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Binary specification of nonsense codons by splicing and cytoplasmic translation

Rolf Thermann, Gabriele Neu-Yilik, Andrea Deters, Ute Frede, Kristina Wehr[1], Christian Hagemeier, Matthias W.Hentze[1,2] and Andreas E.Kulozik[2]

Department of Pediatrics, Charité-Virchow Medical Center, Augustenburger Platz 1, Humboldt University, D-13353 Berlin and [1]Gene Expression Programme, EMBL, Meyerhofstrasse 1, D-69117 Heidelberg, Germany

[2]Corresponding authors

R.Thermann and G.Neu-Yilik contributed equally to this work

Premature translation termination codons resulting from nonsense or frameshift mutations are common causes of genetic disorders. Complications arising from the synthesis of C-terminally truncated polypeptides can be avoided by 'nonsense-mediated decay' of the mutant mRNAs. Premature termination codons in the β-globin mRNA cause the common recessive form of β-thalassemia when the affected mRNA is degraded, but the more severe dominant form when the mRNA escapes nonsense-mediated decay. We demonstrate that cells distinguish a premature termination codon within the β-globin mRNA from the physiological translation termination codon by a two-step specification mechanism. According to the binary specification model proposed here, the positions of splice junctions are first tagged during splicing in the nucleus, defining a stop codon operationally as a premature termination codon by the presence of a 3′ splicing tag. In the second step, cytoplasmic translation is required to validate the 3′ splicing tag for decay of the mRNA. This model explains nonsense-mediated decay on the basis of conventional molecular mechanisms and allows us to propose a common principle for nonsense-mediated decay from yeast to man.

Keywords: dominant β-thalassemia/mRNA stability/nonsense-mediated decay/translation/3′-UTR splicing

Introduction

Nonsense and frameshift mutations introduce premature translation termination codons (PTCs) into the open reading frames (ORFs) of the affected mRNAs and are common causes of genetic disorders. Surprisingly, PTCs usually direct the affected mRNAs to rapid degradation, a process termed nonsense-mediated mRNA decay (NMD). The physiological importance of NMD is related to the reduction in the synthesis of C-terminally truncated proteins, thus avoiding dominant-negative effects of non-functional polypeptides (Maquat, 1995).

PTCs in human β-globin mRNA represent a clinically well-documented example of this beneficial NMD effect. Different PTCs located in exons 1 and 2 direct the affected mRNAs to NMD (Baserga and Benz, 1988; Enssle *et al*., 1993), which is associated with the lack of clinical symptoms in heterozygotes. In contrast, NMD does not occur when PTCs are located in the final exon 3. In these cases, heterozygous patients are clinically affected by an unusual dominant form of β-thalassemia (Thein *et al*., 1990; Hall and Thein, 1994).

In mammals, the mechanism(s) by which PTC-mutated mRNAs are specifically recognized and targeted for decay is largely unknown, and both nuclear and cytoplasmic mechanisms have been implicated to be involved in NMD. A nuclear mechanism is suggested by the following observations. (i) PTC-mutated mRNA but not pre-mRNA is less abundant than wild-type mRNA in nucleus-associated fractions of transfected cells, in the bone marrow of patients with β-thalassemia or in fibroblasts of patients with triose phosphate isomerase (TPI) deficiency (Maquat *et al*., 1981; Daar and Maquat, 1988; Belgrader *et al*., 1993, 1994; Simpson and Stoltzfus, 1994; Kugler *et al*., 1995; Carter *et al*., 1996; Kessler *et al*., 1996). (ii) The fate of PTC-mutated β-globin mRNA can be influenced by the promoter from which it is expressed (Enssle *et al*., 1993). (iii) In some cases, PTCs have been reported to induce alternative splicing events (Naeger *et al*., 1992; Dietz *et al*., 1993; Dietz and Kendzior, 1994; Lozano *et al*., 1994; Dietz, 1997). (iv) Intronic sequences have been implicated to play a role in NMD of mutant TPI mRNAs (Cheng *et al*., 1994). (v) The relative position of a PTC with regard to the exon/intron structure of the unspliced pre-mRNA has been shown to play a crucial role. mRNAs with a PTC in the last exon are usually not subject to NMD (Baumann *et al*., 1985; Baserga and Benz, 1988; Urlaub *et al*., 1989; Thein *et al*., 1990; Mashima *et al*., 1992; Enssle *et al*., 1993; Belgrader and Maquat, 1994; Cheng *et al*., 1994; Carter *et al*., 1996). (vi) The stability of wild-type and PTC-mutated cytoplasmic mRNA was reported to be similar in transfected cells (Humphries *et al*., 1984; Baserga and Benz, 1992; Cheng and Maquat, 1993; Lozano *et al*., 1994).

On the other hand, there are many lines of evidence that implicate a cytoplasmic mechanism in NMD. (i) In transgenic mice with a PTC-mutated human β-globin gene, RNA degradation products were found in the cytoplasm of erythroid bone marrow cells (Lim and Maquat, 1992; Lim *et al*., 1992). (ii) A translational ORF starting with an AUG is necessary for NMD (Naeger *et al*., 1992; Simpson and Stoltzfus, 1994). (iii) NMD can be suppressed by co-transfecting appropriate suppressor tRNAs (Takeshita *et al*., 1984; Belgrader *et al*., 1993) and (iv) by inhibiting global translation (Qian *et al*., 1993; Lozano *et al*., 1994; Menon and Neufeld, 1994; Carter *et al*., 1995), or (v) by alterating the structure of the 5′-untranslated region (5′-UTR) of a PTC-containing mRNA to inhibit translation *in cis* (Belgrader *et al*., 1993; Kugler *et al*., 1995).

Although in principle, both nuclear and cytoplasmic mechanisms could cooperate in NMD, the difficulties in reconciling this complex set of findings have recently led to contradictory models for NMD in mammals. Some of these models involve unorthodox features of gene expression such as nuclear scanning of the ORF (Urlaub *et al*., 1989; Dietz *et al*., 1993; Aoufouchi *et al*., 1996; Carter *et al*., 1996; Li *et al*., 1997). In this report, we have analyzed NMD of human β-globin mRNA. Exploiting specific informative manipulations of mRNAs with and without PTCs, we show that nuclear splicing and cytoplasmic translation co-operate to enact a mechanism that distinguishes physiological from premature translation termination codons. The proposed binary specification model not only explains the findings described here, but can reconcile most experimental data on NMD in mammalian cells on the basis of molecular mechanisms that are consistent with the conventional understanding of the gene expression pathway.

Results

Sequence context is not sufficient for PTC versus physiological translation termination codon definition

Both translation initiation and physiological translation termination (Ter) codons display typical sequence contexts that affect the efficiency of their function (Kozak, 1986; McCaughan *et al*., 1995). We first examined whether PTCs were distinguished from Ter by their direct sequence context or by the position of the translation termination codon relative to other *cis*-acting elements.

The first set of constructs was designed to analyze the role of the Ter sequence context (Figure 1A). A comparison of the wild-type β-globin gene with that bearing a naturally occurring nonsense mutation at position 39 (PTC 39) was used to document NMD in our HeLa cell transfection system. Relative to wild-type, PTC 39 was expressed typically at a 4- to 5-fold lower level (Figure 1B, lanes 1 and 2). When PTC 39 was replaced by the physiological β-globin termination codon Ter including its direct sequence context of 15 flanking nucleotides on both sides (PTC 39-Ter; Figure 1A), NMD was observed, albeit to a somewhat lesser extent (Figure 1B, compare lanes 1 and 3). Replacement of PTC 39 by the physiological stop codon and its 30 immediately flanking nucleotides is thus not sufficient to bypass NMD. When the exon 2 sequences 3′ of PTC 39 were replaced by the entire exon 3 to transfer a wider Ter sequence context into the vicinity of PTC 39 (Figure 1A, PTC 39-E2/3), mRNA expression remained low and was comparable with the level for PTC 39-Ter (Figure 1B, compare lanes 1 and 4). Therefore, exon 3 does not contain dominant sequences that are capable of redefining PTC 39 as a bona fide Ter, or of redirecting the mRNA to a non-NMD pathway. Construct WT-E3-E3-Ter and the control WT-E3-E3-CAA (Figure 1A) were designed to examine the effect of Ter in its physiological exonic context but in an upstream position. The accuracy of the predicted splice events was confirmed by cDNA sequencing of RT–PCR products (data not shown). Both mRNAs were expressed at high levels (Figure 1A, compare lanes 5 and 6 with lane 1), suggesting that the position of PTC/Ter within the penultimate exon may play an important role.

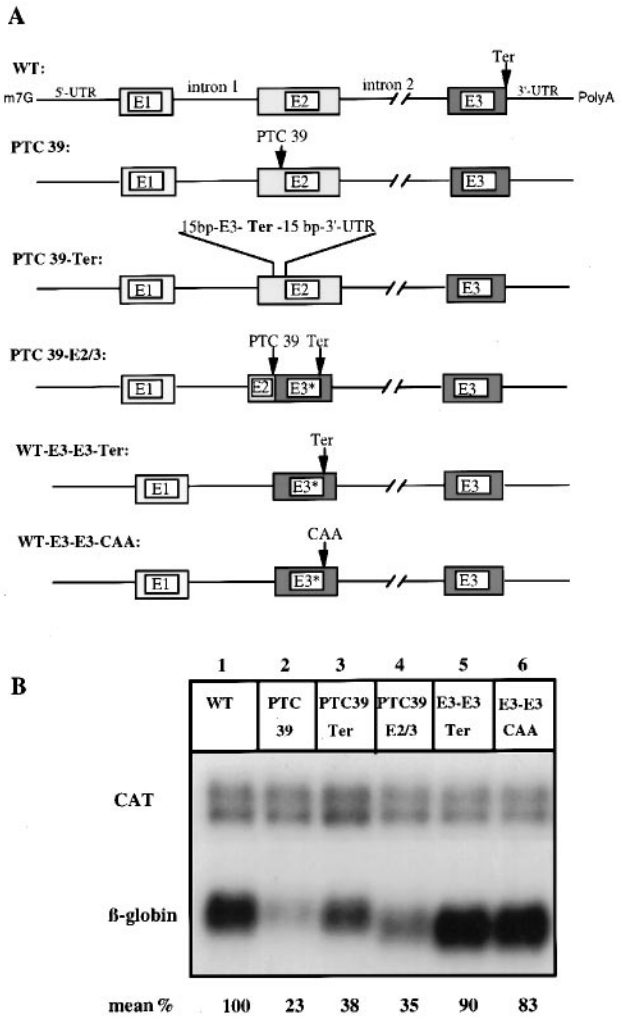


Fig. 1. Sequence context does not exert a dominant influence to distinguish operationally a premature from a physiological termination codon. (**A**) Human β-globin gene constructs used for transfection. The structural modifications and the nomenclature of the constructs are shown diagrammatically. The ORF is represented by boxes, and the untranslated regions and introns by lines (see Materials and methods for details). (**B**) Northern blot of cytoplasmic HeLa cell RNA and hybridization with β-globin and CAT cRNA probes. The percentage values refer to the mean of three independent experiments after normalization for transfection efficiency.

A minimal distance of a PTC mutation from the final intron is critical for nonsense-mediated decay

In most cases, PTCs within the last exon fail to specify NMD. A distinct positional boundary for NMD at ~50 nucleotides upstream of the final intron previously has been established for PTCs in TPI mRNA. If a PTC is located upstream of that boundary, the affected mRNA is degraded. If the PTC is closer to the intron, the mRNA remains stable (Cheng *et al*., 1990, 1994; Cheng and Maquat, 1993). In contrast, the T-cell receptor-β (TCR-β) gene does not exhibit a similar boundary: PTCs as close as eight nucleotides upstream from the last intron specify NMD (Carter *et al*., 1996). The second set of constructs with nested mutations (Figure 2A) was therefore designed to define whether the known positional effects of PTC

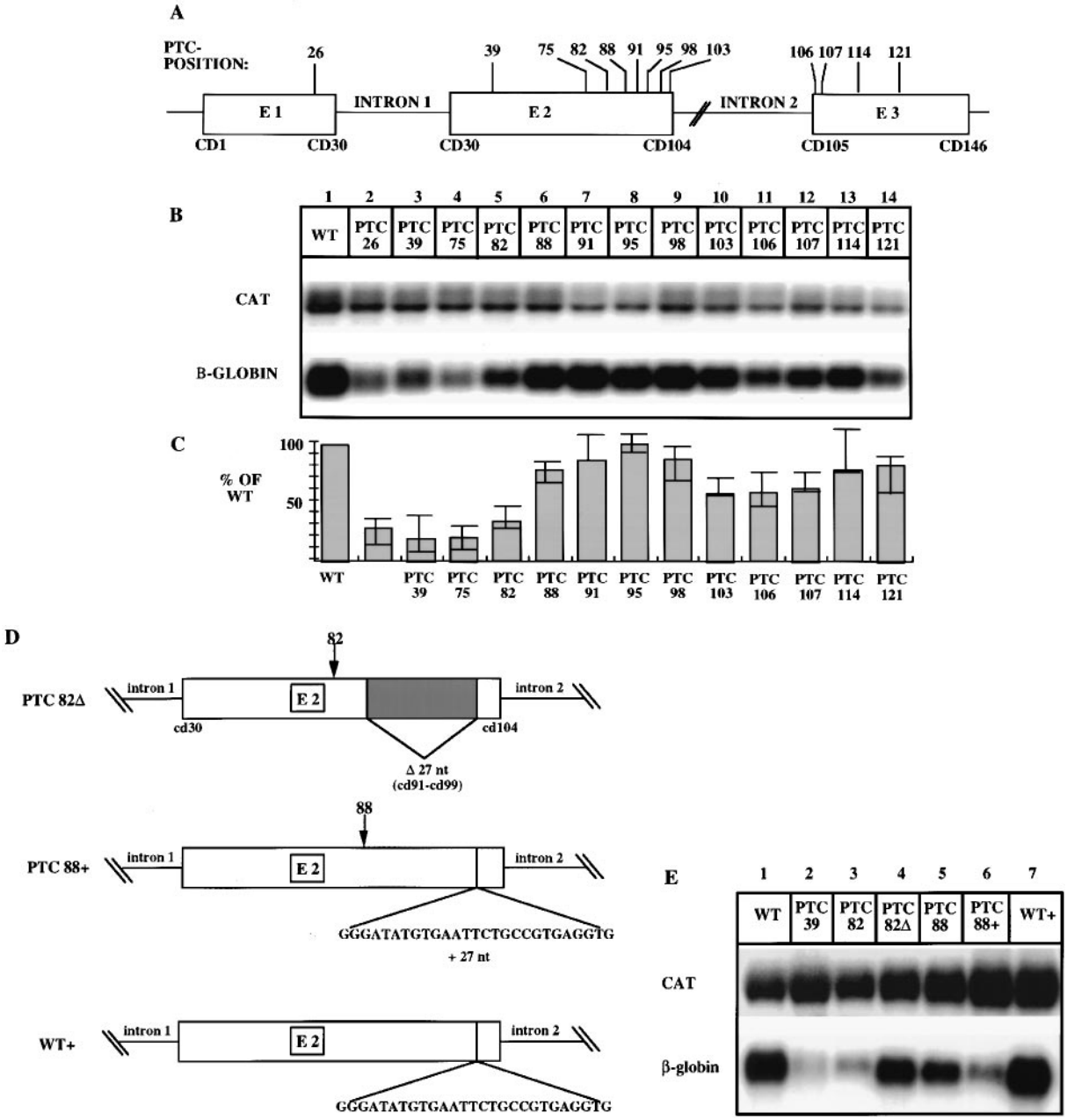


Fig. 2. The distance of the translation termination codon from the final splice junction is critical for NMD. (**A**) Schematic representation of human β-globin gene constructs with the position of nested PTC mutations. (**B**) Northern blot of cytoplasmic RNA of HeLa cells transfected with the constructs shown in (A) and hybridization with β-globin and CAT cRNA probes. (**C**) The column diagram summarizes the results of three independent transfection experiments. Boxes indicate the mean and bars the maximum and minimum values obtained after normalization for transfection efficiency. (**D**) In-frame deletion and insertion constructs shifting the position of PTC 82 towards (PTC 82Δ) and PTC 88 away from the splice junction (PTC 88+). Construct WT+ controls for destabilizing elements within the 27 nucleotide insertion. (**E**) Northern blot of cytoplasmic RNA of transfected HeLa cells and hybridization with β-globin and CAT cRNA probes. The percentage values refer to the mean of three independent experiments after normalization for transfection efficiency.

mutations in the human β-globin gene (Baserga and Benz, 1988; Thein *et al.*, 1990; Enssle *et al.*, 1993) are of the TPI or the TCR-β type.

β-Globin mRNAs with a PTC at codon 26 (in exon 1) and at codons 39, 75 and 82 within the 5′ two-thirds of exon 2 result in NMD, and are expressed at low levels (Figure 2B and C, compare lane 1 with lanes 2–5). In contrast, mRNAs bearing PTCs towards the 3′ end of exon 2 at codons 88, 91, 95, 98 and 103 (Figure 2B and C, lanes 6–10) and those with PTCs in exon 3 at codons 106, 107, 114 and 121 (Figure 2B and C, lanes 11–14) are all expressed at much higher or normal levels. PTCs in the first and in the 5′ region of the second exon thus direct mRNAs to the NMD pathway, whereas PTCs towards the 3′ end of the second exon and in the third exon are associated with high level mRNA expression. This polarity exhibits a clear boundary between 48 and 66 nucleotides upstream of intron 2 (Figure 2), suggesting that the distance between the PTC and the exon 2–intron 2 splice junction may be important. This was tested directly with a set of deletion and insertion constructs (Figure 2D) shifting the PTC towards or away from the boundary.

Construct PTC 82Δ differs from PTC 82 by a 27 nucleotide in-frame deletion, and PTC 88+ differs from PTC 88 by a 27 nucleotide in-frame insertion. In PTC 82Δ, the distance between PTC 82 and the splice junction was thus reduced from 66 to 39 nucleotides, which results in a switch from low to high mRNA expression (Figure 2E, compare lanes 3 and 4). In PTC 88+, the distance between PTC 88 and the splice junction was increased from 48 to 75 nucleotides, which redirects this mRNA to the NMD pathway (Figure 2E, compare lanes 5 and 6). The wild-type control with the same insertion (WT+) is expressed at normal levels (Figure 2E, lane 7), demonstrating that the 27 nucleotide insert *per se* does not harbor destabilizing elements. The distance between the PTC and the final intron thus represents the reference point for a distinct positional polarity of the TPI type.

Splicing in the 3′-UTR subverts a wild-type mRNA to nonsense-mediated decay

The data described above and previous findings (Carter *et al*., 1996) indicate the importance of splicing 3′ of premature translation termination codons for the NMD pathway. They also suggest the provocative possibility that a wild-type mRNA could be subverted to the NMD pathway by the introduction of a spliceable intron at sufficient distance downstream from Ter within the 3′-UTR. The constructs shown in Figure 3A were designed to test this hypothesis directly. Construct WT-SP-Ter contains a heterologous intron plus 15 nucleotides of flanking sequences at both sides 62 nucleotides downstream of Ter. Importantly, and in contrast to previously reported experimental designs (Carter *et al*., 1996; Li *et al*., 1997), the ORF was not altered by this manipulation, and specific *cis*-acting intronic effects such as those reported for TPI RNA (Cheng *et al*., 1994) were excluded here. The controls contain either just the two 15 nucleotides flanking sequences (WT-spf and PTC 39-spf) or the identical intron without splice donor and acceptor signals (WT-sp). Analysis of these constructs (Figure 3B) reveals that: (i) controls WT-spf and PTC 39-spf are comparable with wild-type and PTC 39, respectively (Figure 3B, compare lanes 1 and 2); (ii) the presence of an unspliceable intron in construct WT-sp does not influence the level of wild-type mRNA expression (Figure 3B, compare lanes 1 and 6; note the slower migration of WT-sp mRNA); and (iii) the spliceable intron of construct WT-SP-Ter is removed from the mature mRNA (Figure 3B, compare lanes 3 and 6), resulting in NMD of the WT-SP-Ter mRNA, i.e. the physiological stop codon is operationally redefined as a PTC by the presence of the downstream intron, and an mRNA with a wild-type ORF is directed to the NMD pathway. Note that WT-spf and WT-SP-Ter encode identical mature mRNAs, with the latter but not the former being subject to NMD. The functional relationship between the position of Ter and the 3′ splice event was examined further: (i) with construct WT-SP-CAA that differs from WT-SP-Ter by a Ter→Gln (TAA→CAA) mutation, thus removing the translational termination signal 5′ of the intron inserted into the 3′-UTR; and (ii) with construct WT-SP-CAA-Ter that differs from WT-SP-CAA by a novel Ter 18 nucleotides downstream of the CAA, thus repositioning the translation termination codon to only 44 nucleotides 5′ of the novel

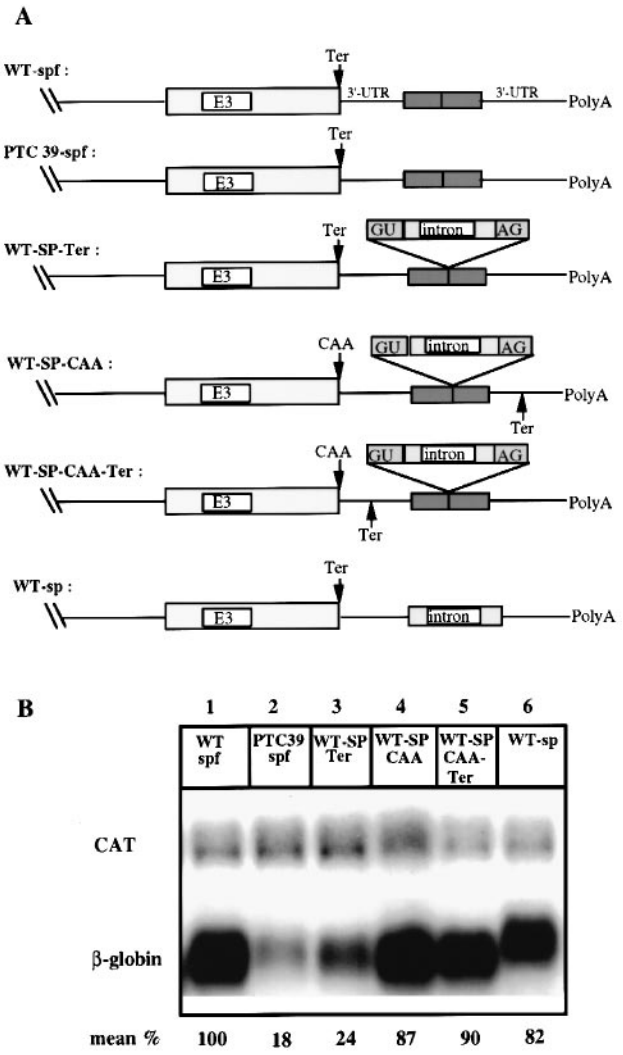


Fig. 3. Splicing in the 3′-UTR directs a wild-type mRNA to the NMD pathway. (**A**) Human β-globin gene constructs with modifications of the 3′-UTR. The presence or absence of GU...AG motifs indicates whether or not the intron was furnished with a splice donor and acceptor site (see Materials and methods for details). (**B**) The Northern blot of cytoplasmic RNA of transfected HeLa cells was hybridized to β-globin and CAT cRNA probes. The percentage values refer to the mean of three independent experiments after normalization for transfection efficiency.

intron, i.e. 3′ of the positional boundary defined in Figure 2 (Figure 3A). Neither mRNA is subject to NMD (Figure 3B, compare lane 1 with lanes 4 and 5), demonstrating that: (i) the positional boundary established for the natural final intron of the β-globin gene (Figure 2) can be translocated when the exon–intron structure of the gene is altered; (ii) the same positional requirements appear to apply to 3′-UTR introns; and (iii) a 3′-UTR intron must be spliceable to subvert a wild-type mRNA to NMD. In conclusion, NMD depends *in cis* on the position of the premature or physiological translation termination codon relative to the most 3′ spliceable intron. These findings also unequivocally identify a nuclear component of the NMD pathway.

Nonsense-mediated decay of mutant mRNAs requires their cytoplasmic translation

Findings from several laboratories have implicated a role for translation or ‘translation-like’ mechanisms in NMD

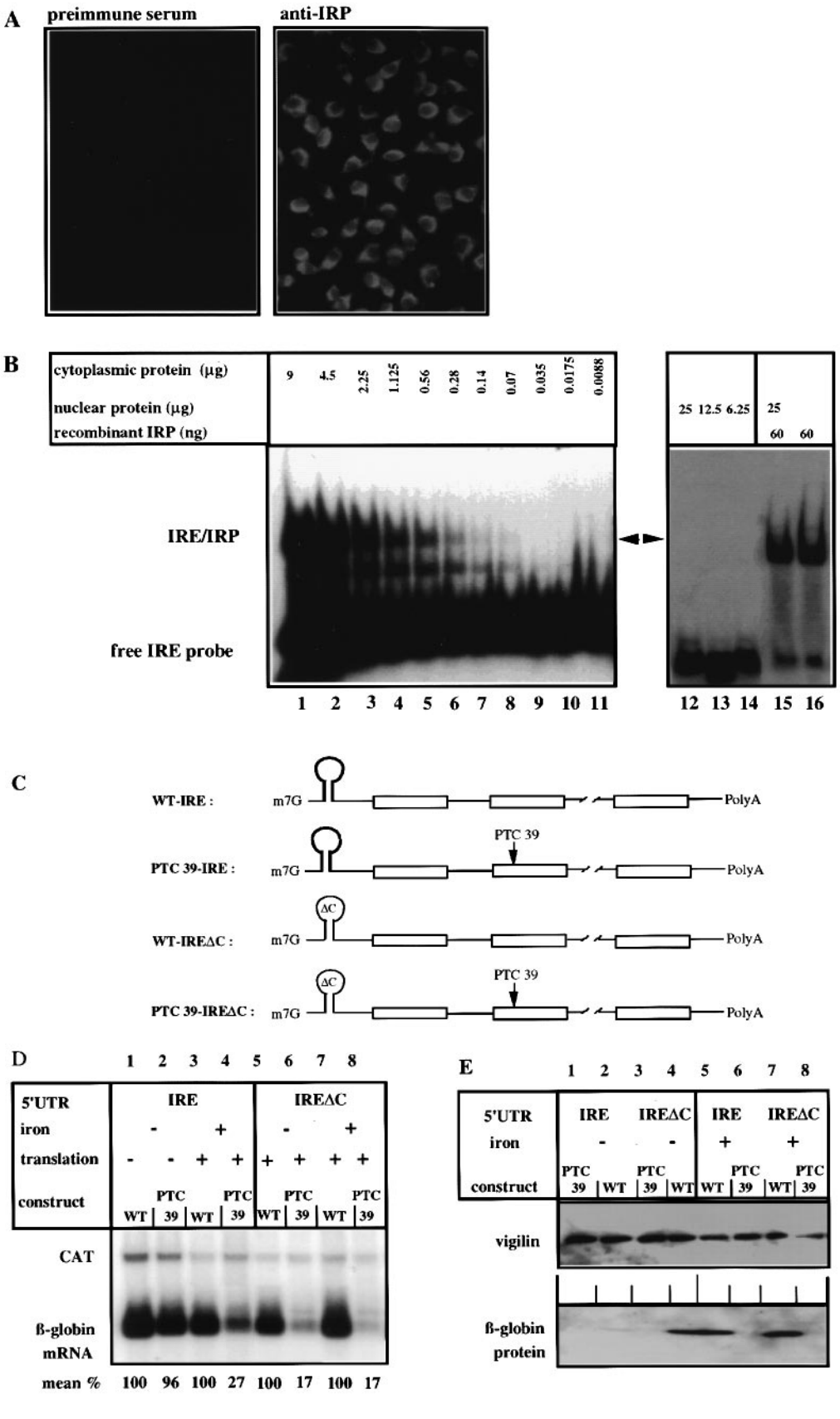
(see above). In studies employing general translation inhibitors (Qian *et al*., 1993; Lozano *et al*., 1994; Menon and Neufeld, 1994; Carter *et al*., 1995) or suppressor tRNAs (Takeshita *et al*., 1984; Belgrader *et al*., 1993), possible pleiotropic effects of these approaches complicate the interpretation of the data. Furthermore, these and other studies using specific translational inhibitory alterations of the 5′-UTRs of PTC mRNAs (Belgrader *et al*., 1993; Kugler *et al*., 1995) could not distinguish unambiguously between conventional cytoplasmic translation and the less orthodox nuclear translation-like mechanisms that are a critical feature of the nuclear scanning model (Carter *et al*., 1996; Li *et al*., 1997).

The interaction between iron-responsive elements (IREs) and iron-regulatory proteins (IRPs) enables the specific translational regulation of mRNAs bearing IREs in their 5′-UTRs (Klausner *et al*., 1993; Hentze and Kühn, 1996). In iron-deficient cells, IRP binding to an IRE specifically inhibits the formation of a 48S pre-initiation complex at the 5′ end of the mRNA, thus blocking an early step of translation (Gray and Hentze, 1994). Elevation of intracellular iron levels inactivates IRP binding and thus permits the translation of the mRNA. IRP-1 (98 kDa) and IRP-2 (105 kDa) are expected to be expressed exclusively in the cytoplasm, because they are too large to traverse the nuclear membrane passively and they do not contain a known nuclear localization signal for active nuclear import. This spatial restriction was assessed directly by immunofluorescence studies of cells with an IRP-1-specific antibody (Pantopoulos *et al*., 1995) that show a cytoplasmic signal excluding nuclei (Figure 4A). Although IRP-1 is the predominant IRP in mammalian cells, this analysis does not exclude the presence of IRP-2 in the nucleus. We therefore performed gel retardation experiments with nuclear and serial dilutions of cytoplasmic extracts from HeLa cells with an IRE probe (Pantopoulos *et al*., 1995) that forms electrophoretically co-migrating complexes with IRP-1 and IRP-2 with equal affinity (Hentze and Kühn, 1996). Binding activity was readily detectable with as little as 0.07 μg of cytoplasmic protein (Figure 4B, lanes 1–8). In contrast, no signal was detectable in the nuclear fraction, even when 25 μg of nuclear protein (i.e. a >350-fold higher amount than 0.07 μg) was used (Figure 4B, lanes 12–14). To exclude non-specific inhibitory effects on complex formation exerted by components in the nuclear extract, we compared complex formation between the IRE probe and recombinant human IRP-1 in assays with or without 25 μg nuclear protein (Figure 4B, lanes 15 and 16). Taken together, these data show that IRP-1 and IRP-2 can be identified readily in the cytoplasm and that neither appears to be present in the nucleus. The introduction of an IRE into the 5′-UTR of human β-globin genes (constructs shown in Figure 4C) thus allows the role of cytoplasmic translation in NMD to be probed specifically. A wild-type β-globin mRNA bearing an IRE within its 5′-UTR (WT-IRE) is stable regardless of the availability of iron (Figure 4D, lanes 1 and 3). In the presence of iron, translation is readily detectable by immunoblotting (Figure 4D, lane 5), whereas hardly any protein can be identified in iron-deficient cells (Figure 4D, lane 2). The iron-dependent regulation of translation is lost when a point-mutated non-functional IRE is introduced into the β-globin 5′-UTR: the mRNA is translated constitutively (Figure 4C, construct WT-IREΔC, Figure 4E lanes 4 and 7) and also is stable (Figure 4D, lanes 5 and 7). In contrast, PTC 39 mRNA bearing a functional IRE (Figure 4C, construct PTC 39-IRE) is subject to NMD when translation is enabled, but escapes NMD in iron-deficient cells where IRP binding inhibits the cytoplasmic translation of the mutated mRNA (Figure 4). The NMD escape is due to translational inhibition rather than to iron deficiency *per se*, because a PTC 39 mRNA bearing a non-functional IRE (Figure 4C, constructs WT-IREΔC and PTC 39-IREΔC) is subject to NMD in both iron-replete and iron-deficient cells (Figure 4D, compare lanes 3 with 4, 5 with 6 and 7 with 8). The β-globin antibodies fail to detect any polypeptide expressed from the PTC 39 mRNAs, hence it was not possible formally to demonstrate translational control of these mRNAs by immunoblotting (Figure 4E, lanes 1, 3, 6 and 8). We therefore analyzed the polysome association (Figure 5) of the mRNAs expressed from the constructs shown in Figure 4C. This analysis confirms that: (i) wild-type β-globin mRNAs are associated preferentially with heavier, and PTC 39 mRNAs with lighter polysomes when translation is enabled, reflecting the differences in length of the respective ORFs (Figure 5A, C and D); and (ii) the IRE–IRP interaction results in an accumulation of the wild-type and the PTC 39 mRNAs at the top of the gradient, showing that they are not associated with ribosomes (Figure 5B). We conclude that specific inhibition of ribosome association and cytoplasmic translation *in cis* prevents the degradation of PTC 39 mRNA. Thus, cytoplasmic translation of the mutated mRNA is necessary for NMD.

Discussion

Cells have evolved mechanisms to distinguish wild-type from mutated mRNAs, and to degrade the latter. These mechanisms can operate when PTCs, but not missense mutations, disrupt the ORF. Nonsense-mediated decay is an ill-understood, yet biologically and medically important, mechanism as demonstrated by mutations in the *smg*

Fig. 4. Cytoplasmic translation is necessary to direct a PTC-mutated mRNA to the NMD pathway. (**A**) Immunofluorescence of B6 cells with pre-immune serum (left) and IRP-1-specific antibody (anti-IRP; right). (**B**) Gel retardation analyses with an IRE probe and serial dilutions of cytoplasmic (lanes 1–11) and nuclear (lanes 12–14) protein from fractionated HeLa cells and recombinant human IRP-1 together with (lane 15) or without (lane 16) nuclear protein. The IRE probe forms co-migrating complexes (arrow) with IRP-1 and IRP-2. (**C**) Wild-type and PTC 39-mutated human β-globin gene constructs with functional (IRE, bold stem loop) and non-functional (IREΔC, thin stem loop) IREs in the 5′-UTR (see Materials and methods for details). (**D**) Northern blot of cytoplasmic RNA of transfected HeLa cells that were grown in medium either supplemented (iron+) or not (iron–) with heme arginate as an iron source. Translation of the constructs containing a functional IRE could thus be regulated specifically (translation– or translation+). Hybridization was performed with β-globin and CAT cRNA probes. The percentage values refer to the mean of three independent experiments after normalization for transfection efficiency. (**E**) Immunoblot of total protein of HeLa cells transfected with the constructs shown in (C). The medium was either supplemented (iron+) or not (iron–) with heme-arginate. Hybridization was performed with human β-globin- and vigilin-specific antibodies. Vigilin is a protein that is expressed independently of the availability of iron and served as a control for equal loading of the lanes.


A
preimmune serum
anti-IRP
B
cytoplasmic protein (µg)
nuclear protein (µg)
recombinant IRP (ng)
9 4.5 2.25 1.125 0.56 0.28 0.14 0.07 0.035 0.0175 0.0088
25 12.5 6.25
25
60 60
IRE/IRP
free IRE probe
1 2 3 4 5 6 7 8 9 10 11
12 13 14 15 16
C
WT-IRE :
PTC 39-IRE :
WT-IREΔC :
PTC 39-IREΔC :
m7G
PolyA
PTC 39
ΔC
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1 2 3 4 5 6 7 8
5'UTR
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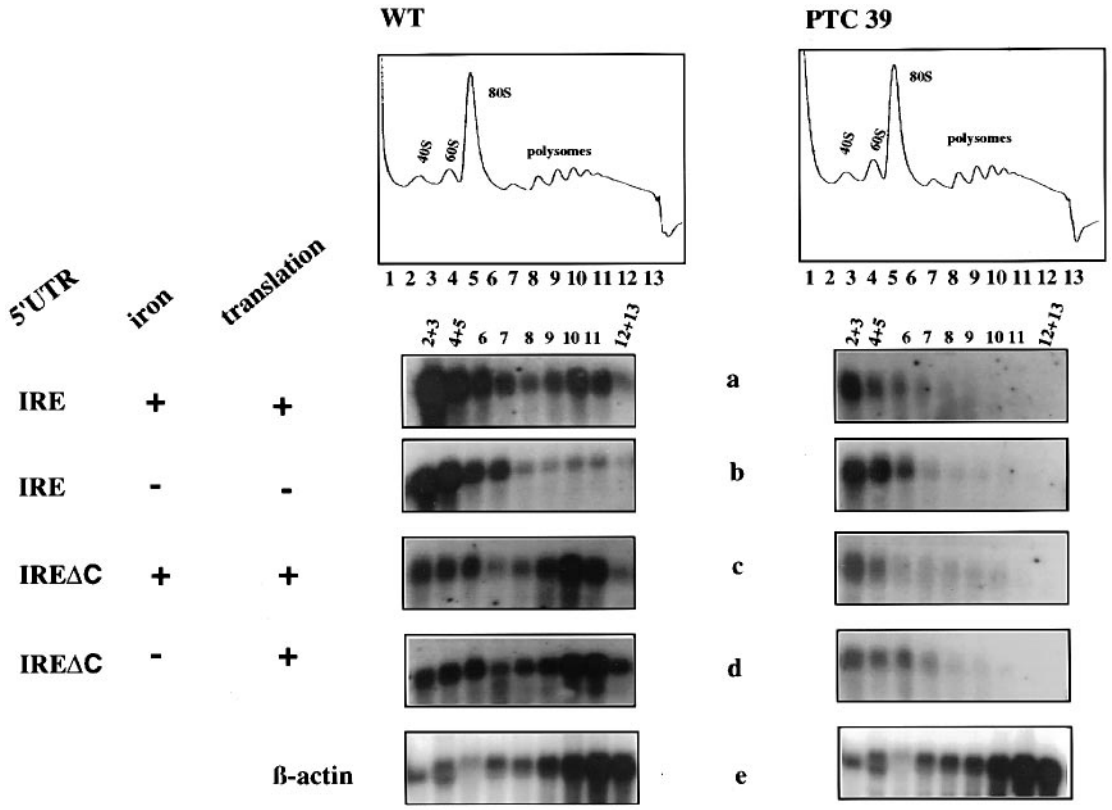

Fig. 5. Translation-competent wild-type and PTC 39 β-globin mRNAs are associated with polysomes. The top panel shows representative gradient profiles of HeLa cells transiently transfected with the constructs described in Figure 4C. The number of the fractions that were used for Northern blotting are indicated below the gradient profiles. The individual panels show Northern blots of the different fractions after hybridization with β-globin- or β-actin-specific probes. The left side represents cells that were transfected with wild-type and the right side those transfectd with a PTC 39-mutated construct. The table indicates the modification of the 5′-UTR, the iron status of the cells and the specific translational competence of the transfected β-globin gene. (**a**) Functional IRE with iron supplementation resulting in translational competence. (**b**) Functional IRE without iron supplementation resulting in translational incompetence. (**c**) Non-functional IRE with iron supplementation. (**d**) Non-functional IRE without iron supplementation. (**e**) Representative blots hybridized with a β-actin-specific probe controlling for the quality of the gradient and general translational competence of the transfected cells.

genes that are required for NMD in *Caenorhabditis elegans*, and display synthetic lethality with nonsense mutations in the *unc-54* myosin heavy chain B gene (Pulak and Anderson, 1993), or by forms of β-thalassemia with symptomatic heterozygotes and a dominant mode of inheritance (Thein *et al*., 1990; Hall and Thein, 1994).

NMD involves different phases: discrimination of a PTC from the physiological stop codon, commitment of the mutated mRNA to NMD, and nucleolytic degradation of the mRNA. Much work has focused on the early phases of NMD. A complex array of findings obtained from various mammalian systems has led to the formulation of several models. In spite of significant progress to distinguish between these models, at least two mutually exclusive models remain (Urlaub *et al*., 1989; Maquat, 1995; Carter *et al*., 1996). Neither of the two models can fully account for the available data (see below). One central issue revolves around the question of whether NMD is a nuclear, a nucleus-associated or a cytoplasmic mechanism. The analyses of the TPI gene suggested that PTC recognition takes place after splicing and that intronic sequences provide a mark that translating ribosomes must approximate or traverse in order to confer mRNA stability (Maquat, 1995). However, the inhibition of translation was shown not to destabilize the mRNA (Belgrader *et al*., 1993). According to the co-translational export model, PTC-mutated mRNA degradation is independent of the recognition of translational sense within the nucleus but occurs while the mRNA is still physically associated with the nucleus. Those PTC-mutated mRNA molecules that escape the nucleus-associated degradation mechanism are stable once they have reached the cytoplasm (Maquat, 1995). In contrast, different variations of 'nuclear scanning' models postulate the existence of a (ribosome-like) structure that scans nuclear (pre-)mRNAs for their translational sense and induces either altered splicing to avoid the PTC or the degradation of PTC-mutated mRNAs within the nucleus (Urlaub *et al*., 1989; Dietz *et al*., 1993; Dietz and Kendzior, 1994; Aoufouchi *et al*., 1996; Carter *et al*., 1996; Li *et al*., 1997).

Our strategy based on PTC mutations of the β-globin gene aimed to unambiguously identify necessary and sufficient components during the discrimination and commitment phase of NMD. The first set of experiments addresses the role of splicing as a nuclear component of the NMD pathway. In the TPI gene, a sequence element in the final intron was implicated in marking the mRNAs *in cis*. NMD depends on the distance between this sequence element and the PTC mutation, but not on splicing *per se* (Cheng *et al*., 1994). In contrast, an analysis of the TCR-β gene implicated a functional role for splicing but no distinct positional effect (Carter *et al*., 1996). The interpretation of

the TCR-β data is complicated by two aspects; the duplication or deletion of exonic and intronic sequences of that particular gene significantly alters the ORFs of the different TCR-β-related mRNAs, and the use of a TCR-β intron rather than a heterologous one. It is difficult, therefore, to exclude a possible effect of specific *cis*-acting intronic or exonic sequences that have been implicated in affecting mRNA stability in other mammalian systems (Cheng *et al*., 1994; Liu and Mertz, 1996).

In the experiments shown in Figure 3, a spliceable intron was inserted into the 3′-UTR of the human β-globin mRNA without changing the structure of the mature mRNA (compare WT-spf with WT-SP-Ter). Moreover, the intron used is unrelated to the β-globin gene, avoiding the inadvertant inclusion of confounding homologous *cis*-acting elements. The results demonstrate that a structurally wild-type mRNA without any changes in its ORF is directed to the NMD pathway by a splicing event in the 3′-UTR (Figure 3). Downstream splicing can therefore serve as a sufficient means for redefining the physiological translation stop codon Ter as a PTC. The operational specification of a PTC includes the requirement for a minimal distance between the termination codon and the 3′-splicing event (Figure 2). This position dependence displays a clear boundary similar to that described for the TPI gene (Cheng *et al*., 1994). The potential mechanistic basis of the boundary effect is discussed below.

The other issue that we directly addressed is the specific role of translation in the cytoplasm. Whereas the introduction of hairpins into the 5′-UTR or the use of suppressor tRNAs (Takeshita *et al*., 1984; Belgrader *et al*., 1993) may interfere with the function of the notional nuclear ribosome-like structure that represents a critical feature of the nuclear scanning model (Carter *et al*., 1996; Li *et al*., 1997), the IRE/IRP system specifically regulates mRNA translation in the cytoplasm (Figure 4A and B; Klausner *et al*., 1993; Hentze and Kuhn, 1996). The design of the experiments shown in Figures 4 and 5 also excludes possible pleiotropic effects of general inhibitors of protein synthesis. Moreover, our strategy exploits the functional (IRE/IRP) rather than preparative (subcellular fractionation) definition of the cytoplasmic compartment. This allows us to distinguish whether the influence of translational features on the NMD pathway is conferred by the conventional cytoplasmic translation apparatus or by a notional translation-like nuclear mechanism (Carter *et al*., 1996; Li *et al*., 1997) and also avoids uncertainties about the purity of nuclear preparations. We show that conventional cytoplasmic translation is a necessary component to validate a PTC-mutated β-globin mRNA for the NMD pathway (Figures 4 and 5).

Our results suggest a binary specification model for the discrimination and commitment phase of NMD (Figure 6). In the first step, splicing induces the tagging of the position of (former) introns. It is conceivable that such a tag could be a spliceosome-associated protein that remains bound to, and escorts the mRNA into the cytoplasm (Visa *et al*., 1996). In the exceptional cases where additional specific *cis*-acting sequences (Cheng *et al*., 1994) can substitute for the presence of a spliceable downstream intron (such as the TPI mRNA), the tag (protein) may be recruited by these specific sequences. During or after nuclear export, the second step requires cytoplasmic translation. If translation is blocked, the tags are not encountered and the mRNA remains stable (Figure 6, pathway b). If the tags are encountered by elongating ribosomes within the ORF (as would happen with tags representing all former introns upstream of termination codons), the mRNA is not validated for the NMD pathway. In this case, the tags may remain 'neutral', could be 'approved' or removed, and the mRNA is not degraded (Figure 6, pathway a). If mRNA translation occurs and a tag 3′ of the translation stop codon is identified (as would happen with PTCs in all but the final exon and with 3′-UTR intron tags), the tag is validated for NMD and the mRNA targeted for degradation (Figure 6, pathway c). If PTC mutations are located at an insufficient distance from the final tag (Figure 2) (Baumann *et al*., 1985; Urlaub *et al*., 1989; Thein *et al*., 1990; Mashima *et al*., 1992; Enssle *et al*., 1993; Belgrader and Maquat, 1994; Cheng *et al*., 1994), this may be (falsely) 'approved' or removed by the leading edge of the elongating ribosome, which thus plays an mRNA protective role. The factor or the complex that validates downstream tags for NMD remains to be identified, but could well represent (components of) a ribosomal post-termination complex (Ruiz-Echevarria *et al*., 1998). It is possible that such a complex could contain homologs of the UPF proteins (Applequist *et al*., 1997) which are required for NMD in yeast and, interestingly, have been shown to be ribosome associated (Ruiz-Echevarria *et al*., 1996).

This binary specification model can explain or account for many previous, seemingly contradictory findings that were decribed above. Our model can also explain a recent report demonstrating that translation re-initiation after a premature stop codon results in the escape of TPI mRNA from the NMD pathway (Zhang and Maquat, 1997). One of the most puzzling aspects of NMD has been the nuclear or cytoplasmic site of PTC identification and the frequently nucleus-associated site of mRNA degradation. Our findings functionally assign the relevant translational activity to the cytoplasm, and thus do not support some of the unconventional aspects of the nuclear scanning model. The nuclear association of PTC mRNA degradation in some (but not all) cases of NMD is consistent with the binary specification model. We suggest that some mRNPs undergoing nuclear export have an inherent transient affinity for the preparatively defined 'nuclear fraction' while being accessible to the cytoplasmic translation apparatus on the cytoplasmic side of the nuclear envelope.

The residual levels of PTC-mutated mRNAs of commonly 10–30% of the wild-type levels may be explained by a $<$100% efficiency of tagging and/or by tag dissociation. The normal cytoplasmic stability of the remaining 10–30% PTC mRNAs that bear no tag is predicted by the binary specification model. An observation that can be explained by neither the binary specification nor by any of the other models is the effect of PTC mutations on splice-site choice (Dietz *et al*., 1993; Dietz and Kendzior, 1994; Dietz, 1997) or the splicing process *per se* (Lozano *et al*., 1994; Aoufouchi *et al*., 1996), which may be induced by another PTC-dependent mechanism.

In yeast, NMD has been perceived to be enacted by a mechanism which is profoundly different from that used in multicellular eukaryotes. NMD in yeast is splicing independent, cytoplasmic and apparently dependent on a

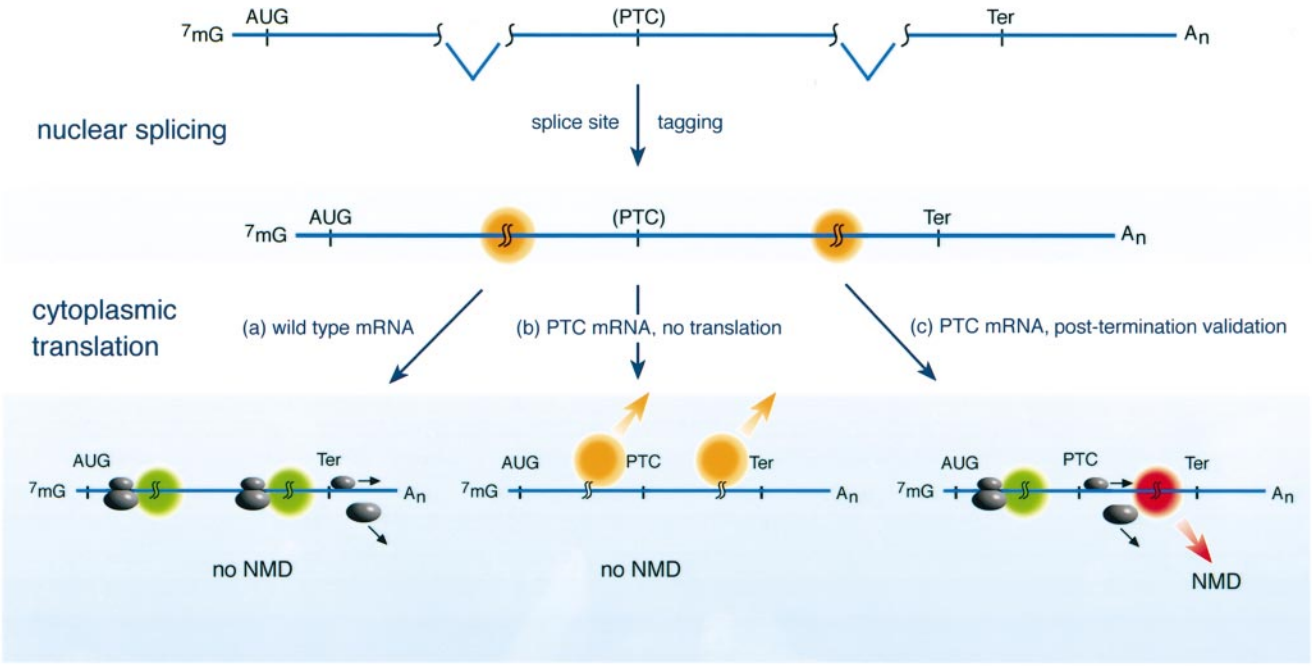

Fig. 6. The binary specification model of NMD. The details of the model are described in the text.

specific 'downstream sequence element' (DSE) (Ruiz-Echevarria *et al*., 1996). Interestingly, the DSE is thought to require recognition by a ribosomal post-termination complex (Ruiz-Echevarria *et al*., 1998). The binary specification model provides a conceptional bridge between NMD in yeast and mammals. Hypothetically, the mammalian splicing tag could be substituted by the DSE (or a DSE-binding protein) in *Saccharomyces cerevisiae*, where introns are rare. All subsequent steps of NMD in yeast and man could then conceivably be alike. Further characterization of the splicing tag and of the function of the DSE represent experimental tests of this hypothetical prediction.

In conclusion, we present specific evidence that the discrimination and commitment to degrade PTC-mutated human β-globin mRNAs involves the co-operation of conventional nuclear and cytoplasmic molecular mechanisms. Our findings and their interpretation raise the possibility of a common principle for NMD from yeast to man. Further implications of our findings relate to the common usage of introns in the 3′-UTRs of expression vectors in molecular biology and gene therapy, which may warrant re-evaluation.

Materials and methods

Plasmid constructs

The constructs shown in Figures 1A–3A contain a 4.4 kb human β-globin gene with a linked SV40 enhancer inserted as a *Not*I fragment into pBlueskriptSK II+ (Stratagene) (Enssle *et al*., 1993). The wild-type and the PTC 39 constructs are derived from a healthy proband and from a patient with homozygous β-thalassemia, respectively. Construct PTC 39 was modified by overlap extension PCR (Pogulis *et al*., 1996) to generate construct PTC 39-Ter, in which the stop codon at position 39 (TAG) and the 15 bp flanking both sides were replaced by the physiological stop codon (TAA) including 30 bp of its immediate flanking sequences. In construct PTC 39-E2/3, exon 2 sequences 3′ of PTC 39 were replaced in-frame by a PCR fragment containing exon 3 including the physiological translation termination codon and a novel splice donor at the 3′ end. In construct WT-E3-E3-Ter, the entire exon 2 is replaced in-frame by a PCR fragment containing the entire coding sequence of exon 3, termed exon 3*. Construct WT-E3-E3-CAA differs from WT-E3-E3-Ter by a TAA→CAA mutation of the translation termination codon of exon 3*.

Constructs PTC 26, PTC 75, PTC 82, PTC 91, PTC 95, PTC 98, PTC 103, PTC 106, PTC 107, PTC 114 and PTC 121 were created by site-directed mutagenesis (Hagemeier, 1996). Construct PTC 88 was generated by replacing the sequences in the wild-type construct by a PCR fragment derived from a proband with heterozygous β-thalassemia carrying a ΔG frameshift mutation at codon 82/83, which results in a TGA PTC at codon 88 (Vetter *et al*., 1997).

Constructs PTC 82Δ and PTC 88+ were generated by site-directed mutagenesis (Hagemeier, 1996). Construct PTC 82Δ differs from construct PTC 82 by a 27 bp deletion of codons 91–99. In construct PTC 88+, a 27 bp fragment was inserted into construct PTC 88 in-frame at codon 99. Construct WT+ differs from PTC 88+ by the absence of the PTC 88 mutation.

In the constructs shown in Figure 3A, the *Apa*I and *Xho*I sites of the Bluescript polylinker were deleted. New *Apa*I and *Xho*I restriction sites were then inserted by site-directed mutagenesis into the 3′-UTR at a position 41 bp downstream of the physiological stop codon. Construct WT-SP-Ter was generated by introducing the MINX-intron (Zillmann *et al*., 1988) with 30 bp flanking sequences including its splice donor and acceptor sites into the *Apa*I and *Xho*I sites in the 3′-UTR. Construct WT-SP-CAA differs from WT-SP-Ter by a TAA→CAA mutation of Ter. The novel in-frame Ter of this construct is located 41 bp downstream of the inserted intron. Construct WT-SP-CAA-Ter differs from WT-SP-CAA by a new TAA that was created by site-directed mutagenesis at an in-frame position 18 bp downstream of the former Ter. Construct WT-sp differs from construct WT-SP-Ter by the absence of the intron flanking sequences including the splice donor and acceptor sites. Construct WT-spf differs from WT-SP-Ter by the absence of the intron, but retains the intron flanking sequences in the 3′-UTR. In construct PTC 39-spf, the WT CAG at position 39 of construct WT-spf was replaced by a TAG PTC.

The constructs shown in Figure 4A contain the human β-globin gene fragment as described above in a pGEM-5Zf (+) expression vector (Promega). In constructs WT-IRE and PTC 39-IRE, a 35 bp fragment containing a functional IRE (Hentze *et al*., 1987) was inserted by site-directed mutagenesis (Hagemeier, 1996) into the 5′-UTR 37 nucleotides downstream of the cap structure of the wild-type and the PTC 39 constructs (Figure 1A) (Goossen and Hentze, 1992). Constructs WT-IREΔC and PTC 39-IREΔC differ from the IRE constructs by a deletion of a single cytosine residue, which functionally inactivates the IRE (Hentze *et al*., 1988).

The structure of all constructs was confirmed by DNA sequence analysis of the insert including the promotor and the entire transcribed region from the cap site to the polyadenylation signal.

Cell culture and transfections

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) under standard conditions. Cells were transiently transfected by calcium phosphate precipitation (Ausubel *et al*., 1994) with 20 μg of the test constructs and 15 μg of an *Escherichia coli* chloramphenicol acetyltransferase (CAT) gene cloned into the multiple cloning site of the plasmid pSG5 (Green *et al*., 1988), which served as a control for transfection efficiency. Cells were washed after 20 h and harvested 24 h later.

For the experiments with the IRE-containing constructs, culture media were supplemented with 100 μM of the iron source heme arginate (Leiras, Turku, Finland) 8 h after washing. For iron starvation, it was sufficient to grow the cells in unmodified medium that contained only 0.25 μmol of Fe^{2+}. At 16 h after addition of heme arginate, i.e. 24 h after transfection, the cells were harvested.

Isolation and analysis of RNA

Total cytoplasmic RNA was purifed from the supernatant of homogenized cells with TRIzol reagent (Gibco-BRL). Northern blot analysis was performed as previously described (Enssle *et al*., 1993) with 1–2 μg of total cytoplasmic RNA or, when fractions of polysome gradients were analyzed, with half of each fraction. Blots were hybridized using *in vitro* transcribed ^{32}P-labeled antisense cRNA β-globin and CAT probes.

The template for the β-actin probe was a 383 bp human β-actin cDNA PCR fragment, which was subjected to single strand PCR in the presence of [^{32}P]dCTP (Konat *et al*., 1994). Hybridization was carried out at 65°C overnight.

The signals were quantified by imaging in a GS-250 Molecular Imager (Bio-Rad). β-Globin mRNA levels are expressed as a percentage of wild-type after normalization for transfection efficiency.

Indirect immunofluorescence

B6 cells were grown on coverslips in DMEM. Coverslips were washed twice with phosphate-buffered saline (PBS), and cells were permeabilized with 0.5% Triton X-100 in CKS buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES pH 6.8, 3 mM $MgCl_2$, 1 mM EGTA) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) for 2 min on ice. Subsequently, cells were fixed with 3.7% paraformaldehyde in CKS buffer for 10 min at room temparature, followed by three 5 min washes in PBS. Labeling with rabbit polyclonal antibodies raised against recombinant human IRP-1 (Pantopoulos *et al*., 1995) or pre-immune serum as a specificity control was performed according to standard methods. Samples were examined and photographed in a Zeiss Axioskop fluorescence microscope.

Gel retardation experiments

These were performed exactly as previously described (Pantopoulos and Hentze, 1995).

Cell fractionation

Cytoplasmic and nuclear fractions from HeLa cells used for IRE/IRP bandshift analyses were prepared essentially as described (Kugler *et al*., 1995), with the following modifications: the lysis buffer contained 0.5% Triton X-100, 0.2% Na deoxycholate instead of 0.5% NP-40 and was supplemented with 1 mM dithiothreitol (DTT). Cells of a 10 cm dish were lysed in 1 ml of lysis buffer. After cell lysis, the nuclei were pelleted and washed once with lysis buffer containing detergent and once with buffer without detergent. Nuclei were then centrifuged (15 000 *g*, 45 min, 4°C) through a 1.8 M sucrose cushion in reticulocyte standard buffer (Kugler *et al*., 1995). The pellet was resuspendend in 250 μl of lysis buffer without detergent and lysed by freeze–thawing.

Polysome gradients

Polysome gradients were performed as described previously (Kessler *et al*., 1996) but with minor modifications. Briefly, cells were washed twice with cold PBS, containing 50 μg/ml cycloheximide, harvested and lysed in 1 ml of cold lysis buffer plus 50 μg of cycloheximide/ml. Nuclei and cell debris were removed by centrifugation at 10 000 *g* for 10 min. The supernatant was loaded onto 13 ml 10–50% sucrose gradients in the same buffer but without detergents and cycloheximide. The gradients were centrifuged for 2 h at 36 000 r.p.m. in an SW 40 rotor at 4°C. Thirteen fractions were collected from the top of the gradient. UV absorbance at 254 nm was measured continously. SDS was added to each fraction to a final concentration of 0.5%. Subsequently, fractions were subjected to phenol extraction. The extracted RNA was analyzed by Northern blotting as described above. Transfection efficiency was determined by assaying for CAT activity.

Immunoblotting

Cytoplasmic protein was extracted after lysis in cold lysis buffer plus 10 μg/ml PMSF as described for polysome gradients. Equal amounts of cells were analyzed on 5–15% SDS–polyacrylamide gradient gels. The proteins were electroblotted onto PVDF membranes (Immobilon, Schleicher and Schuell) using a semi-dry apparatus. Membranes were stained with Ponceau S to ensure that the transfer was complete and uniform, and then blocked with 5% skimmed milk in Tris-buffered saline (TBS) plus 0.05% Tween-20. The primary β-globin antibody was raised in rabbits against the N-terminal 13 amino acids of human β-globin (Eurogentec, Seraing, Belgium). Immunoblotting was performed using standard conditions and a 1:2000 dilution of the antibody. The vigilin antibody was a rabbit antiserum against the 15 C-terminal amino acids of human vigilin and was diluted 1:6000. The secondary antibody (anti-rabbit IgG coupled to horseradish peroxidase; Dianova) was diluted 1:3000. Specific staining was detected using a bioluminescence kit (Boehringer Mannheim) according to the manufacturer's instructions.

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