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Transcriptional Silencing of Nonsense Codon-containing Immunoglobulin μ Genes Requires Translation of Its mRNA*

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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 in the 5′-untranslated region. Furthermore, RNA interference-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.

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)[3] 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 are 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 mini-μ constructs were stably integrated into the genome of HeLa cells. In addition of triggering NMD, the transcription rate of PTC-containing (PTC+) mini-μ genes was also reduced under these conditions (13). This PTC-specific transcriptional silencing is accompanied by a change in the chromatin state toward 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 raise to this PTC+ transcript, we found that overexpression of the small interfering RNase (siRNAse) 3′hExo completely suppressed NMTGS (13). This suggests the involvement of small interfering RNAs (siRNAs) or siRNA-like molecules in NMTGS, but all attempts to detect these putative siRNAs were not successful so far.[4]

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, polymerase 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 *Schizosaccharomyces 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

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[3] The abbreviations used are: NMD, nonsense-mediated mRNA decay; IRE, iron-responsive element; NMTGS, nonsense-mediated transcriptional gene silencing; PTC, premature translation-termination codon; RNAi, RNA interference; RT, reverse transcription; UTR, untranslated region; MOPS, 4-morpholinepropanesulfonic acid; GAPDH, glyceraldehyde phosphate dehydrogenase; ChIP, chromatin immunoprecipitations; SB, sodium butyrate; TSA, trichostatin A; IRP, iron regulatory protein.

[4] M. Bühler, R. Gudipati, and O. Mühlemann, unpublished information.

induce the RNAi-mediated transcriptional silencing system. On the other hand, a genetic link between NMD factors and RNAi has been documented both in *Caenorhabditis elegans* (15) and *Arabidopsis thaliana* (16). Mutations in the NMD factors SMG-2, SMG-5, or SMG-6 of *C. elegans* resulted in worms that still responded to double-stranded RNA-induced gene silencing but that had lost the persistence of RNAi-mediated silencing (15). Similarly, *A. thaliana* 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+ mini-μ transcripts by the insertion of an iron-responsive element (IRE) into the 5′-UTR of the mini-μ 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 pβmini-μ-IRE and pβmini-μ-mutIRE plasmids were generated by insertion of a double-stranded oligonucleotide encoding the IRE or the mutIRE sequence into the SalI site of pβmini-μwt and pβmini-μter310 (12), respectively. The inserted sequence was 5′-TAGCAA-CCCGGGTTTCCTGCTTCAACAGTGCTTGGACGGAA-CCC-3′ (excluding the SalI 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 manufacturer's 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. Before 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 1× MOPS and 1% formaldehyde. RNA was transferred to a positively charged nylon membrane (Roche Diagnostics) in 0.5× MOPS by 1 h of wet blotting in a genie blotter (Idea Scientific, Minneapolis). After UV cross-linking of the RNA to the nylon filter, prehybridization and hybridization were carried out in 6× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), 5× Denhardt's reagent, and 0.5% SDS at 60 °C. Pre-hybridization was with 50 μg/ml denatured salmon sperm DNA and 100 μg/ml denatured calf thymus DNA. For hybridization, 100 ng of μter310 mutIRE DNA was labeled with [α-^{32}P]dCTP using the Ready-To-Go™ DNA labeling kit (Amersham Biosciences). After overnight hybridization, membranes were washed twice with 2× SSC, 0.2% SDS and twice with 0.2× 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 *Escherichia coli* transformed with pBluescript KS(−) containing the antisense sequences of human glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA and mini-μ and infected with helper phage M13K07 (New England Biolabs). For nuclei preparation, ~4×10^7 cells were harvested and centrifuged at 4 °C with 300 × *g* for 8 min. The cells were then resuspended in 6 ml of ice-cold nuclei lysis buffer (3 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 0.5% IgePalCAG30) under gentle vortexing and incubated for 5 min on ice. The nuclei were pelleted at 4 °C with 500 × *g* for 5 min, resuspended in 6 ml of nuclei lysis buffer, centrifuged again as above, resuspended in 250 μl of nuclei storage buffer (40% glycerol, 5 mM $MgCl_2$, 100 nM EDTA, 2.5 mM Tris HCl pH 8.25, 5 mM dithiothreitol, 0.4 units of RNasIn (Promega)), and stored in liquid nitrogen. Per run-on reaction, ~10^7 nuclei were incubated with 11 MBq of [α-^{32}P]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 magnesium 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 of single-stranded DNA probe had been immobilized per dot. The nylon membrane was loaded with the single-stranded DNA dots using a Bio-Rad assemble vacuum system, cross-linked with 120 kJ (Stratalinker), and dried in the air. The membranes were prehybridized for 3 h in 6× SSC, 5× Denhardt's, 0.5% SDS, 20 ng/μl herring sperm DNA, and 100 ng/μl *E. coli* tRNA. Hybridization conditions were 6× SSC, 5× 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 h at 60 °C. The filter strips were washed twice at 65 °C in 1× 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 Biotechnology Inc., 17-295) according to the manufacturer's protocol. 5 μg of 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 quantify mini-μ and endogenous β-globin. The TaqMan assays span the 5′ splice site of mini-μ exon C2 and the 5′ splice site of endogenous β-globin exon 2 (sequences are available upon request). For each antibody, the relative amount of immunoprecipitated mini-μ DNA was normalized to that of endogenous β-globin DNA and to input DNA and calculated as "-fold enrichment" relative to mini-μter310 IRE using the formula $(IP_{\mu}/input_{\mu})/(IP_{\beta glob}/input_{\beta glob})$. Finally, these relative -fold enrichment of mini-μ for each of the three H3K9 methylation-specific antibodies was normalized to the relative -fold enrichment of mini-μ determined with the α-H3 antibody.

Inhibition of Histone Deacetylases and Quantitative Real-time RT-PCR—Between 2 and 3 × 10^5 cells were seeded in 6-well plates and treated the next day with 15 mM sodium butyrate (SB; Upstate) or 200 ng/ml trichostatin A (TSA; Sigma) for 24 h. Subsequently, total cellular RNA was isolated using Absolutely RNA RT-PCR Miniprep kit (Stratagene), and

1 μg of RNA was reverse-transcribed in 50 μl of Stratascript first strand buffer in the presence of 0.4 mM dNTPs, 300 ng of random hexamers, 40 units of RNasIn (Promega), and 50 units of Stratascript reverse transcriptase (Stratagene). For real-time RT-PCR, reverse-transcribed material corresponding to 40 ng of RNA was amplified in 25 μl of 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 h after transfection, untransfected cells were eliminated by culturing the cells in the presence of 1.5 μg/ml puromycin for 48 h. Cells were then washed in phosphate-buffered saline and incubated in puromycin-free medium in the presence or absence of 15 mM SB for another 24 h. Total cellular RNA was isolated, and whole cell lysates for Western blotting were prepared 96 h 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 0.25×10^4–2×10^5 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 AffiniPure goat anti-mouse IgG (μ chain-specific, Jackson ImmunoResearch), and 1:400-diluted supernatant of the mouse hybridoma cell line Y12, which produces a monoclonal antibody against human Sm proteins (19). 1:4000-Diluted horseradish peroxidase (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 Biosciences) 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—To further investigate NMTGS of our Ig-μ minigene (12) (hereafter called mini-μ), we have introduced the IRE of the human ferritin H chain (20) into the 5′-UTR of mini-μ, giving rise to the mini-μ IRE constructs (Fig. 1*A*, *upper panel*). The IRE is an endogenous regulatory sequence that allows specific controlling of 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 48 S preinitiation 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. 1*B* and data not shown). We have tried to induce the translation of the mini-μ 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 mini-μ, which cannot bind to the IRP (20) and, thus, allows translation (Fig. 1*A*, *lower panel*).

We transfected HeLa cells with mini-μ 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 that had these different mini-μ constructs stably integrated into the genome. Quantification of a Western blot with a 3-fold serial dilution shows about a 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. 1*C* 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. 1*C*). 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 mini-μ constructs efficiently inhibits NMD (23)[5], 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 histone deacetylase inhibitors SB or TSA and determined the relative mini-μ mRNA levels by real-time RT-PCR. 18 S rRNA served as the endogenous control for normalization in these experiments. As shown in Figs. 1, *D* and *E*, the relative mRNA levels of μter310 mutIRE increased between 5- and 8-fold in cells treated with SB or TSA compared with 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 mini-μ 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. 1*D*), which indicates that μter310 mutIRE is subjected to NMTGS to an extent that is comparable with the previously observed NMTGS of mini-μ constructs that have no insertions in the 5′-UTR (13). In con-

[5] L. Stalder, unpublished results.

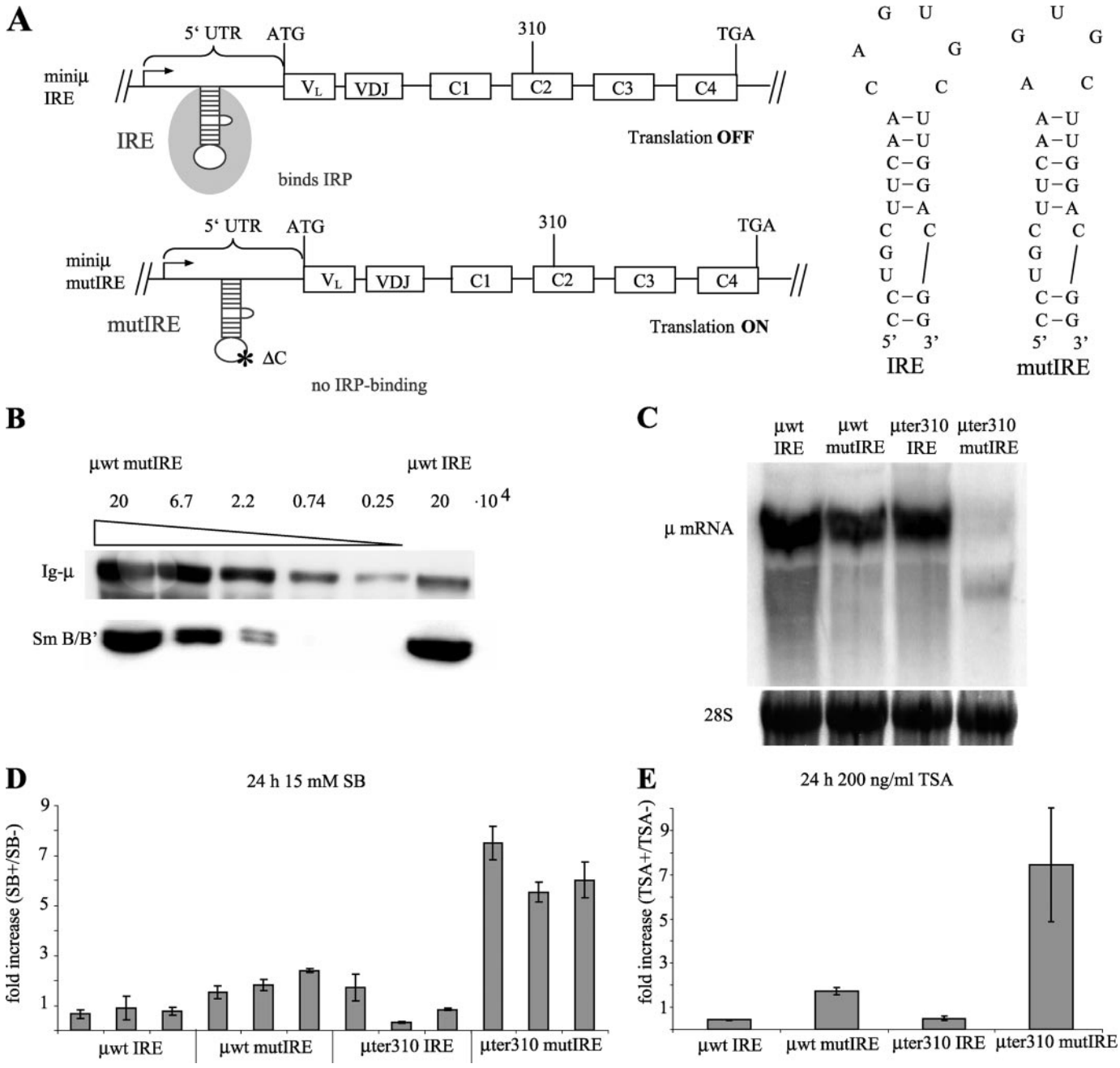

FIGURE 1. **Transcriptional silencing of mini-μter310 requires translation of the mini-μter310 mRNA.** *A*, schematic representation of the Ig-μ constructs mini-μ IRE and mini-μ mutIRE. These constructs are under the control of the human β-actin promoter and contain a functional or mutant IRE in the 5′-UTR (represented as *stem-loop structures*). Coding regions are represented as *white boxes*, and the 5′-UTR, introns, and the 3′-UTR are represented 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 mini-μ 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 mini-μwt IRE compared with mini-μwt mutIRE. *C*, Northern blot analysis of the RNA from HeLa cells stably expressing the indicated mini-μ constructs using a ^{32}P-labeled probe to detect μ mRNA. As a loading control, the 28 S 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 mini-μ constructs were treated with 15 mM SB (*D*) or 200 ng/ml TSA (*E*) for 24 h, and relative RNA levels were measured by real-time RT-PCR. Relative mini-μ mRNA levels were normalized to 18 S rRNA, and the ratio of mini-μ mRNA in treated compared with untreated cells is shown as -fold increase. Average values and S.D. from three real-time PCR measurements of one typical experiment are shown. For each mini-μ reporter construct, the results from three independently generated polyclonal cell pools are shown in *D*.

trast, 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—To determine the transcription rate of the μter310 IRE and μter310 mutIRE constructs and the effect of histone deacetylase inhibition on these transcription rates in the poly-

clonal HeLa cell pools, nuclei of SB-treated or untreated cell pools were isolated and assayed by nuclear run-on analysis (Fig. 2*A*). The ^{32}P-labeled nuclear RNA was isolated and hybridized to dots of single-stranded antisense mini-μ DNA, antisense 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 the control for background hybridization and was subtracted from the signal obtained for mini-μ and GAPDH. The net signal of mini-μ 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 Fig. 2*B*. The relative mini-μ gene copy number in the different cell pools was determined from genomic DNA by real-time PCR (data not shown).

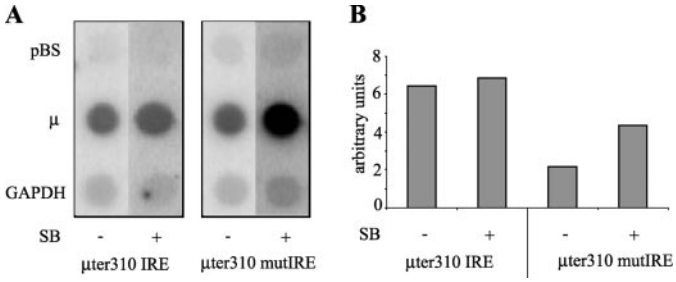

FIGURE 2. **Nuclear run-on assay with nuclei from polyclonal populations of HeLa cell stably expressing the indicated mini-μ constructs.** *A*, HeLa cells stably expressing the indicated μter310 constructs were treated with 15 mM SB for 24 h (+) or not (−), and nuclei were isolated subsequently. After the incubation of the nuclei with ^{32}P-labeled UTP, the ^{32}P-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 mini-μwt sequence; pBS containing the antisense sequence of the GAPDH cDNA. *B*, the amount of ^{32}P in each dot was determined by PhosphorImager scanning, and the *histogram* represents the quantified signal intensity for mini-μ 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.

NMTGS Is Accompanied by Histone H3 Lysine 9 Methylation—We next wanted to analyze the chromatin state of mini-μ in the stable polyclonal cell pools. It is well documented that transcriptional silencing often is accompanied by methylation of histone H3 lysine 9 (H3K9; for review, see Ref. 24), and we have previously shown that the silenced state of the PTC+ Ig-μ is accompanied by increased monomethylation of H3K9 (13). Therefore, we performed 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 cross-linked with formaldehyde, the genomic DNA was sheared to an average size of 500 base pairs, and the DNA was then immunoprecipitated. The amount of mini-μ-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 mini-μ and input mini-μ is depicted in Fig. 3, *A–C*, as -fold enrichment, and μter310 IRE was set to 1.

These nuclear run-on experiments revealed a 4.5-fold lower transcription rate of μter310 mutIRE compared with 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 mini-μter310 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 histone deacetylase inhibitors.

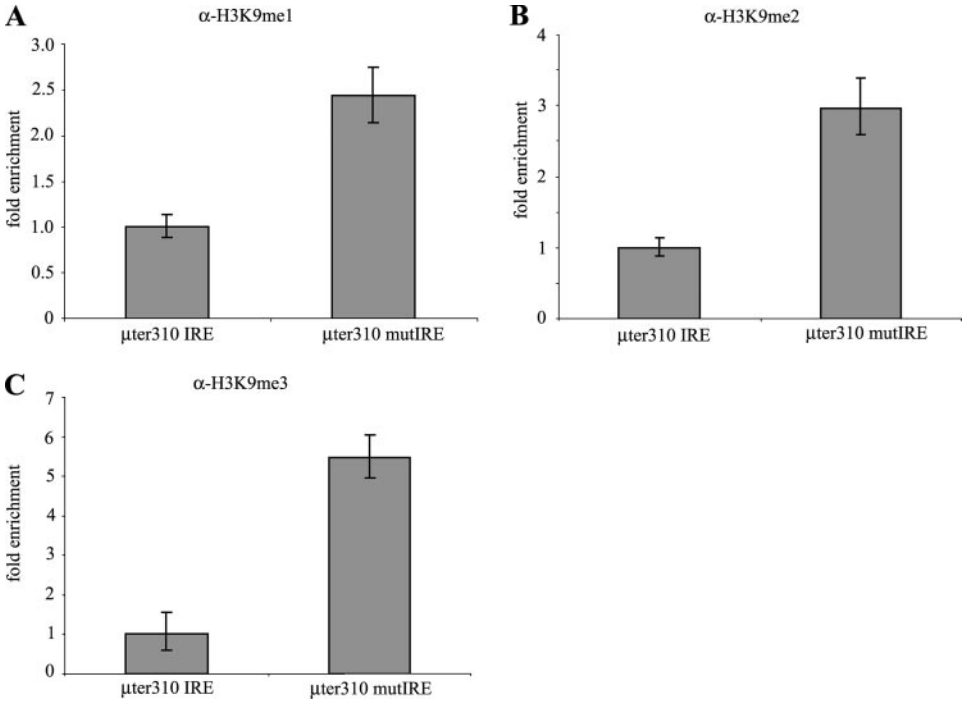

FIGURE 3. **NMTGS correlates with increased histone H3 lysine 9 methylation.** *A–C*, ChIP assays were performed with antibodies specific for H3K9me1 (*A*), H3K9me2 (*B*), and H3K9me3 (*C*). ChIP with a H3-specific rabbit polyclonal antibody that does not discriminate between different posttranslational modifications (α-H3) was used for normalization of the results to the total level of histone H3. After formaldehyde cross-linking of cells expressing mini-μter310 IRE or mini-μter310 mutIRE, sheared genomic DNA fragments of an averaged size of 500 base pairs were co-precipitated with the antibodies indicated. The relative amount of mini-μ 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 mini-μ 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 S.D. from three real-time PCR runs of one ChIP experiment are shown.

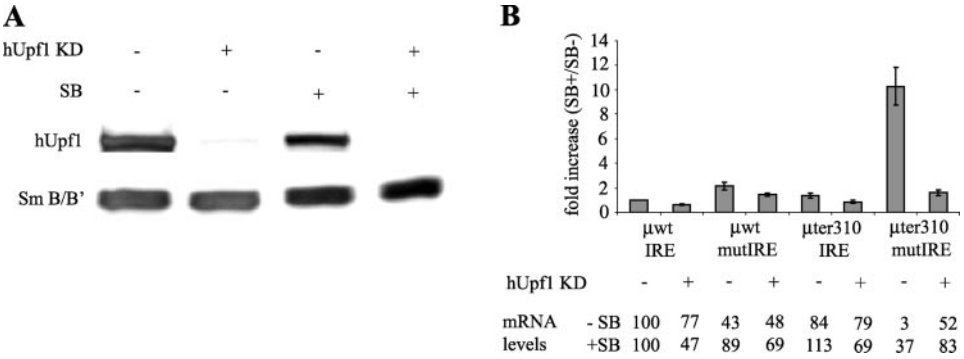

FIGURE 4. **Upf1 is required for NMTGS.** HeLa cells stably expressing the indicated mini-μ constructs were transfected with plasmids encoding short hairpin RNAs (shRNAs) to target Upf1 (*KD+*) or an shRNA encoding a scrambled sequence with no predicted target in human cells (*KD−*). 72 h after transfection half of the cells were treated with 15 mM SB for 24 h (*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 mini-μ mRNA by real-time RT-PCR. Relative mini-μ mRNA levels were determined and normalized to 18 S rRNA for each sample, and the average numbers are indicated in the *lower part* of the *panel*. The ratio of relative mini-μ mRNA in treated compared with untreated cells is shown as -fold increase in the histogram. Average values and S.D. from three real-time PCR runs of one typical experiment are shown.

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 mini-μ IRE or the mini-μ mutIRE constructs were transfected with pSUPERpuro plasmids encoding short hairpin RNAs (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 h. 72 h after transfection the cells were treated with SB for 24 h and then harvested. The relative mRNA levels of the mini-μ reporter genes and of Upf1 were analyzed by real-time RT-PCR and normalized to the relative levels of endogenous 18 S rRNA. A representative Western blot with extracts from μwt IRE-expressing cells under all four experimental conditions is shown in Fig. 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 with the corresponding controls (hUpf KD−) (data not shown). In agreement with earlier results (Fig. 1*D*), the mRNA level of μter310 mutIRE increased on average 10-fold upon treatment with SB in the control knockdown (Fig. 4*B*). In contrast, the mRNA level increased 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 mini-μ 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, 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. Because recognition of a PTC necessitates decoding of an mRNA open reading frame and because 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. 1, *D* and *E*, 2, and 4*B*). 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)[5] and because in our experimental system

the PTC+ mini-μ 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, as 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 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. 4*B*). 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 4 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 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 (for review, see Ref. 4). Our data also imply 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.

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Transcriptional Silencing of Nonsense Codon-containing Immunoglobulin μ Genes Requires Translation of Its mRNA

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