Transcriptional Silencing of Nonsense Codon - Containing Immunoglobulin μ Genes Requires Translation of its mRNA

Lukas Stalder & Oliver Mühlemann*
Institute of Cell Biology, University of Berne, Baltzerstrasse 4, CH-3012 Bern, Switzerland

Running title: NMTGS requires translation

* Corresponding author. E-mail: oliver.muehlemann@izb.unibe.ch
Telephone: +41 31 631 4627, Fax: +41 31 631 4616.

Eukaryotes have evolved quality control mechanisms that prevent the expression of genes in which the protein coding potential is crippled by the presence of a premature translation-termination codon (PTC). In addition to nonsense-mediated mRNA decay (NMD), a well-documented posttranscriptional consequence of the presence of a PTC in an mRNA, we recently reported the transcriptional silencing of PTC-containing immunoglobulin (Ig) μ and γ minigenes when they are stably integrated into the genome of HeLa cells. Here we demonstrate that this transcriptional silencing of PTC-containing Ig-μ constructs requires active translation of the cognate mRNA, as it is not observed under conditions where translation of the PTC-containing mRNA is inhibited through an iron responsive element (IRE) in the 5′ UTR. Furthermore, RNAi-mediated depletion of the essential NMD factor Upf1 not only abolishes NMD, but also reduces the extent of nonsense-mediated transcriptional gene silencing (NMTGS). Collectively, our data indicate that NMTGS and NMD are linked, relying on the same mechanism for PTC recognition, and that the NMTGS pathway branches from the NMD pathway at a step after Upf1 function.

Introduction
To recognize mistakes during the molecular processes involved in gene expression and to prevent production of faulty gene products, several quality control mechanisms have evolved (1). Among those, nonsense-mediated mRNA decay (NMD) represents a translation-dependent posttranscriptional process that selectively degrades mRNAs with premature translation-termination codons (PTCs), thereby preventing the synthesis of potentially deleterious truncated proteins (2-5). Recent transcriptome analysis of yeast, drosophila and mammalian cells revealed that, in addition to serving as a quality control system, NMD also influences the steady-state level of 5 – 15 % of the physiological mRNAs, suggesting an important role of NMD in fine-tuning gene expression (5-9). The exact mechanisms of PTC recognition and subsequent mRNA degradation are not yet understood und
NMTGS requires translation currently under intensive investigation in many laboratories (10,11). The three evolutionary conserved proteins Upf1/SMG-2, Upf2/SMG-3 and Upf3/SMG-4 (2) are undoubtedly the key NMD factors involved in PTC recognition in all eukaryotes, whereas additional factors function as NMD regulators in metazoans (4).

During our studies of NMD with reporter gene constructs derived from the mouse Sp6 heavy chain gene expressed under the control of the human β-actin promoter (12), we made an unexpected observation when these so-called miniµ constructs were stably integrated into the genome of HeLa cells. In addition of triggering NMD, the transcription rate of PTC-containing (PTC+) miniµ genes was also reduced under these conditions (13). This PTC-specific transcriptional silencing is accompanied by a change in the chromatin state towards reduced acetylation and increased mono- and dimethylation of lysine 9 on histone H3, and we dubbed the term “nonsense-mediated transcriptional gene silencing” (NMTGS) to describe our observations. Among the six NMD reporter gene constructs we have tested so far, only the Ig-µ and the Ig-γ minigene showed NMTGS (13), indicating that NMTGS depends on a gene-specific signal that might be confined to immunoglobulin heavy chain encoding genes. In a first attempt to elucidate the intriguing molecular pathway by which a translation signal (the PTC) can feed back to inhibit the transcription of the gene that gives rise to this PTC+ transcript, we found that over-expression of the siRNase 3′hExo completely suppressed NMTGS (13). This suggests the involvement of siRNAs or siRNA-like molecules in NMTGS, but all attempts to detect these putative siRNAs were not successful so far (Marc Bühler, Rajani Gudipati and O.M., unpublished).

Although the biological relevance of NMTGS is still unclear, our observations appear to fit into a puzzle of reminiscent results recently reported from other experimental systems. On the one hand, pol II transcription from pericentromeric repeat regions or certain intergenic loci was found to be silenced by a mechanism that involves small RNAs, components of the RNAi machinery and chromatin remodeling factors in a variety of different eukaryotes ranging from S. pombe to mammals and plants (14). From that point of view, nonsense mRNAs that trigger NMTGS may also represent a kind of non-coding transcripts with the potential to induce the RNAi-mediated transcriptional silencing system. On the other hand, a genetic link between NMD factors and RNAi has been documented both in C. elegans (15) and A. thalina (16). Mutations in the NMD factors SMG-2, SMG-5 or SMG-6 of C. elegans resulted in worms that still responded to dsRNA-induced gene silencing, but that had lost the persistence of RNAi-mediated silencing (15). Similarly, A. thalina with a mutation in Upf1/SMG-2 are defective in silencing an inverted repeat construct (16). It remains to be seen in the future if and how this yet unsolved puzzle will fit together.

To explore the hypothesized link between the translation-dependent PTC recognition step in NMD and NMTGS, we inhibited translation of our PTC+ miniµ transcripts by the insertion of an iron responsive element (IRE) into the 5′ UTR of the miniµ construct. Using this approach, we demonstrate here that NMTGS depends on the translation of the PTC+ mRNA and
that it involves the essential NMD factor Upf1. Together, these data corroborate the mechanistic link between NMD and NMTGS and suggest that both processes rely on the same mechanism for PTC recognition and branch from each other at a step after Upf1 function.

Experimental procedures

Plasmids and cell lines - The pβminiµ-IRE and pβminiµ-mutIRE plasmids were generated by insertion of a double stranded oligonucleotide encoding the IRE or the mutIRE sequence into the Sal I site of pβminiµwt and pβminiµter310 (12), respectively. The inserted sequence was 5’-TAGCAACCCGGGTTTCCTGCTTC AACAGTGCTTGGACGGAACCC-3’ (excluding the Sal I sites), the deleted C in mutIRE is underlined. Correct orientation of the inserts was confirmed by sequencing. HeLa cells were transfected with Lipofectamine (Invitrogen) or Dreamfect (OZ Biosciences) according to manufacturers protocol, and polyclonal populations of stably transfected HeLa cells were obtained by selection with 0.5 - 1.0 mg/ml G418 (Roche Diagnostics) for 10 - 14 days. Prior to analysis, the cells were cultured in the absence of G418 for at least 2 weeks.

Northern blot analysis - Total cellular RNA (15 µg) was separated on a 1.2 % agarose gel containing 1x MOPS and 1 % formaldehyde. RNA was transferred to positively charged nylon membrane (Roche) in 0.5x MOPS by 1 hour wet blotting in a genie blotter (Idea Scientific, Minneapolis). Following UV crosslinking to the nylon filter, prehybridization and hybridization were carried out in 6x SSC, 5x Denhardt’s reagent and 0.5 % SDS at 60 °C. Prehybridization was with 50 µg/ml denatured salmon sperm DNA and 100 µg/ml denatured calf thymus DNA. For hybridization, 100 ng muter310 mutIRE DNA was labeled with α-32P dCTP using the Ready-To-Go™ DNA labeling Kit (Amersham). After overnight hybridization, membranes were washed twice with 2x SSC/0.2 % SDS and twice with 0.2x SSC/0.1 % SDS at 60 °C before exposure to a PhosphorImager screen.

Nuclear run-on analysis - Nuclear run-on analysis was performed essentially as described in Bühler et al. (13). Single stranded DNA was isolated from E. coli transformed with pBluescript KS(-) containing the antisense sequences of human GAPDH cDNA and miniµ and infected with helper phage M13K07 (New England Biolabs). For nuclei preparation, approximately 4 x 10⁷ cells were harvested and centrifuged at 4 °C with 300 x g for 8 min. The cells were then resuspended in 6 mL ice-cold nuclei lysis buffer (3 mM MgCl₂, 10 mM Tris HCl pH 7.5, 10 mM NaCl, 0.5% IgePalCAG30) under gentle vortexing and incubated 5 min on ice. The nuclei were pelleted at 4 °C with 500 x g for 5 min, resuspended in 6 mL nuclei lysis buffer, centrifuged again as above, resuspended in 250 µL nuclei storage buffer [40% glycerol, 5 mM MgCl₂, 100 nM EDTA, 2.5 mM Tris HCl pH 8.25, 5 mM DTT, 0.4 U RNasin (Promega)], and stored in liquid nitrogen. Per run–on reaction, approximately 10⁷ nuclei were incubated with 11 MBq α32P–UTP (0.834 µM), 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.834 µM UTP, 120 mM KCl and 2.5 mM Mg–acetate under 500 rpm shaking for 30 min at 30 °C. Subsequently, total RNA was isolated using “Absolutely RNA RT–PCR miniprep Kit” (Stratagene) and hybridized
to a positively charged nylon membrane (Roche Diagnostics) on which 5 µg ssDNA probe had been immobilized per dot. The nylon membrane was loaded with the ssDNA dots using a Biorad assemble vacuum system, crosslinked with 120 kJ (Stratalinker) and dried in the air. The membranes were pre-hybridized for 3 hours in 6 x SSC, 5 x Denhardt’s, 0.5 % SDS, 20 ng/µL herring sperm DNA and 100 ng/µL E.coli tRNA. Hybridization conditions were 6 x SSC, 5 x Denhardt’s, 0.5 % SDS, 10 ng/µL herring sperm DNA, 50 ng/µL E.coli tRNA. The isolated nuclear RNA was denatured at 90 °C for 5 min, mixed with the hybridization buffer and hybridized for 24 hours at 60 °C. The filter strips were washed twice at 65 °C in 1x SSC, 0.1 % SDS for 15 min. Signals were visualized and quantified by PhosphorImager scanning.

Chromatin immunoprecipitations (ChIP) - ChIP was performed from formaldehyde-fixed, stably transfected HeLa cells using a ChIP assay kit (Upstate, 17-295) according to the manufacturer’s protocol. 5 µg polyclonal rabbit α-H3K9me1, α-H3K9me2, α-H3K9me3 or α-H3 antibody (Upstate) were used. For each real-time PCR measurement (see below), 40 ng of input DNA or immunoprecipitated DNA was used to quantitate miniµ and endogenous β-globin. The TaqMan assays span the 5’ splice site of miniµ exon C2 and the 5’ splice site of endogenous β-globin exon 2, respectively (sequences available upon request). For each antibody, the relative amount of immunoprecipitated miniµ DNA was normalized to that of endogenous β-globin DNA and to input DNA, and calculated as “fold enrichment” relative to miniµter310 IRE using the formula (IP_{miniµ}/input_{miniµ})/(IP_{β-glob}/input_{β-glob}). Finally, these relative “fold enrichment” of miniµ for each of the three H3K9 methylation-specific antibodies was normalized to the relative “fold enrichment” of miniµ determined with the α-H3 antibody.

Inhibition of HDACs and quantitative real-time RT-PCR – Between 2 and 3 x 10^5 cells were seeded in 6-well plates and treated the next day with 15 mM SB (Upstate) or 200 ng/mL TSA (Sigma) for 24 hours. Subsequently, total cellular RNA was isolated using “Absolutely RNA RT – PCR miniprep Kit” (Stratagene) and 1 µg RNA was reverse transcribed in 50 µL Stratascript first strand buffer in the presence of 0.4 mM dNTPs, 300 ng random hexamers, 40 U RNasin (Promega), and 50 U Stratascript reverse transcriptase (Stratagene). For real-time RT-PCR, reverse-transcribed material corresponding to 40 ng RNA was amplified in 25 µL Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) with specific primers and TaqMan probes (sequences are available upon request) by using the ABI SDS7000 Sequence Detection System.

RNAi-mediated Upf1 knockdown - Knockdown of Upf1 was induced by transfection of pSUPERpuro plasmids targeting two different sequences in hUpf1 (17). Starting 24 hours after transfection, untransfected cells were eliminated by culturing the cells in the presence of 1.5 µg/mL puromycin for 48 hours. Cells were then washed in PBS and incubated in puromycin-free medium in the presence or absence of 15 mM SB for another 24 hours. Total cellular RNA was isolated and whole cell lysates for western blotting were prepared 96 hours post transfection. The efficiency of the knockdown was
assessed on the mRNA level by real-time RT-PCR (not shown) and on the protein level by western blotting.

**Western blotting** - Whole cell lysates corresponding to 0.25 x 10^4 – 2 x 10^5 cells per lane were electrophoresed on a 10 % SDS-PAGE. Proteins were transferred to Optitran BA-S 85 reinforced nitrocellulose (Schleicher & Schuell) and probed with 1:2500 diluted polyclonal rabbit anti-hUpf1 antiserum (18), 1:500 diluted affinitypure goat anti–mouse IgG (µ chain specific, Jackson ImmunoResearch), 1:400 diluted supernatant of the mouse hybridoma cell line Y12, which produces a monoclonal antibody against human Sm proteins (19). 1:4000 diluted HRP-conjugated donkey anti-goat or 1:2500 diluted HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-mouse IgG (Promega) was used as secondary antibody. ECL+ Plus western blotting detection system (Amersham) was used for detection and signals were visualized on a Luminescent Image Analyzer LAS-1000 (Fujifilm).

## Results

**Inhibited translation of Ig-µ mRNA with an iron responsive element in the 5’ untranslated region.**

In order to further investigate NMTGS of our Ig-µ minigene (12) (hereafter called minιµ), we have introduced the iron responsive element (IRE) of the human ferritin H chain (20) into the 5’UTR of minιµ, giving rise to the minιµ IRE constructs (Fig. 1A, upper panel). The IRE is an endogenous regulatory sequence, which allows controlling specifically the translation in response to the intracellular iron concentration, for example the translation of ferritin and transferrin receptor mRNA (21). The IRE forms a bulged stem loop structure in the 5’UTR that binds the iron regulatory proteins (IRPs) when the intracellular iron concentration is low. Under these conditions, the IRP bound to the IRE inhibits the formation of a 48S pre-initiation complex at the 5’ end of the mRNA, thus blocking translation initiation at an early step (22). Increasing the iron concentration in the cells leads to dissociation of the IRP, thus permitting the mRNA for translation. Under standard cell culture conditions, the IRP is bound to the IRE and translation is strongly inhibited (Fig 1B and data not shown). We have tried to induce the translation of the minιµ IRE with several iron sources, but the induction was neither efficient nor reliable (data not shown). As a control for our experiments, we therefore inserted a mutated IRE (mutIRE) with a single nucleotide deletion in the loop into the 5’UTR of minιµ, which cannot bind to the IRP (20) and thus allows translation (Fig. 1A, lower panel).

We transfected HeLa cells with minιµ constructs harboring a PTC at amino acid 310 (µter310) or not (µwt) and containing either the functional IRE (IRE) or the mutated version (mutIRE), and selected polyclonal pools which had these different minιµ constructs stably integrated into the genome. Quantification of a western blot with a 3 – fold serial dilution shows about 30 – fold less Ig-µ protein in cells expressing µwt IRE than cells expressing µwt mutIRE, even though the mRNA level of µwt mutIRE was consistently 2 – fold lower than that of µwt IRE (Fig. 1C and data not shown). This demonstrates that the translation of µwt IRE mRNA is efficiently inhibited. In cells expressing
the μter310 IRE or μter310 mutIRE construct, we could not detect any Ig-μ protein (data not shown).

Insertion of the IRE into μter310 restored the otherwise hardly detectable mRNA levels of this PTC+ transcript to mRNA levels similar to those obtained with μwt constructs (Fig. 1C). Our previous study showed that the low steady-state mRNA level of stably integrated μter310 constructs into the genome of HeLa cells is the net result of both posttranscriptional mRNA degradation (i.e. NMD) and NMTGS (13). IRE-mediated translation inhibition of transiently transfected PTC+ β-globin and minιμ constructs efficiently inhibits NMD [(23) and L.S., unpublished results], but the almost complete recovery of the μter310 IRE mRNA level suggested that NMTGS might also be inhibited by preventing translation of the μter310 mRNA.

**Translation of the Ig-μ PTC+ mRNA is required for NMTGS.**

To address if NMTGS depends on translation of its cognate mRNA, we needed an assay that allows monitoring of transcriptional effects without affecting NMD. We have previously shown that NMTGS involves chromatin remodeling and that the transcriptionally silenced state can be reversed by inhibition of histone deacetylases (13). Therefore, we treated the cells with the HDAC inhibitors sodium butyrate (SB) or trichostatin A (TSA) and determined the relative minιμ mRNA levels by real–time RT–PCR. 18S rRNA served as endogenous control for normalization in these experiments. As shown in Figures 1D and 1E, the relative mRNA levels of μter310 mutIRE increased between 5 and 8 – fold in cells treated with SB or TSA compared to untreated cells. To rule out that different average transgene copy numbers or differences in genomic integration sites could account for the observed effects, three individual polyclonal cell pools were generated for each minιμ construct by independent transfections. Although some variations between the results obtained from the three corresponding cell pools were observed, only the three cell pools expressing μter310 mutIRE showed a strong mRNA increase upon SB treatment (Fig. 1D), which indicates that μter310 mutIRE is subjected to NMTGS to an extent that is comparable with the previously observed NMTGS of minιμ constructs that have no insertions in the 5’UTR (13). In contrast, the relative mRNA levels of translation-suppressed μter310 IRE changed only marginally (less than 2.5 – fold up or down) when cells were treated with SB or TSA. Likewise, changes of less than 2.5 – fold were observed for μwt IRE and μwt mutIRE mRNA levels upon SB- or TSA-treatment. Collectively, these results indicate that translation of the PTC+ mRNA is a prerequisite for NMTGS to occur and confirms the PTC–specificity of NMTGS.

**The transcription rate of PTC+ Ig-μ is translation-dependent.**

In order to determine the transcription rate of the μter310 IRE and μter310 mutIRE constructs and the effect of HDAC inhibition on these transcription rates in the polyclonal HeLa cell pools, nuclei of SB-treated or untreated cell pools were isolated and assayed by nuclear run-on analysis (Fig. 2A). The 32P – labeled nuclear RNA was isolated and hybridized to dots of single–stranded antisense minιμ DNA, antisense glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA, and
pBluescript DNA immobilized on nylon filter strips and the radioactivity bound to each dot was quantified by PhosphorImager scanning. The pBluescript signal served as control for background hybridization and was subtracted from the signal obtained for miniμ and GAPDH. The net signal of miniμ was normalized to the net GAPDH signal, and to the relative average transgene copy number of the corresponding polyclonal cell pool, and is displayed in the histogram in Figure 2B. The relative miniμ gene copy number in the different cell pools was determined from genomic DNA by real-time PCR (data not shown).

These nuclear run-on experiments revealed a 4.5 – fold lower transcription rate of μter310 mutIRE compared to the transcription rate of μter310 mutIRE. This reduced transcription of μter310 mutIRE could be increased by treatment of the cells with SB to a similar rate as measured for μter310 IRE, whereas the transcription rate of μter310 IRE did not alter significantly upon SB treatment. This translation-dependent reduction of miniμter310 transcription strongly indicates that NMTGS requires translation of the cognate PTC+ transcript. The results of the run-on assays confirm the data obtained by real-time RT-PCR (Fig. 1 D and E) showing that NMTGS can be reversed by HDAC inhibitors.

**NMTGS is accompanied by histone H3 lysine 9 methylation.**

We next wanted to analyze the chromatin state of miniμ in the stable polyclonal cell pools. It is well documented that transcriptional silencing often is accompanied by methylation of histone H3 lysine 9 [H3K9; reviewed in (24)], and we have previously shown that the silenced state of the PTC+ Ig-μ is accompanied by increased mono-methylation of H3K9 (13). Therefore we performed chromatin immunoprecipitation (ChIP) assays using antibodies specific for mono-, di- or tri-methylated H3K9 (α-H3K9me1, α-H3K9me2, α-H3K9me3) and as a control with an antibody against all forms of H3. HeLa cells expressing the μter310 IRE or the μter310 mutIRE constructs were crosslinked with formaldehyde, the genomic DNA was sheared to an average size of 500 base pairs, and the DNA was then immunoprecipitated. The amount of miniμ containing DNA in the immunoprecipitated material and in the cell lysate before immunoprecipitation (input) was determined relative to the amount of endogenous β-globin by real-time PCR. Additionally, the values were normalized to the amount of total H3, to rule out possible differences between the cell pools. The ratio between the relative amounts of immunoprecipitated miniμ and input miniμ is depicted in Figure 3 A – C as “fold enrichment”, and μter310 IRE was set to 1.

In general, μter310 mutIRE is more associated with methylated H3K9 than μter310 IRE, which is in agreement with previous results that also revealed a correlation between reduced transcription and increased H3K9me1 (13). Our data show that the actively translated μter310 mutIRE is about 2.5 – fold more associated with H3K9me1, about 3 – fold more associated with H3K9me2, and about 5 – fold more associated with H3K9me3 than the non-translated μter310 IRE.
NMTGS depends on the NMD factor Upf1.

Because NMTGS and NMD are triggered by the same signal, namely translation termination at a PTC, we hypothesized that NMD factors involved in PTC recognition would also be required for NMTGS. Although the exact mechanism of PTC recognition is not yet understood, it is clear that Upf1 plays a central role in this process. Upf1 has recently been shown to be a component of the so called SURF – complex (Smg1-Upf1-eRF1-eRF3), which is thought to assemble at ribosomes that terminate at PTCs (25). Therefore, we depleted Upf1 from the cells by RNAi and tested if this affects NMTGS.

HeLa cells expressing the miniµ IRE or the miniµ mutIRE constructs were transfected with pSUPERpuro plasmids encoding shRNAs that target Upf1 mRNA for RNAi-mediated degradation (Fig. 4, hUpf1 KD +) or encoding a “scrambled” shRNA with no predicted target in human cells (hUpf1 KD -) (17). Untransfected cells were eliminated by treating the cells with puromycin for 48 hours. 72 hours after transfection, the cells were treated with SB for 24 hours and then harvested. The relative mRNA levels of the miniµ reporter genes and of Upf1 were analyzed by real–time RT–PCR and normalized to the relative levels of endogenous 18S rRNA. A representative western blot with extracts from µwt IRE-expressing cells under all four experimental conditions is shown in Figure 4 A to document that Upf1 was very efficiently depleted irrespectively if the cells were treated with SB or not. The relative Upf1 mRNA levels in all Upf1 knockdown samples (hUpf1 KD +) were reduced at least 10 - fold compared to the corresponding controls (hUpf1 KD -) (data not shown). In agreement with earlier results (Fig. 1D), the mRNA level of µter310 mutIRE increased on average 10 – fold upon treatment with SB in the control knockdown (Fig. 4B). In contrast, the mRNA level increases only marginally (1.5 – fold) upon SB in Upf1-depleted cells, suggesting that Upf1 is required for NMTGS. As expected, the mRNA levels of the translation-inhibited µter310 IRE and of the PTC – free miniµ constructs (IRE and mutIRE), which all are not subjected to NMTGS and NMD, change only marginally upon SB treatment (between 0.6 and 2 – fold). This result shows that NMTGS is lost when PTC recognition (and as a consequence NMD) is abrogated and indicates a mechanistic link between NMTGS and NMD.

Discussion

The recent discovery that the presence of a PTC in a gene can lead to a transcriptional silencing of this gene that exhibits chromatin characteristics typically found in heterochromatic regions and that appears to involve components of the RNAi machinery was unexpected and exciting at the same time (13). Although the physiological significance of this observation is not yet understood, it immediately raised some intriguing mechanistic questions. One of these questions, which we have addressed in this study here, concerns the relationship between NMTGS and NMD.

So far, the evidence that NMTGS is really PTC-specific was based on correlation: We have observed transcriptional silencing only with PTC+ Ig-µ and Ig-γ minigene constructs, but not with missense or silent mutations in these genes.
or with more sophisticated “frameshift-shift-back” constructs (13). It was therefore important to test the postulated PTC-specificity of NMTGS by an additional approach. Since recognition of a PTC necessitates decoding of an mRNA’s open reading frame, and since translating ribosomes are the only known cellular entities with this function, it is expected that a PTC-specific process must depend on translation. To test this hypothesis, the use of unspecific translation inhibitors like cycloheximide seemed not suitable in our experimental system, because (i) of the pleiotropic effects of such drugs and because (ii) we do not know, in which phase of NMTGS translation might be involved. It could be that translation is only necessary for initiation of NMTGS, but not for the maintenance of the silenced state. Therefore, we decided to use the IRE-system, which allowed us to selectively inhibit translation of our NMTGS reporter mRNA. We could demonstrate that under such conditions, NMTGS is not observed (Figs. 1D, 1E, 2 and 4B). Thus we conclude that transcriptional silencing of a PTC+ gene requires translation of the PTC+ mRNA that it encodes.

Because this IRE-mediated inhibition of translation also abrogates NMD [(23), and LS, unpublished results], and because in our experimental system the PTC+ miniµ reporter genes are subjected to both NMD and NMTGS, it was important to use assays that allow to specifically detect transcriptional effects. Among the available assays, nuclear run-on analysis (Fig. 2) is the most direct method to detect differences in transcription rates, but it has the disadvantage that it requires large amounts of nuclei (10^7 per reaction). Therefore, nuclear run-ons are not feasible for certain experiments, like for example RNAi-mediated knockdowns. Thus, as an alternative method to distinguish between transcriptional (NMTGS) and posttranscriptional (NMD) effects on the steady-state mRNA level, we have established an assay that involves treatment of the cells with histone deacetylase inhibitors. We have previously demonstrated that SB does not interfere with NMD and that the measured changes in mRNA levels mirror the changes detected by run-on analysis (13).

The finding that NMTGS depends on translation immediately suggested that NMD and NMTGS are mechanistically linked and share the same mechanism for PTC recognition. Consistent with this proposition, µter310 mutIRE mRNA levels did not anymore increase significantly upon SB treatment in cells devoid of Upf1, indicating that the transcriptional silencing of µter310 mutIRE was lost as a consequence of the Upf1 knockdown (Fig. 4B). We cannot exclude that Upf1 indirectly regulates NMTGS, but it is much more likely that it is directly involved, given its characterization as an essential NMD factor involved in PTC recognition (3,4). The fact that transcription of the µter310 mutIRE gene is silenced in the cells at the beginning of the experiment and that its transcription activity increases during the four days of the Upf1 knockdown further suggests that Upf1 is not only required to establish NMTGS, but also for maintaining it. In mechanistic terms, this implies that the entire pathway from PTC recognition to establishment of the transcriptionally silenced chromatin state must be constantly active in order to maintain NMTGS. If true, this should...
facilitate future identification of additional factors involved in NMTGS.

In summary, the data presented in this study support a model in which NMD and NMTGS represent two different pathways to down-regulate gene expression in response to the detection of an improper translation termination event at a premature termination codon. The result that translation and Upf1 are essential for NMTGS suggests that NMD and NMTGS use the same machinery for PTC recognition, since there is compelling evidence that Upf1 has a crucial role in the discrimination between correct and improper translation termination [reviewed in (4)]. Our data also implies that NMTGS branches from the NMD pathway after Upf1 function. This suggests that the signal for an mRNA to elicit NMTGS would then comprise the recognition of a PTC in combination with a yet unidentified, gene-specific cis-acting element. Clearly, this is only the beginning and much more work is necessary to further elucidate the mechanisms downstream of PTC recognition leading to mRNA degradation and sometimes to transcriptional silencing of the affected gene.

Acknowledgements
We thank Fabio Mohn and Marc Bühler for help and advice during the initial stage of this project, Niels Gehring and Andreas Kulozik for their support with the IRE translation regulation system, Jens Lykke-Andersen for the α-hUpf1 antibody, and the laboratory of Daniel Schümperli for providing Y12 antibody. L.S. was supported by a fellowship from the Roche Research Foundation and O.M. is paid by the Max Cloëtta Foundation. This work was further supported by the Kanton Bern and by grants to O.M. from the Swiss National Science Foundation and the Helmut Horten Foundation.

Abbreviations
IRE: Iron Responsive Element
NMD: Nonsense-Mediated mRNA Decay
NMTGS: Nonsense-Mediated Transcriptional Gene Silencing
PTC: Premature translation Termination Codon
RNAi: RNA interference
SURF: Smg1-Upf1-eRF1-eRF3-complex
RT-PCR: Reverse transcription followed by polymerase chain reaction

References
1. Fasken, M. B., and Corbett, A. H. (2005) Nat Struct Mol Biol 12(6), 482
2. Maquat, L. E. (2004) Nat Rev Mol Cell Biol 5(2), 89-99
3. Behm-Ansmant, I., and Izaurralde, E. (2006) Genes Dev 20(4), 391-398
4. Amrani, N., Sachs, M. S., and Jacobson, A. (2006) Nat Rev Mol Cell Biol 7(6), 415
5. Lelivelt, M. J., and Culbertson, M. R. (1999) Mol Cell Biol 19(10), 6710-6719

NMTGS requires translation
6. He, F., Li, X., Spatrick, P., Casillo, R., Dong, S., and Jacobson, A. (2003) *Mol Cell* **12**(6), 1439-1452
7. Mendell, J. T., Sharifi, N. A., Meyers, J. L., Martinez-Murillo, F., and Dietz, H. C. (2004) *Nat Genet* **36**(10), 1073-1078
8. Rehwinkel, J., Letunic, I., Raes, J., Bork, P., and Izaurralde, E. (2005) *Rna* **11**(10), 1530-1544
9. Wittmann, J., Hol, E. M., and Jack, H. M. (2006) *Mol Cell Biol* **26**(4), 1272-1287
10. Buhler, M., Steiner, S., Mohn, F., Paillusson, A., and Muhlemann, O. (2006) *Nat Struct Mol Biol* **13**(5), 462-464
11. Amrani, N., Ganesan, R., Kervestin, S., Mangus, D. A., Ghosh, S., and Jacobson, A. (2004) *Nature* **432**(7013), 112-118
12. Buhler, M., Paillusson, A., and Muhlemann, O. (2004) *Nucleic Acids Res* **32**(11), 3304-3315
13. Buhler, M., Mohn, F., Stalder, L., and Muhlemann, O. (2005) *Mol Cell* **18**(3), 307-317
14. Bernstein, E., and Allis, C. D. (2005) *Genes Dev* **19**(14), 1635-1655
15. Domeier, M. E., Morse, D. P., Knight, S. W., Portereiko, M., Bass, B. L., and Mango, S. E. (2000) *Science* **289**(5486), 1928-1931
16. Arciga-Reyes, L., Wootton, L., Kieffer, M., and Davies, B. (2006) *The Plant Journal* **47**(3), 480-489
17. Paillusson, A., Hirschi, N., Vallan, C., Azzalin, C. M., and Muhlemann, O. (2005) *Nucleic Acids Res* **33**(6), e54
18. Lykke-Andersen, J., Shu, M. D., and Steitz, J. A. (2000) *Cell* **103**(7), 1121-1131
19. Lerner, E. A., Lerner, M. R., Janeway, C. A., Jr., and Steitz, J. A. (1981) *Proc Natl Acad Sci U S A* **78**(5), 2737-2741
20. Hentze, M. W., Caughman, S. W., Casey, J. L., Koeller, D. M., Rouault, T. A., Harford, J. B., and Klausner, R. D. (1988) *Gene* **72**(1-2), 201-208
21. Hentze, M. W., Muckenthaler, M. U., and Andrews, N. C. (2004) *Cell* **117**(3), 285-297
22. Gray, N. K., and Hentze, M. W. (1994) *Embo J* **13**(16), 3882-3891
23. Thermann, R., Neu-Yilik, G., Deters, A., Frede, U., Wehr, K., Hagemeier, C., Hentze, M. W., and Kulozik, A. E. (1998) *Embo J* **17**(12), 3484-3494
24. Peters, A. H., and Schubeler, D. (2005) *Curr Opin Cell Biol* **17**(2), 230-238
25. Kashima, I., Yamashita, A., Izumi, N., Kataoka, N., Morishita, R., Hoshino, S., Ohno, M., Dreyfuss, G., and Ohno, S. (2006) *Genes Dev* **20**(3), 355-367

NMTGS requires translation
Figure Legends.

FIGURE 1. Transcriptional silencing of miniμter310 requires translation of the miniμter310 mRNA. A, Schematic representation of the Ig-μ constructs miniμ IRE and miniμ mutIRE. These constructs are under the control of the human β-actin promoter and contain a functional or mutant IRE in the 5’ UTR (represented as stem-loop structures). Coding regions are represented as white boxes, the 5’ UTR, introns and the 3’ UTR as lines. The start codon (ATG), the termination codon (TGA) and amino acid position 310 are indicated, and the arrow depicts the transcription start site. PTC-free miniμ constructs are denoted “μwt”, constructs with the PTC-generating point mutation (GGA to TGA) at amino acid position 310 are named “μter310”. Sequence and secondary structure of the IRE and the mutIRE are shown on the right. B, Western blot analysis of the μwt encoded Ig-μ polypeptide and Sm B/B’ in a serially diluted cell lysate of HeLa miniμwt IRE compared to miniμwt mutIRE. C, Northern blot analysis of the RNA from HeLa cells stably expressing the indicated miniμ constructs, using a 32P-labeled probe to detect μ mRNA. As a loading control, the 28S rRNA band from the ethidium bromide-stained gel before blotting is shown in the lower panel. D and E, HeLa cells stably expressing the indicated miniμ constructs were treated with 15 mM sodium butyrate (SB; D) or 200 ng/mL Trichostatin A (TSA; E) for 24 hours, and relative RNA levels were measured by real–time RT–PCR. Relative miniμ mRNA levels were normalized to 18S rRNA, and the ratio of miniμ mRNA in treated compared to untreated cells is shown as fold increase. Average values and standard deviations from 3 real-time PCR measurements of one typical experiment are shown. For each miniμ reporter construct, the results from three independently generated polyclonal cell pools are shown in D.

FIGURE 2. Nuclear run–on assay with nuclei from polyclonal populations of HeLa cell stably expressing the indicated miniμ constructs. A, HeLa cells stably expressing the indicated μter310 constructs were treated with 15 mM SB for 24 h (+) or not (-), and nuclei were isolated subsequently. After the incubation of the nuclei with 32P-labeled UTP, the 32P–labeled RNA was isolated and hybridized to the following single stranded DNA spotted in nylon membrane strips: the empty pBluescript KS(-) vector (pBS), which served as a control for nonspecific background hybridization, pBS containing the antisense miniμwt sequence, and pBS containing the antisense sequence of the glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA. B, The amount of 32P in each dot was determined by PhosphorImager scanning and the bars histogram represents the quantified signal intensity for miniμ normalized to the GAPDH signal and to the average number of Ig-μ genes in each cell pool. Relative average transgene copy numbers were determined from genomic DNA by real-time PCR and were 1 and 4.7 for the cell pools μter310 IRE and μter310 mutIRE, respectively. Each bar represents the average value of two independent run-on experiments.

FIGURE 3. NMTGS correlates with increased histone H3 lysine 9 methylation. A – C, Chromatin immunoprecipitation (ChIP) assays were performed with antibodies NMTGS requires translation
Specific for H3K9me1 (A), H3K9me2 (B), and H3K9me3 (C). ChIP with a H3-specific rabbit polyclonal antibody that does not discriminate between different posttranslational modifications (α-H3) was used for normalization of the results to the total level of histone H3. After formaldehyde crosslinking of cells expressing miniµter310 IRE or miniµter310 mutIRE, sheared genomic DNA fragments of an averaged size of 500 base pairs were co-precipitated with the antibodies indicated. The relative amount of miniµ and endogenous β-globin DNA in the immunoprecipitated fraction and in the fraction before immunoprecipitation was quantified by real–time PCR. The relative enrichment of immunoprecipitated miniµ DNA, normalized to the enrichment of endogenous β-globin DNA and to total H3, are represented as “fold enrichment”. Miniµter310 IRE was set to 1. Average values and standard deviations from 3 real-time PCR runs of one ChIP experiment are shown.

**FIGURE 4. Upf1 is required for NMTGS.** HeLa cells stably expressing the indicated miniµ constructs were transfected with plasmids encoding shRNAs to target Upf1 (KD +) or an shRNA encoding a “scrambled” sequence with no predicted target in human cells (KD -). 72 hours after transfection, half of the cells were treated with 15 mM SB for 24 hours (SB+), and the other half received normal medium (SB-). A, Analysis of Upf1 protein levels in the µwt-expressing cells by western blot using polyclonal α-hUpf1 antiserum (18). As a loading control, the protein Sm B/B’ was detected using the monoclonal antibody Y12 (19). B, Quantification of the miniµ mRNA by real-time RT–PCR. Relative miniµ mRNA levels were determined and normalized to 18S rRNA for each sample and the average numbers are indicated in the lower part of the panel. The ratio of relative miniµ mRNA in treated compared to untreated cells is shown as fold increase in the histogram. Average values and standard deviations from 3 real-time PCR runs of one typical experiment are shown.
FIGURE 1

A

B

C

D

E

NMTGS requires translation
NMTGS requires translation
NMTGS requires translation
Transcriptional silencing of nonsense codon-containing immunoglobulin genes requires translation of its mRNA

Lukas Stalder and Oliver Mühlemann

*J. Biol. Chem.* published online April 11, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M610595200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts