomes present at the 3′ end of the mRNA. These are subsequently released for recycling by tmRNA (SsrA), a unique molecule having properties of both tRNA and mRNA (Muto *et al*, 1998; Abo *et al*, 2000; Karzai *et al*, 2000). The tRNA-like region of tmRNA donates alanine to the stalled peptide chain using the empty A-site of ribosomes, whereas the mRNA-like region of tmRNA displaces the nonSTOP mRNA and directs the addition of a short peptide tail to the C-terminus of the polypeptide. The resulting carboxyl-terminal tag targets the chimeric protein for proteolysis. It has also been recently reported that the tmRNA system facilitates degradation of nonSTOP mRNAs (Yamamoto *et al*, 2003).

On the other hand, yeast has evolved a mechanism distinct from the tmRNA system to ensure that aberrant proteins from nonSTOP mRNAs are not produced. NonSTOP mRNAs in which ribosomes are stalling at the 3′ end are recognized by Ski7 and directed for degradation mediated by the exosome (Frischmeyer *et al*, 2002; van Hoof *et al*, 2002; Wagner and Lykke-Andersen, 2002). Although it was shown that translation of nonSTOP mRNAs is substantially repressed in yeast (Inada and Aiba, 2005), Meaux and van Hoof (2006)

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Received: 22 November 2006; accepted: 15 March 2007; published online: 19 April 2007

were able to detect significant protein production from nonSTOP mRNAs.

A loss of two nucleotides removes the termination codon from the mitochondrial *RNA14* transcript in a mitochondrial cytopathy, and results in the production of a nonSTOP mRNA in mitochondria (Temperley *et al*, 2003). Jesina *et al* (2004) reported that protein production from nonSTOP *RNA14* is reduced, suggesting that translation of nonSTOP mRNA is repressed in human mitochondria. Chrzanowska-Lightowlers *et al* (2004), however, reported that functional polypeptides are produced from nonstop *RNA14* mRNA. Thus, the functional properties of nonSTOP mitochondrial mRNAs remain ill-defined and the fate of nonSTOP mRNA produced in the nucleus has not been examined in mammalian cells.

In this report, we investigated the fate of nonSTOP mRNAs in mammalian cells. Although the expression level of nonSTOP mRNA was not altered, translation of nonSTOP mRNA was significantly repressed at a post-initiation step. *In vivo* run-off assays and RNAse protection assays suggest that a ribosome is present at the 3′ end of the nonSTOP mRNA. In addition, several lines of experiments indicate that translation of nonSTOP mRNA is halted before completion of full-length polypeptides. These results support a model whereby a stalled ribosome at the 3′ end of a nonSTOP mRNA causes translational arrest by blocking upstream ribosome flow.

Results

Translational repression of nonSTOP mRNA in vivo

A GFP-based reporter gene lacking a termination codon (nonSTOP GFP) was constructed using a PCR-based amplification strategy (Figure 1A). The wild-type GFP gene encodes an open reading frame (ORF) of 720 bp with a 24 bp 3′ untranslated region, whereas the nonSTOP GFP gene encodes a 744 bp ORF. Monitoring fluorescence by microscopy revealed a large reduction in the production of GFP in cells transfected with the nonSTOP GFP construct, relative to cells that had received the wild-type GFP reporter (STOP GFP) (upper panels in Figure 1B). To ensure similar transfection

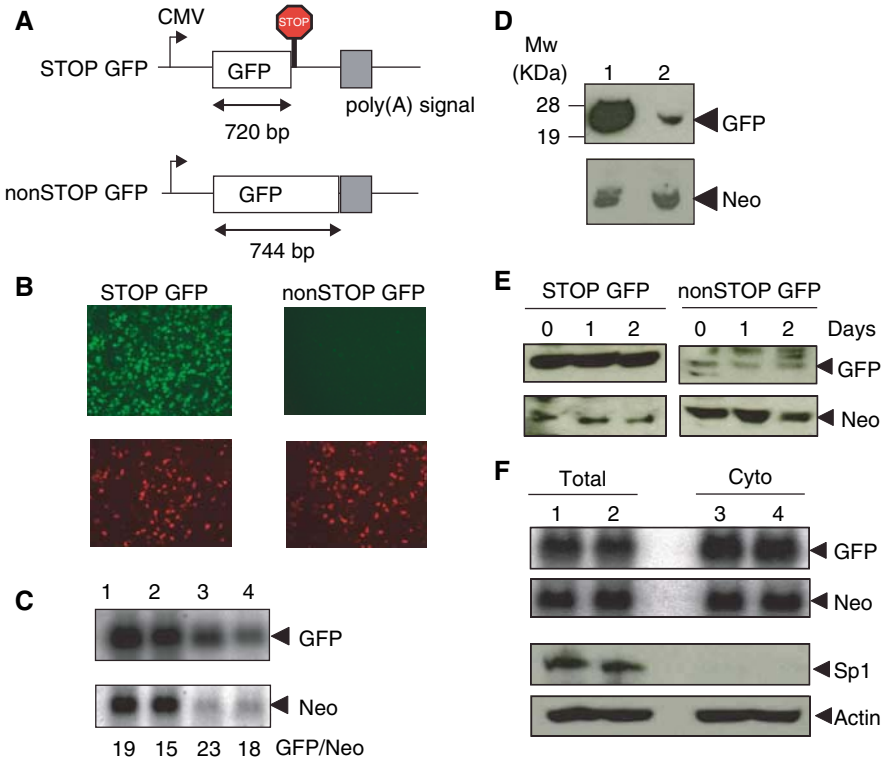

Figure 1 Expression of nonSTOP GFP in HeLa cells. (**A**) Schematic drawing of GFP reporter genes. Right-angled arrows indicate the CMV promoter. Open boxes indicate the coding frame and shaded boxes indicate the polyadenylation signal. The presence of the GFP termination codon is indicated by a STOP sign. The lengths of the ORFs are indicated. (**B**) Fluorescence microscopic observation of cells transfected with the indicated pEGFP-N1-derived plasmid and pDsRed2 plasmid. Upper panels are results of observation with the Green fluorescence filter set (U-MGFP, Olympus). Lower panels are results of observation with the red fluorescence filter set (U-MWIG, Olympus). (**C**) Northern blot analysis of total RNA extracted from HeLa cells transfected with either pGFP STOP (lane 1 and 3) or pGFP nonSTOP (lanes 2 and 4) plasmid. In all, 1 μg reporter plasmid (lanes 1 and 2) or 100 ng plasmid (lanes 3 and 4) was transfected into cells. Total RNA (10 μg) was resolved on a 1.2% agarose–formaldehyde gel and probed with ^{32}P-labeled GFP (upper panel) or neomycin phosphotransferase (Neo) (lower panel). The relative expression levels of GFP mRNA normalized to Neo mRNA are indicated below the panels. (**D**) Western blot hybridization analysis of extracts prepared from HeLa cells transfected with either the pGFP STOP (lane 1) or pGFP nonSTOP (lane 2) plasmid. Extracts were resolved by SDS–PAGE, transferred to Immobilon P and blotted to detect GFP (upper panel) or neomycin phosphotransferase (Neo) (lower panel). (**E**) Assessing stability of GFP protein synthesized from pGFP STOP or pGFP nonSTOP reporter. Cycloheximide (100 μg/ml) was added to transfected cells 24 h post-transfection, at which time extracts were prepared from cells (0, 1, or 2 days) and analyzed by Western blot analysis. (**F**) Subcellular localization of nonSTOP mRNA. Total RNA was prepared from the cytosolic extract of transfected cells and processed for Northern blot hybridization analysis with the ^{32}P-GFP probe (top panel) or a ^{32}P-Neo probe (second panel from top). Total cellular extract and cytosolic extract were processed for Western blot analysis to assess the quality of the fractionation procedure and were probed for the presence of Sp1 (nuclear) (third panel from top) or actin (cytoplasmic) (bottom panel).

efficiencies and eliminate the possibility of a trans-acting inhibitor being produced from nonSTOP GFP, pDsRed2 was cotransfected and expression from this construct was similar in the two transfections (lower panels in Figure 1B). Northern blot analysis revealed that the steady-state amount of nonSTOP GFP mRNA was not significantly reduced relative to wild-type STOP GFP mRNA (Figure 1C). There was no significant difference between the half-life of STOP mRNA and that of nonSTOP mRNA, as judged by Northern blot analysis performed with the transcription inhibitor, actinomycin D (data not shown). Western blot analysis showed a significant reduction in GFP production from the nonSTOP GFP mRNA (upper panel in Figure 1D, compare lane 2 to 1). In contrast, production of neomycin phosphotransferase from the same expression vector was unaltered (lower panel in Figure 1D). Taken together, these results suggest that the absence of a termination codon dose not significantly affect mRNA steady-state levels, but rather inhibits protein expression levels *in vivo*.

Because translation products from nonSTOP mRNAs (nonSTOP protein) should contain an extended peptide sequence at the C-terminus caused by translation of sequences downstream of the deleted termination codon, there is the possibility that such a nonSTOP protein is produced, but then rapidly degraded. To test for the possible existence of such a mechanism, protein stability was examined using the translation inhibitor cycloheximide (Figure 1E). The amount of nonSTOP GFP and wild-type GFP proteins were not significantly changed during 2 days after addition of cycloheximide (Figure 1E), suggesting that degradation of nonSTOP GFP protein was not accelerated *in vivo*.

Messenger RNA export from the nucleus is monitored and regulated by a cell. Inhibition of nonSTOP mRNA transport from nucleus to cytoplasm could explain the decreased production of nonSTOP proteins. To test this possibility, steady-state abundance of STOP and nonSTOP GFP mRNAs were determined in the cytoplasmic fraction (Figure 1F). Subcellular fractionation studies showed a similar distribution of the two mRNAs in the cytoplasm, suggesting that export of nonSTOP transcript into the cytoplasm is not impaired. Taken together, these results strongly suggest that translation of nonSTOP GFP mRNA is repressed in the cytoplasm.

In order to test whether translational repression of nonSTOP mRNA could be observed with other cistrons, we examined the consequences of termination codon absence on expression of the *Firefly* luciferase reporter gene. As shown in Figure 2, luciferase activity expressed from nonSTOP *Firefly* luciferase (nonSTOP Luc) gene was reduced substantially (Figure 2A), while the steady-state level of nonSTOP *Firefly* luciferase transcripts was similar to that of wild-type *Firefly* luciferase mRNA having a *bona fide* termination codon (STOP Luc) (Figure 2B and D). Western blot analysis (Figure 2C) ruled out the formal possibility that addition of an extended C-terminal sequence inhibited luciferase activity of nonSTOP *Firefly* protein that was responsible for the observed decrease in luciferase activity.

Inhibition of protein synthesis by cycloheximide indicated that protein stability of nonSTOP luciferase was similar to that of wild-type luciferase protein *in vivo* (Figure 2E). Furthermore, to examine the potential involvement of proteasome-mediated protein degradation in eliminating nonSTOP products, two different proteasome inhibitors, ALLN (Griscavage *et al*, 1995; Debiasi *et al*, 1999) and MG132 (Meriin *et al*, 1998; Steinhilb *et al*, 2001), were used during expression of nonSTOP luciferase. The addition of proteasome inhibitors failed to increase the expression of the nonSTOP *Firefly* luciferase product (Figure 2F). Apoptosis was induced upon prolonged exposure (3 days) of cells to proteasome inhibition and was noted with ALLN and MG132 when the experiment was extended to this period, indicating that the inhibitors were acting as expected (data not shown). These results confirm the former conclusions obtained with GFP, that low level expression of nonSTOP protein is not a consequence of altered protein degradation. Translational repression of nonSTOP GFP reporter mRNAs was observed in other mammalian cells, including 293, A549, and MDA231 cells (data not shown). These results lead us to conclude that translational repression is a general property of nonSTOP transcripts in mammalian cells.

Translational repression of nonSTOP mRNA in an in vitro translation system

In vitro translation systems, such as rabbit reticulocyte lysates, are very useful for mechanistic studies. At first, we confirmed that the cap structure and poly(A)-tail stimulated the translation efficiency of *in vitro* synthesized mRNA having stop codons in rabbit reticulocyte lysates (data not shown). Next, we compared protein production from nonSTOP Luc mRNA versus STOP Luc mRNA. Luciferase activity produced from nonSTOP *Firefly* luciferase mRNA was ~5% of luciferase activity produced from STOP *Firefly* luciferase mRNA (Supplementary Figure 1A), whereas *Renilla* luciferase mRNA translation, added as an internal control, was expressed at similar levels in both reactions (data not shown). Consistent with this observation, production of ^{14}C-labeled full-length polypeptide from nonSTOP mRNA was not detectable by SDS–PAGE analysis (Supplementary Figure 1B). Thus, translation of nonSTOP RNA is repressed in the *in vitro* rabbit reticulocyte translation system.

NonSTOP transcripts associate with polysomes

To investigate whether nonSTOP mRNAs associate with ribosomes *in vivo*, mRNA distribution in polysomes was analyzed by sucrose density gradient separation. Cytoplasmic extracts of cells expressing either wild-type STOP Luc or nonSTOP Luc mRNAs were separated by ultracentrifugation on 10–50% linear sucrose gradients. Both STOP Luc mRNAs and nonSTOP Luc mRNAs were clearly distributed in polysome fractions (third panels from the top in Figure 3A and B). In addition, the bulk of nonSTOP Luc mRNA was distributed in slightly lighter fractions, compared to STOP Luc mRNAs (Figure 3C). As a control, sedimentation of an internal GAPDH control mRNA did not significantly change (bottom panels in Figure 3A and B). On the other hand, both STOP Luc mRNAs and nonSTOP Luc mRNAs were redistributed in the ribosome-free fractions by EDTA treatment, indicating that nonSTOP Luc mRNAs were associated with ribosomes (data not shown). Moreover, distribution of nonSTOP GFP mRNAs was also lighter than that of STOP GFP mRNA in polysome fractions consistent with the results shown here for the luciferase reporters (data not shown). This different distribution in polysome fractions between STOP mRNAs and nonSTOP mRNAs could indicate that (i)

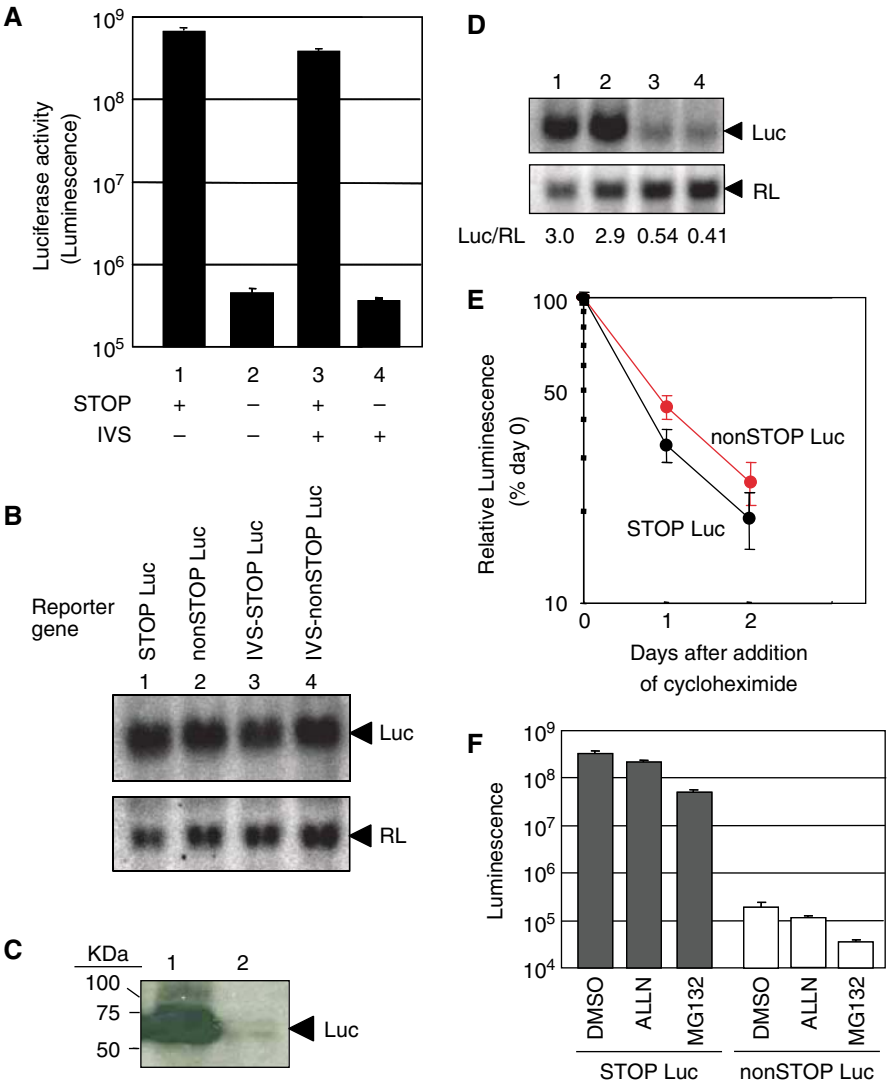

Figure 2 Expression of nonSTOP *Firefly* luciferase in HeLa cells. (**A**) Luciferase assays were performed and *Firefly* luciferase activities (Luc) were normalized to cotransfected *Renilla* luciferase activities. Lane 1: pGL STOP plasmid encoding wild-type *Firefly* luciferase gene; lane 2: pGL nonSTOP plasmid encoding nonSTOP *Firefly* luciferase gene lacking a termination codon; lane 3: pGL-IVS STOP plasmid in which a chimeric intron (IVS) is inserted into the 5′ UTR of wild-type *Firefly* luciferase gene; lane 4: pGL-IVS nonSTOP plasmid in which an IVS fragment is inserted into the 5′ UTR of nonSTOP *Firefly* luciferase gene. There were no differences in expression of the cotransfected *Renilla* luciferase among the four conditions. Error bars represent standard deviations of four independent experiments. (**B**) Northern blot analysis of 10 μg total RNA prepared from HeLa cells transfected with pGL STOP (lane 1), pGL nonSTOP (lane 2), pGL-IVS STOP (lane 3), or pGL-IVS nonSTOP (lane 4) with ^{32}P-*Firefly* luciferase cDNA probe (Luc, upper panel) or ^{32}P-*Renilla* luciferase cDNA probe (RL, lower panel). All cells were cotransfected with pRL CMV for normalization of transfection efficiency. (**C**) Western blot hybridization analysis of 10 μg total protein prepared from HeLa cells transfected with pGL STOP (lane 1) or pGL nonSTOP (lane 2) with an anti-luciferase antibody. (**D**) Northern blot analysis of total RNA extracted from HeLa cells transfected with either pGL STOP (lane 1 and 3) or pGL nonSTOP (lane 2 and 4) plasmid. All cells were cotransfected with pRL CMV. A total of 1 μg reporter plasmid (lanes 1 and 2) or 100 ng plasmid (lanes 3 and 4) was transfected into cells growing on 35 mm dish. Total RNA (10 μg) resolved on an agarose gel was probed by Northern blotting with ^{32}P-labeled probes (upper panel: *Firefly* luciferase (Luc); lower panel: *Renilla* luciferase (RL)). The relative expression levels of Luc mRNA normalized to RL mRNA are indicated below the panels. (**E**) Determining luciferase activity following a block in translation with cycloheximide. *Firefly* luciferase activities were measured and normalized to the total amount of protein. Relative luciferase activities were calculated by normalizing *Firefly* luciferase activity at the indicated time points to the *Firefly* luciferase activity at day 0. Error bars represent standard deviations of four independent experiments. (**F**) Effect of inhibition of proteasome on the steady-state amount of luciferase activity. Cells transfected with either pGL STOP or pGL nonSTOP plasmid were exposed to 10 μM ALLN or 10 μM MG132, 24 h after transfection, incubated for an additional 22 h, at which point luciferase activity was measured. *Firefly* luciferase activity was normalized to cotransfected *Renilla* luciferase activity. There was no difference in expression level of cotransfected *Renilla* luciferase under any condition. Error bars represent standard deviations of four independent experiments.

the translation initiation efficiency of nonSTOP mRNAs is decreased relative to that of STOP mRNAs, (ii) movement of ribosomes on nonSTOP mRNA is slower than on STOP mRNAs, or (iii) dissociation of ribosomes from nonSTOP mRNA occurs faster than from STOP mRNA.

Cap-independent translation of nonSTOP mRNA is repressed

If the translational repression of nonSTOP mRNA occurs at a step post-initiation, then functionally bypassing important processes for normal cap-dependent initiation

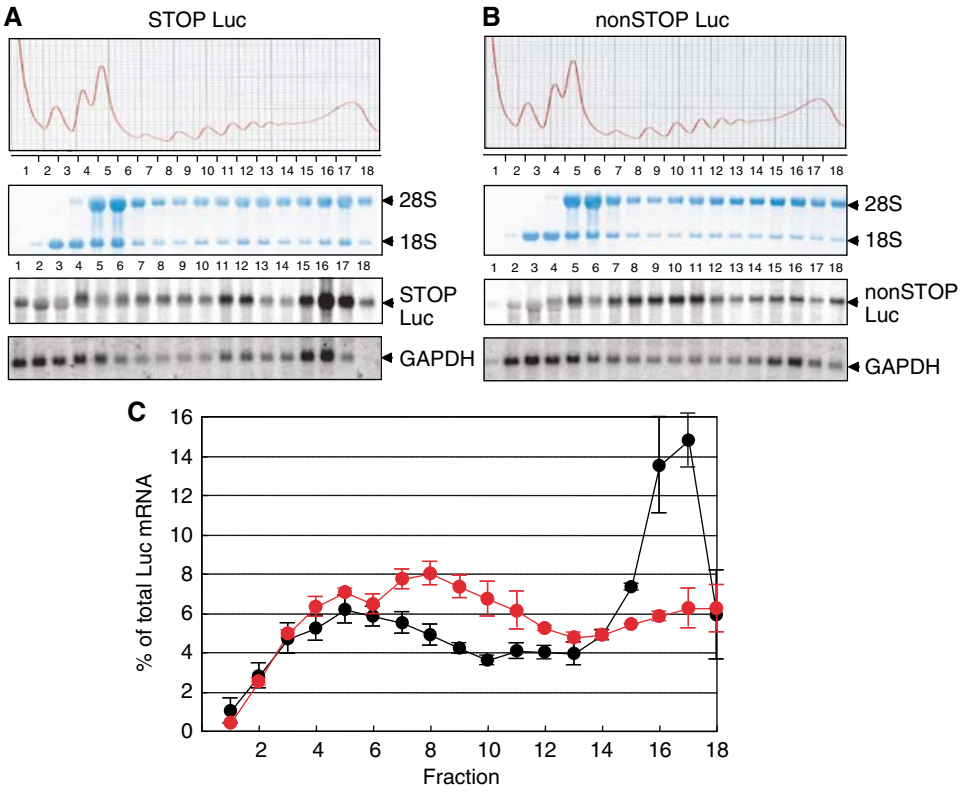

Figure 3 Polysome profiling of luciferase mRNAs. HeLa cells were transfected with either pGL STOP (**A**) or pGL nonSTOP plasmid (**B**), and extracts prepared in the presence of cycloheximide. Extracts were resolved by velocity sedimentation on 10–50% linear sucrose gradients and fractionated with continuous measurement of absorbance at 254 nm (top panels). RNA samples prepared from the indicated fractions were stained with methylene blue (second panels from the top) and analyzed by Northern blot hybridization using ^{32}P-Luc probe (third panels from the top) or ^{32}P-GAPDH probe (bottom panels). (**C**) The signal intensity from each luciferase signal was quantitated and normalized to the total signal intensity across all fractions. Black and red circles represent wild-type and nonSTOP luciferase mRNAs, respectively. Each value shown represents the mean ± standard deviation (s.d.) obtained from four independent experiments.

should not affect the repression. To test this prediction, bicistronic luciferase vectors containing the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV) were utilized (Figure 4A). Initiation from the ECMV-derived IRES does not involve scanning and does not require eIF1, eIF1A, or the eIF4E subunit of eIF4F, permitting ECMV IRES-driven translational initiation to bypass cap-dependent translational initiation (Pestova *et al*, 2001; Sonenberg and Dever, 2003). These reporters were transfected into HeLa cells and luciferase assays performed the following day to determine whether translational repression of nonSTOP mRNA also extended to IRES-mediated initiation. As shown in Figure 4, luciferase activity from IRES-initiated translation of nonSTOP *Firefly* luciferase was significantly reduced without a concomitant reduction in mRNA expression (Figure 4B and C). These experiments were extended by transfecting uncapped mRNAs into HeLa cells and examining luciferase activity. In the absence of a cap structure, translation of nonSTOP mRNA was also reduced *in vivo* (Figure 4D) and *in vitro* (data not shown). These results suggest that translation repression of nonSTOP mRNA occurs, irrespective of whether initiation is cap dependent or cap independent.

Run-off translation of fractionated polysomes in vitro

There are several reported examples where translation is regulated at the level of elongation (Theodorakis *et al*, 1988; Chiba *et al*, 1999; Browne and Proud, 2002). We therefore questioned if elongation rates were reduced on nonSTOP mRNA. Run-off translation of mRNA associating with ribosomes is one method by which to assess translational competence post-initiation (Vayda, 1995). Polysomes were isolated from cells in the absence of cycloheximide and resuspended in rabbit reticulocyte lysates containing the initiation inhibitor aurintricarboxylic acid (Theodorakis *et al*, 1988; Vayda, 1995). In this way, synthesis of polypeptides that were initiated *in vivo* is completed *in vitro*, in the absence of *de novo* initiation event. Messenger RNA containing polysomes (Figure 3, fraction #13) was used to program an *in vitro* translation extract followed by monitoring of luciferase activity. Quantitative PCR analysis showed that these fractions contained similar amounts of STOP and nonSTOP *Firefly* luciferase mRNAs (data not shown). As shown in Figure 5, STOP Luc mRNA associating with polysomes expressed luciferase activity for 120 min when used to program rabbit reticulocyte lysate. On the other hand, nonSTOP Luc mRNA expressed very little luciferase activity and did not produce luciferase over 120 min. These results are consistent with a model in which the step of translational repression is post-initiation. Moreover, this result suggests that repression of nonSTOP mRNA cannot be accounted for by reduced elongation rates on nonSTOP mRNA since no significant expression of nonSTOP luciferase was observed over the time period.

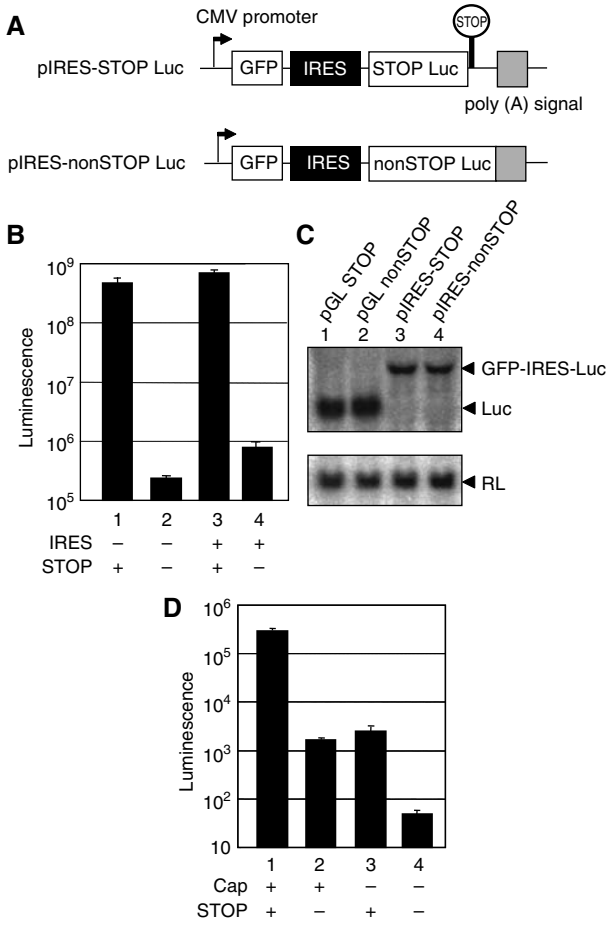

Figure 4 Repression of cap-independent translation of nonSTOP mRNA. (**A**) Schematic drawing of reporter genes. Open boxes indicate ORFs. Black boxes indicate an internal ribosome entry site (IRES) from encephalomyocarditis virus (ECMV). Shaded boxes indicate a polyadenylation signal. The termination codon of *Firefly* luciferase gene is indicated by a STOP sign. (**B**) Luciferase assays were performed following transfection of HeLa cells and *Firefly* luciferase activities (Luc) were normalized to cotransfected *Renilla* luciferase activities. Lane 1: pGL STOP; lane 2: pGL nonSTOP; lane 3: pIRES-STOP Luc; lane 4: pIRES-nonSTOP Luc. Error bars represent standard deviations of four independent experiments. (**C**) Northern blot analysis of 10 μg total RNA prepared from HeLa cells transfected with pGL STOP (lane 1), pGL nonSTOP (lane 2), pIRES-STOP Luc (lane 3), or pIRES-nonSTOP Luc (lane 4) and probed with ^{32}P-*Firefly* luciferase probe (Luc, upper panel) or ^{32}P-*Renilla* luciferase probe (RL, lower panel). (**D**) Transfection of *in vitro* synthesized RNAs into HeLa cells. Measured *Firefly* luciferase activities of HeLa cells transfected with *Firefly* luciferase mRNAs were normalized to the activity of cotransfected *Renilla* luciferase mRNA. Lane 1: capped wild-type *Firefly* luciferase mRNA; lane 2: capped nonSTOP *Firefly* luciferase mRNA; lane 3: uncapped wild-type *Firefly* luciferase mRNA; lane 4: uncapped nonSTOP *Firefly* luciferase mRNA. There was no difference in the expression level of cotransfected *Renilla* mRNA under the four conditions. Error bars represent standard deviations of four independent experiments.

Translation of nonSTOP RNA produced significant amount of short polypeptide in vitro

Results from polysome profiling and *in vitro* run-off translations could be accounted for by invoking premature dissociation of ribosomes from the mRNA during translation, but prior to completion of translation elongation. If ribosomes are actively released from nonSTOP mRNA during the elongation process, then a significant amount of truncated polypeptides

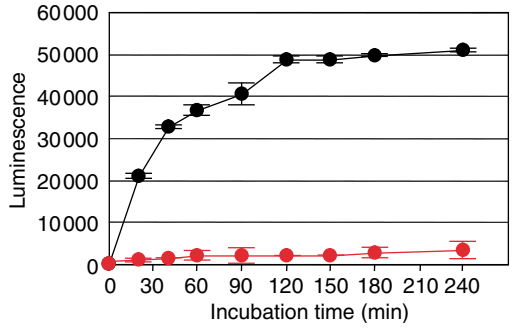

Figure 5 *In vitro* run-off translation programmed with mRNA associating with polysomes. Polysomal fraction (#13 from Figure 3) containing either wild-type *Firefly* luciferase (STOP Luc) or nonSTOP *Firefly* luciferase (nonSTOP Luc) mRNA was prepared by sucrose gradient centrifugation in the absence of cycloheximide, and used to program rabbit reticulocyte lysate at 30°C in the presence of the 50 μg/ml aurintricarboxylic acid, an initiation inhibitor. Small aliquots were removed and luciferase activities were measured at the indicated time. Luciferase activities obtained are presented as the mean values ± experimental errors, which was obtained from two independent experiments. Black and red circles represent luciferase activity produced from reactions containing polysome-associated STOP Luc and nonSTOP Luc mRNA, respectively.

should be produced. Translation of STOP Luc mRNA produced significant amounts of luciferase activity (Figures 6A). Interestingly, ^{14}C-leucine incorporation into trichloroacetic acid-insoluble material from nonSTOP Luc mRNA was similar to that from STOP Luc mRNA (Figure 6B). Moreover, immunoprecipitates of translational products obtained from mRNA encoding N-terminal FLAG-tagged nonSTOP luciferase yielded a smeared signal as assessed by SDS–PAGE analysis (Figure 6C). These results indicate that nonSTOP mRNA is competent for translation but produce full-length polypeptides.

Run-off translation assay in vivo using hippuristanol, a specific eIF4A inhibitor

Former studies in yeast proposed that ribosomes are stalled at the 3′ end of nonSTOP mRNA. To test whether this was also the case for mammalian systems, we determined the distribution of nonSTOP mRNAs in polysome fractions following inhibition of translation initiation by hippuristanol, a compound that prevents eIF4A-mediated translation initiation (Bordeleau *et al*, 2006). Cells expressing either STOP Luc mRNA or nonSTOP Luc mRNA were treated with 5 μM hippuristanol for 0 or 10 min and polysomes were monitored by sucrose density gradient centrifugation. A 10 min exposure of cells to hippuristanol is sufficient to completely dissociate polyribosomes from mRNAs (Petersen *et al*, 2006) (Figure 7A). Northern analysis showed that inhibition of translation initiation by hippuristanol resulted in STOP Luc mRNAs being distributed predominantly in ribosome-free fractions (fractions #2 to #5 in Figure 7B and C) at 10 min after addition of hippuristanol. In contrast, distribution of nonSTOP Luc mRNA following treatment of hippuristanol was shifted towards fractions #3 to #6. GAPDH mRNA was distributed in ribosome-free fractions in extracts expressing either STOP Luc or nonSTOP Luc mRNA after hippuristanol treatment and found to have a similar distribution

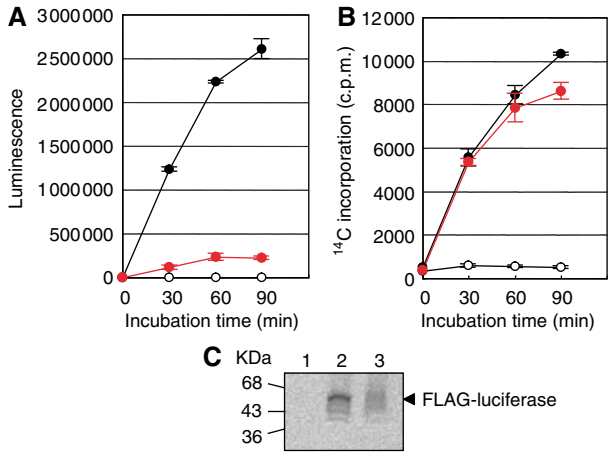

Figure 6 Short truncated polypeptides are produced from nonSTOP mRNA *in vitro*. *In vitro* synthesized mRNA coding FLAG-tagged *Firefly* luciferase (STOP Luc) or FLAG-tagged nonSTOP *Firefly* luciferase (nonSTOP Luc) were incubated at 30°C with rabbit reticulocyte lysate supplemented with ^{14}C-leucine and all necessary components for translation. (**A**) Small aliquots were sampled and *Firefly* luciferase activities were measured at the indicated times. Luciferase activities are shown as the mean ± experimental error, which was obtained from two independent experiments. Black and red circles represent the result of STOP Luc mRNA and nonSTOP Luc mRNA, respectively. White circles are the results from control translations lacking input mRNA. (**B**) ^{14}C-label incorporation into acid-insoluble material. Sampled small aliquots were spotted on 3MM paper, and then incubated with 5% trichloroacetic acid solution at 80°C for 30 min, washed, and then quantitated by liquid scintillation counting. Radioactivity counts are shown as the mean values ± experimental errors, which were obtained from two independent experiments. Black and red circles represent acid-insoluble ^{14}C-label incorporation produced from STOP Luc mRNA and nonSTOP Luc mRNAs, respectively. White circles represent the result of control reactions lacking mRNA. (**C**) *In vitro* translation reactions performed with no mRNA (lane 1), FLAG-*Firefly* luciferase mRNA (lane 2), or FLAG-nonSTOP *Firefly* luciferase mRNA (lane 3) were processed for immunoprecipitation using an anti-FLAG antibody (M2 mAb, SIGMA). Immunoprecipitates were analyzed by SDS–PAGE and the signals visualized using a BAS-2500 imaging analyzer.

(Figure 7B). Hence, complexes harboring nonSTOP mRNA are of a different nature than those harboring a STOP mRNA.

Ribosomes are stalled at the poly(A) region of nonSTOP mRNA

If ribosomes are stalled at the poly(A) region of nonSTOP mRNA, then the poly(A) region should be protected from nuclease digestion and nuclease-resistant poly(A) fragments should be detectable by gel electrophoresis. *In vitro* synthesized mRNAs harboring a ^{32}P-labeled poly(A) tail were used to program an *in vitro* translation reaction, followed by micrococcal nuclease digestion (Figure 8). Although no RNA fragments of defined size were obtained from extracts containing STOP Luc mRNA, nonSTOP mRNA produced a nuclease-resistant ^{32}P-labeled poly(A) fragment of ~40 nt length (Figure 8A and B, compare lanes 4 to 3), which corresponds in size to ribosome-protected fragments determined in previous studies (Kozak and Shatkin, 1977; Wolin and Walter, 1988). Nuclease-resistant poly(A) fragments from nonSTOP mRNA disappeared upon incubation with puromycin (Figure 8A, compare lane 8 to lane 4), indicating that appearance of nuclease-resistant poly(A) was mediated by ribosomes. Nuclease-resistant poly(A) fragments produced from nonSTOP mRNA was not diminished after 60 min incubation in the presence of aurintricarboxylic acid, an initiation inhibitor (Figure 8A and B, compare lane 6 to lane 4). Incubation in the presence of hippuristanol also did not affect the production of ~40 nt poly(A) fragment from nonSTOP mRNA (Figure 8B, compare lane 8 to lane 6). These results indicate that a ribosome(s) is stalled on the poly(A) tail of nonSTOP mRNA.

Discussion

To our knowledge, this is the first systematic and comprehensive study to examine the fate of nonSTOP mRNAs in mammalian cells. We found that translation of nonSTOP mRNA is significantly reduced and not a consequence of decreased mRNA levels (Figures 1 and 2). Cytoplasmic accumulation of nonSTOP mRNA does not appear to be impaired (Figure 1F) and degradation rates of nonSTOP proteins did not appear to be elevated (Figures 1E, 2E, and 2F). A substantial amount of nonSTOP mRNA was associated with polysomes (Figure 3) and cap-independent translation of nonSTOP mRNA was also repressed (Figure 4). Protein production from polysome-associated nonSTOP mRNA was repressed *in vitro* (Figure 5). Taken together, these results strongly suggest that nonSTOP mRNAs have incurred a block in translation at a step that is post-initiation.

Our finding that nonSTOP mRNA distributed to lighter polysome fractions at steady state, in comparison to wild-type STOP mRNA (Figure 3), suggested that elongation rates were decreased or translating ribosomes were prematurely dissociating from nonstop mRNA. An *in vitro* run-off assay, performed over the course of 120 min, indicated that the former possibility was unlikely (Figure 5). Rather, the production of short polypeptides from nonSTOP mRNA, as judged by incorporation of ^{14}C-amino acid into TCA-insoluble material at a rate that was similar to that of control STOP mRNA (Figure 6B), suggests that ribosomes are actively translating the mRNA but being prematurely released before completing translation. The appearance of low molecular weight polypeptides produced from nonSTOP mRNA is consistent with this idea (Figure 6C). Moreover, nonSTOP mRNA shifted into lighter fractions within 10 min of exposure of cells to the initiation inhibitor hippuristanol, suggesting that ribosomes are easily and rapidly dissociating from nonSTOP mRNA (Figure 7). The fact that the nonSTOP mRNA appears in a set of fractions that are slightly heavier than STOP mRNA, from hippuristanol-treated cells, is consistent with studies from yeast indicating the presence of a stalled ribosome(s) on nonSTOP mRNA (Figure 7). Additionally, production of ribosome-protected nuclease-resistant poly(A) fragments provided a direct evidence of stalled ribosomes at the poly(A) tail of nonSTOP mRNA (Figure 8). We therefore propose a model whereby a stalled ribosome at the 3′ end of mRNA leads to translational repression by inhibiting completion of subsequent upstream translation events leading to enhanced ribosome release. It has been reported that growing polypeptide chains interact with several ribosome-bound factors during the elongation process, such as chaperons and the conserved heterodimeric nascent polypeptide-associated complex (NAC) that are thought to be monitoring the status of translation (Craig *et al*, 2003;

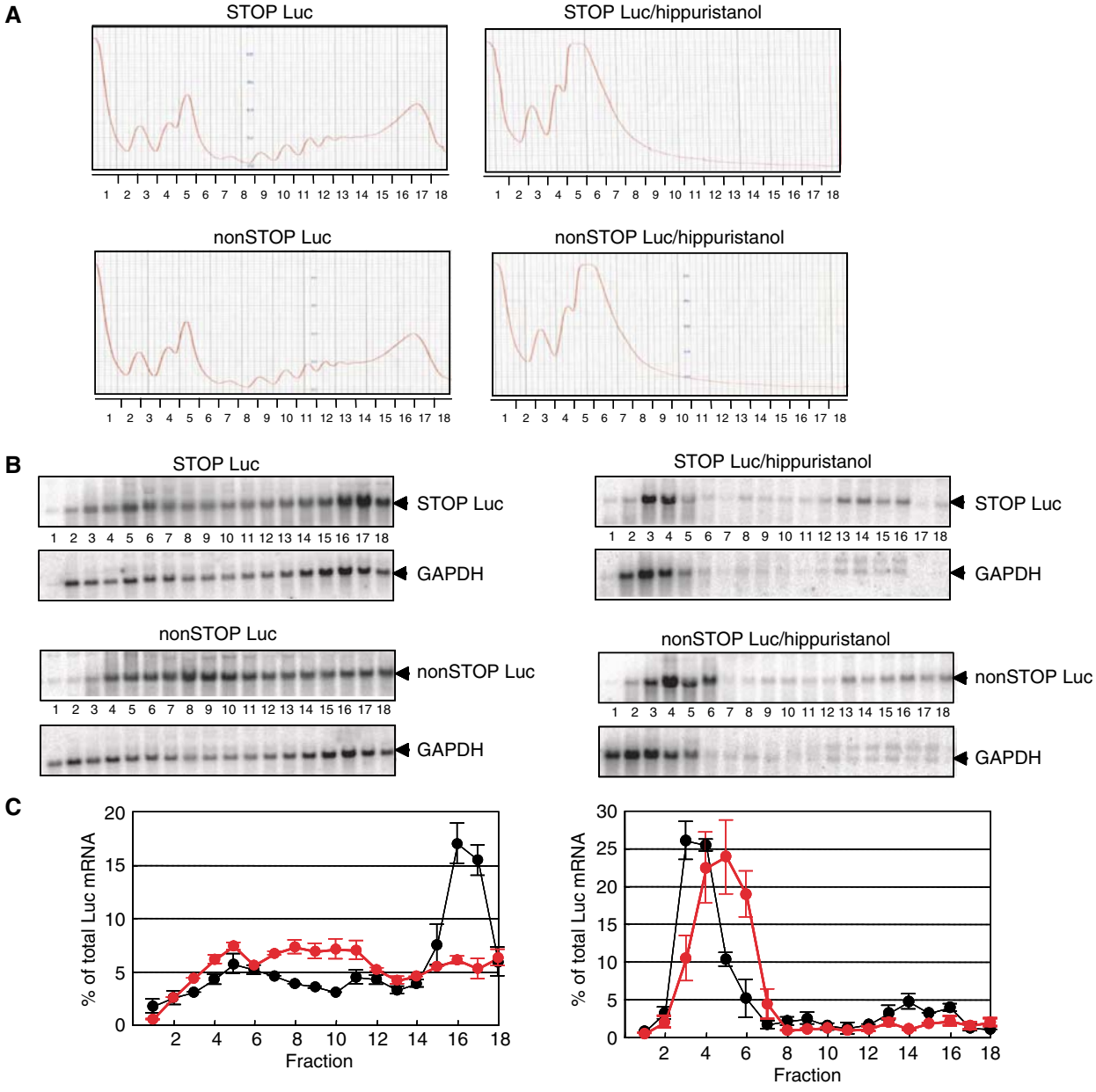

Figure 7 Inhibition of translation *in vivo* indicates that translating nonSTOP mRNA is distinct from STOP mRNA. (**A**) HeLa cells transfected with either pGL STOP (top panels) or pGL nonSTOP (bottom panels) were treated with 5 μM hippuristanol for 0 (left panels) or 10 min (right panels) before cell lysis and separation of polyribosomes. Absorbance at 254 nm profiling during fractionation is shown. (**B**) Collected fractions were subjected to Northern blot analysis with ^{32}P-Luc or ^{32}P-GAPDH probe. STOP Luc indicates signals of wild-type luciferase mRNA expressed from pGL STOP plasmid. NonSTOP Luc indicates signals of nonSTOP luciferase mRNA expressed from pGL nonSTOP plasmid. (**C**) Luciferase mRNA signals normalized to the total signal across all fractions in Figure 7B (left panel: no hippuristanol; right panel: 5 μM hippuristanol for 10 min). Each value is shown as the mean value ± experimental errors, which were obtained from two independent experiments. Black and red circles represent STOP Luc and nonSTOP Luc mRNA, respectively.

Hundley *et al*, 2005). In addition, certain components of NAC are involved in the translational repression of several mRNAs at a step post-initiation in *Dorosophila* (Markesich *et al*, 2000; Braat *et al*, 2004). We therefore speculate that NAC or NAC-like associating factors interacting with nascent polypeptides may be involved in translational repression of nonSTOP mRNA by monitoring whether the termination process is finished properly or not.

It has been reported that translation of nonSTOP mRNA is repressed in yeast (Inada and Aiba, 2005). However, former studies showed that nonSTOP mRNAs are distributed in heavier fractions compared to STOP mRNAs when fractionated on polysomes. Moreover, the distribution of nonSTOP mRNA in polysomes was not significantly altered 30 min post-initiation inhibition, although wild-type STOP mRNAs redistributed into ribosome-free fractions after inhibition of initiation. These results are consistent with ribosomes accumulating and stalling on nonSTOP mRNAs. In contrast, our study indicates a different mechanism at work in mammalian cells since nonSTOP mRNAs are redistributed into

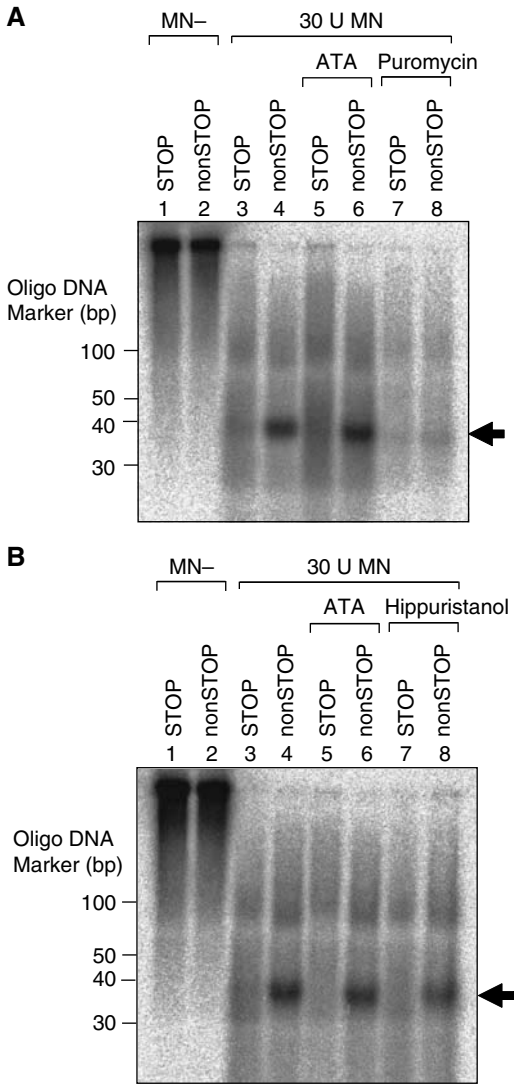

Figure 8 ^{32}P-labeled poly(A) fragments protected by ribosomes on nonSTOP mRNA upon micrococcal nuclease (MN) treatment. (**A**) *In vitro* synthesized *Firefly* luciferase mRNA having a ^{32}P-labeled poly(A) tail (STOP Luc + ^{32}P-poly(A); lanes 1, 3, 5, and 7) or nonSTOP *Firefly* luciferase mRNA having a ^{32}P-labeled poly(A) tail (nonSTOP Luc + ^{32}P-poly(A); lanes 2, 4, 6, and 8) was incubated with 20 μl rabbit reticulocyte lysate for 60 min at 30°C. Following *in vitro* translations, either aurintricarboxylic acid (ATA) (50 μM; lanes 5 and 6) or puromycin (200 μM; lanes 7 and 8) were added and then incubated for 60 min. Upon addition of 1 mg/ml cycloheximide and 40 mM $CaCl_2$, the mixture was digested with 30 units micrococcal nuclease for 15 min at 30°C (lanes 3–8). After termination of the nuclease digestion by the addition of 160 mM EGTA, 0.5% SDS and 1 mg/ml proteinase K were added and the mixture was incubated for 30 min at 37°C. RNA was extracted by water-saturated phenol/chloroform and precipitated with ethanol in the presence of 0.2 mg/ml glycogen. The resulting pellet was resuspended in RNA running solution (10 M urea, 1 × TBE and 0.1% BPB) and analyzed by electrophoresis in a 8 M urea, 10% polyacrylamide gel and the signal visualized using a BAS-2500 imaging analyzer. The undigested mRNAs (lanes 1 and 2) represent 1/5th the amount of samples loaded in the other lanes. (**B**) Examination of the effect of two different initiation inhibitors for the appearance of ribosome-protected poly(A) fragments. Indicated luciferase mRNAs harboring a ^{32}P-labeled poly(A) tail were used to program an *in vitro* translation reaction, then incubated additionally in the presence of either aurintricarboxylic acid (50 μM; lanes 5 and 6) or hippuristanol (10 μM; lanes 7 and 8). After terminating the reaction by addition of cycloheximide, RNAs were digested and analyzed as mentioned above. The arrows at the right position of panels indicate the position of ~40 nt poly(A) fragments.

light polysomes after inhibition of initiation. The reason(s) for this mechanistic difference is (are) not clear.

For nonSTOP mRNA, where translation would proceed into the 3′ untranslated region of an mRNA, one might expect to observe an increase in the size of the encoded protein. This was not the case in our experiments (Figure 2D), and is likely because the length of the extended sequence downstream of the nonSTOP GFP-coding region leading up to the poly(A) addition signal is only 24 nucleotides, a difference that would add on an additional eight amino acids and that would not be observed by SDS–PAGE (Figure 1A). Since our data indicate that the poly(A) tail is protected from RNAse digestion on the nonSTOP mRNA (Figure 8), ribosomes are clearly paused in this region. Since AAA (from the poly(A) tract) encodes for lysine, it maybe that Lys-tRNA becomes limiting during translation of the poly(A) region and this leads to the observed translational arrest.

Stability of nonSTOP mRNA in mammalian cells

Previous reports have shown that nonSTOP transcripts are degraded in yeast (Frischmeyer *et al*, 2002; van Hoof *et al*, 2002; Inada and Aiba, 2005) contrary to what our results indicate for the fate of similar transcripts in mammalian cells. It has been reported that the amount of plasmid used in transfection studies can impact on the stability of mRNA expressed from them (Nanbu and Nagamine, 1997). We therefore examined the expression level of nonSTOP mRNA using a low amount of plasmid for transfection (100 ng reporter plasmid per 35 mm dish) and found that there were no significant differences between the amount of STOP or nonSTOP mRNA even when low amounts of plasmid were transfected (Figures 1C and 2D). Next, we employed a second independent reporter gene, *Firefly* luciferase and found no differences in steady-state levels of nonSTOP and STOP *Firefly* luciferase mRNA (Figure 2B), indicating that our results are not cistron dependent. Frischmeyer *et al* (2002) reported that steady-state levels of nonSTOP transcripts expressed from a nonSTOP-Ter-poly(A)-β-gluc minigene was reduced by 50% compared to the level of wild-type transcript produced from a Ter-poly(A)-β-gluc minigene. The expression level of mutant ribosomal protein (rp) S19 mRNA lacking a stop codon was also reported to be lower than that of wild-type rpS19 mRNA in Diamond–Blackfan anemia (Chatr-Aryamontri *et al*, 2004). One of the significant differences between the reporter genes utilized in our studies, and those in previous reports, is the presence of introns in the latter. It is well known that splicing affects the fate of mRNAs transcribed in nuclei, including stability through nonsense-mediated decay. Therefore, we examined the effect of splicing on RNA stability for the reporter gene used in this study. However, steady-state amounts of nonSTOP mRNA were not decreased even though our reporter gene has splice sites (Figure 2A and B and Supplementary Figure 2). The location of the splice sites or specific *cis*-acting elements in the β-gluc minigene or rp S19 gene may have impacted on the results obtained.

Ribosome stalling at the 3′ end of nonSTOP mRNA is a conserved mechanism to mark nonSTOP transcript in eukaryotes

As shown in Figure 7, we found that nonSTOP mRNAs were distributed in very light polysomal fractions even after suffi-

cient time was allowed for ribosomes translating wild-type STOP mRNA to complete one round of translation. Moreover, ribosome-protected nuclease-resistant poly(A) fragments were produced from nonSTOP mRNA in an *in vitro* translation reaction (Figure 8). These results strongly suggest the presence of ribosomes stalled at the 3′ end of the nonSTOP mRNA, consistent with data from yeast studies (Frischmeyer *et al*, 2002; van Hoof *et al*, 2002; Inada and Aiba, 2005). This aberrant presence of a ribosome stalled at the 3′ end of the mRNA likely prevents completion of translation on nonSTOP mRNA. In this way, the translation machinery can recognize aberrant mRNAs lacking in-frame termination codons and cellular pathways recognizing this kind of abnormality is conserved in eukaryotes. However, it seems like that the subsequent inhibitory mechanism by which upstream ongoing translation is affected differs between yeast and mammalian cells, as mentioned above. Premature termination of translation through enhanced ribosome dissociation from mRNA (Ribosome drop-off model) has been proposed in microRNA-mediated translational repression (Petersen *et al*, 2006) and peptidyl-tRNA dissociation from ribosomes during translation is known to occur in prokaryotes (Manley, 1978). Thus, there are several reports suggesting 'ribosome drop-off-like mechanism' for translational repression, and this kind of regulation appears more common than previously suspected.

Regulation of endogenous gene expression by nonSTOP translational repression

RNA quality control systems not only mute aberrant mRNAs that harbor mutations or errors in transcription or processing but also regulate the expression of normal endogenous genes. For example, NMD regulates the expression of naturally occurring transcripts that represent ~10% of the transcriptome in yeast (Lelivelt and Culbertson, 1999; He *et al*, 2003), worm (Mitrovich and Anderson, 2000; Mitrovich and Anderson, 2005), fly (Rehwinkel *et al*, 2005), and mammalian cells (Mendell *et al*, 2004). Thus, it is reasonable to assume that the cellular mechanism recognizing nonSTOP mRNAs regulates the expression of naturally occurring transcripts. In fact, it was reported that certain genes produce natural nonSTOP transcripts, such as *CBP1*, *AEP/ATP13*, *RNA14*, and *SIR1* in yeast (Mayer and Dieckmann, 1989; Finnegan *et al*, 1991; Minvielle-Sebastia *et al*, 1991; Stone *et al*, 1991), *nad6* and *ccmC* in plants (Raczynska *et al*, 2006), and GHR in chicken (Oldham *et al*, 1993). The expression of these genes might be regulated by a mechanism such as described herein for recognizing nonSTOP mRNAs. Moreover, it was shown that there is genetic interaction between the [Psi^+] phenotype and the mechanism recognizing nonSTOP mRNAs in yeast (Wilson *et al*, 2005). Recently, it was proposed that degradation of pseudogenes by the NMD pathway is implicated in protein/gene evolution (Mitrovich and Anderson, 2005). Future studies will determine the importance of the nonSTOP-mediated RNA surveillance pathway not only for muting aberrant transcripts but also in the regulation and potential evolution of natural occurring transcripts.

Materials and methods

Cell culture, transfection and measuring luciferase activity

HeLa cells were grown in DMEM supplemented with 10% FBS. Cells were transfected with Lipofectamine 2000 (Invitrogen) or Transfectam (Promega) according to the manufacturer's instructions. The day after transfection, cells were lysed by Passive Lysis Buffer (Promega), and *Firefly* and *Renilla* luciferase activity were measured using the Dual-Luciferase Assay kit (Promega). The activity of *in vitro* synthesized mRNAs was determined by translation in rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions. All transfection experiments, with both DNA and RNA, were performed at least three independent times, each time in tetraplicate.

Polysome profile analysis

Cells ($\sim 5 \times 10^6$ per 10 cm dish) were washed twice with ice-cold phosphate-buffered saline containing 100 μg/ml cycloheximide and lysed directly on the plate by the addition of hypotonic lysis buffer (100 μg/ml cycloheximide, 1 mM dithiothreitol, 200 U/ml RNase inhibitor (Promega), 1.5 mM KCl, 2.5 mM $MgCl_2$, 5 mM Tris–HCl pH 7.4, 1% Triton X-100, and 1% deoxycholate). The lysate was collected and treated as described (Jefferies *et al*, 1994). Briefly, 500-μl aliquots of extracts were layered onto linear sucrose gradients (10–50% in 80 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris–HCl pH 7.4, and 1 mM dithiothreitol), which were prepared in 14×89 mm polyallomer tubes and centrifuged in a SW40Ti Beckmann rotor at 36 000 r.p.m. for 2 h at 4°C. The gradient was then fractionated using a Piston Gradient Fractionator (BioComp). Polysome profiles were generated by continuous measurement of the absorbance at 254 nm. RNA from each fraction was isolated and analyzed by Northern blot hybridization as described. The addition or omission of cycloheximide did not change the polysome profile (data not shown).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We thank Dr Kozo Tomita (AIST) for helpful suggestions and discussions. We also thank Drs Hiroaki Imataka (Riken, GSC), Toshiyuki Hamada (AIST), Mahito Nakanishi (AIST), Masanao Oda (AIST), Tomoko Okada (AIST), Kaoru Suzuki (AIST), Toshifumi Inada (Nagoya University), Hiroji Aiba (Nagoya University), and Kazuhisa Sekimizu (University of Tokyo) for fruitful suggestions and encouragements. We are grateful to Dr Yoshikuni Nagamine (Friedrich Miescher Institute; FMI), Ms Fumiko Iwamato (FMI), and Mr Teppei Yamaguchi (FMI) for helpful supports. This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports and Technology of Japan.

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