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Internal ribosome entry sequence-mediated translation initiation triggers nonsense-mediated decay

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In eukaryotes, a surveillance pathway known as nonsense-mediated decay (NMD) regulates the abundance of messenger RNAs containing premature termination codons (PTCs). In mammalian cells, it has been asserted that the NMD-relevant first round of translation is special and involves initiation by a specific protein heterodimer, the nuclear cap-binding complex (CBC). Arguing against a requirement for CBC-mediated translation initiation, we show that ribosomal recruitment by the internal ribosomal entry sequence of the encephalomyocarditis virus triggers NMD of a PTC-containing transcript under conditions in which ribosome entry from the cap is prohibited. These data generalize the previous model and suggest that translation *per se*, irrespective of how it is initiated, can mediate NMD.

INTRODUCTION

In eukaryotes, a conserved surveillance pathway known as nonsense-mediated decay (NMD) regulates the abundance of messenger RNA containing premature termination codons (PTCs). It has been shown that both splicing (Carter *et al*, 1995; Maquat & Li, 2001; Neu-Yilik *et al*, 2001; Brocke *et al*, 2002) and translation (Belgrader *et al*, 1993; Thermann *et al*, 1998) are required for mammalian NMD to occur.

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During splicing the position of excised introns is marked by deposition of a protein complex known as the exon junction complex (EJC), 20–24 nucleotides (nt) upstream of each exon–exon junction (Le Hir *et al*, 2000), and stop codons seem to be validated as 'normal' if they are no more than 50–55 nt upstream of the last exon–exon junction in an mRNA (Maquat, 2004). Presumably, during translation termination, the position of the EJC relative to the encountered stop codon is crucial, and stop codons that are further upstream than 50 nt of the last exon–exon junction are recognized as abnormal, resulting in transcript degradation.

Different studies suggest that mammalian NMD is only triggered during early rounds of translation, perhaps even during the first round. Turnover measurements of a PTC-containing triosephosphate isomerase mRNA indicated increased decay only at early time points (<90 min) after a transcription pulse (Belgrader *et al*, 1994). A nonsense-mutated dihydrofolate reductase transcript showed a half-life that was identical to that of the wild-type (WT) transcript 2–14 h after transcription inhibition (Urlaub *et al*, 1989). Furthermore, most studies comparing the abundance of mutant and WT β-globin mRNAs found no apparent difference in degradation rates from 3 to 50 h after transcription inhibition (Humphries *et al*, 1984; Takeshita *et al*, 1984; Baserga & Benz, 1992), also suggesting that preferential degradation of the PTC-containing form is an early event. One explanation for these findings might be that the EJC is stripped off transcripts during the first round of translation (Dostie & Dreyfuss, 2002).

In mammalian cells, translation initiation on mature, capped messages involves recruitment of the 43S ribosome by initiation factors. A critical protein for cap-mediated translation is the cytoplasmic cap-binding protein, eIF4E, which binds to the 7-methylguanosine cap of the transcript as a subunit of the eIF4F initiation complex (Grifo *et al*, 1983). In addition to eIF4E, mammalian cells contain a protein heterodimer that binds to the 7-methylguanosine cap structure. This heterodimer, known as the nuclear cap-binding complex (CBC), is located primarily in the nucleus, where it stimulates splicing of the first intron from pre-mRNAs and facilitates export of mature mRNAs from the nucleus
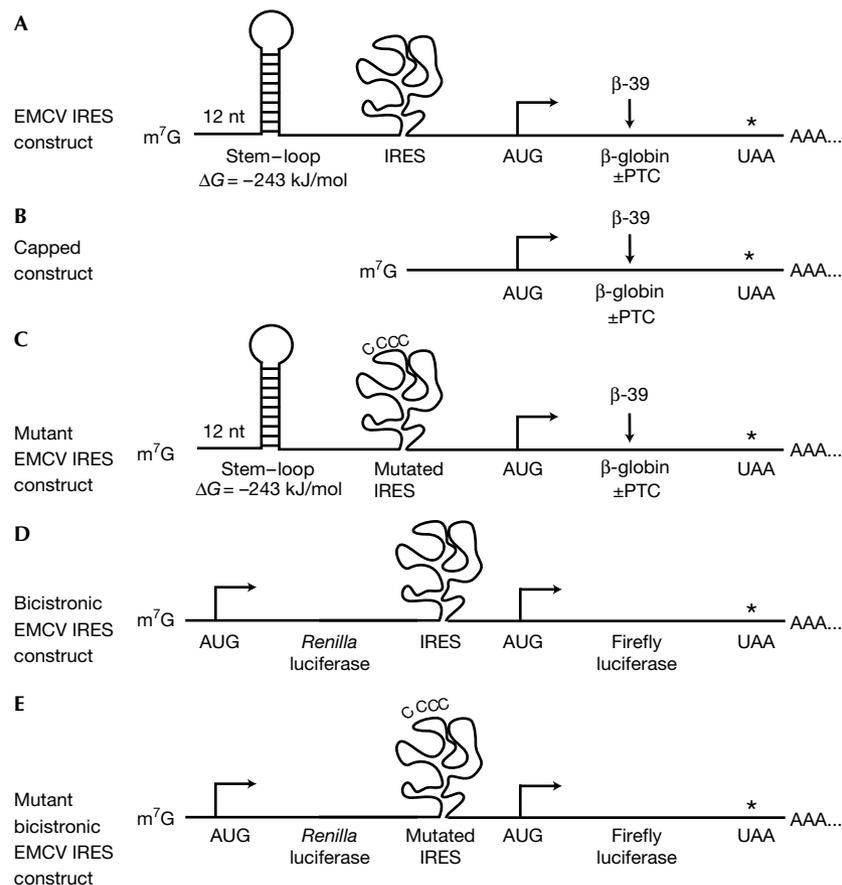
Fig 1 | Constructs used in this study. Construct (**A**) contains a stem–loop structure to prevent ribosomal read-through, the EMCV IRES to provide internal translation initiation and the β-globin gene as a reporter for NMD. Construct (**B**) was used as a control for cap-dependent translation, and contains the β-globin gene as an NMD reporter. Construct (**C**) contains the same features as construct (**A**), except that the IRES is mutated. Bicistronic construct (**D**) contains a *Renilla* luciferase reporter for cap-dependent translation and a firefly luciferase reporter for IRES-dependent translation. Bicistronic construct (**E**) contains the same features as construct (**D**), except that the IRES is mutated. EMCV, encephalomyocarditis virus; IRES, internal ribosome entry sequence; NMD, nonsense-mediated decay.

(Izaurralde *et al*, 1995). Yeast CBC is dispensable for both translation (Baron-Benhamou *et al*, 2003) and NMD (Kuperwasser *et al*, 2004; Gao *et al*, 2005), and shows only weak translation initiation factor activity (Fortes *et al*, 2000); translation initiation factor activity has not yet been shown for the CBC in other eukaryotes. Nevertheless, NMD in mammalian cells has been proposed to take place exclusively during a first ('pioneer') round of translation that occurs while transcripts are still bound by the nuclear CBC rather than by eIF4E (Ishigaki *et al*, 2001; Lejeune *et al*, 2002). Moreover, it has been suggested that the 80 kDa subunit of CBC promotes the interaction of NMD factors with PTC-containing mRNAs (Hosoda *et al*, 2005).

Alternatively, NMD could be triggered during translation, irrespective of CBC-mediated ribosomal recruitment. To test this alternative model, we used the internal ribosome entry sequence (IRES) of the encephalomyocarditis virus (EMCV). This IRES is capable of recruiting ribosomes directly. It drives translation efficiently independent of a 7-methylguanosine cap structure (Jang *et al*, 1988) and under conditions in which cytoplasmic cap-dependent translation is inhibited (Jang *et al*, 1989). We

demonstrate that cap-independent translation by the EMCV IRES causes NMD of a PTC-containing transcript, even when ribosomal read-through from the cap is prohibited. These data support a model in which translating ribosomes recruited independently of either of the cap-binding proteins are sufficient to meet the translation requirement for transcript degradation by NMD.

RESULTS AND DISCUSSION
Translation from the EMCV IRES mediates efficient NMD
To investigate whether translation initiated from the EMCV IRES element can mediate NMD, the EMCV IRES was cloned upstream of either the WT β-globin gene or an NMD-sensitive β-globin construct containing a nonsense mutation (NS) at codon 39 (Fig 1A). To hinder potential read-through of ribosomes recruited by cap-binding proteins and to ensure that only the IRES would be used for translation initiation, a stable stem–loop structure was inserted 12 nt downstream of the cap (Fig 1A). Insertion of such a stem–loop decreases ribosomal read-through by >96% (De Gregorio *et al*, 1999). After transient transfection into HeLa cells, the mRNA levels of the IRES-containing constructs were
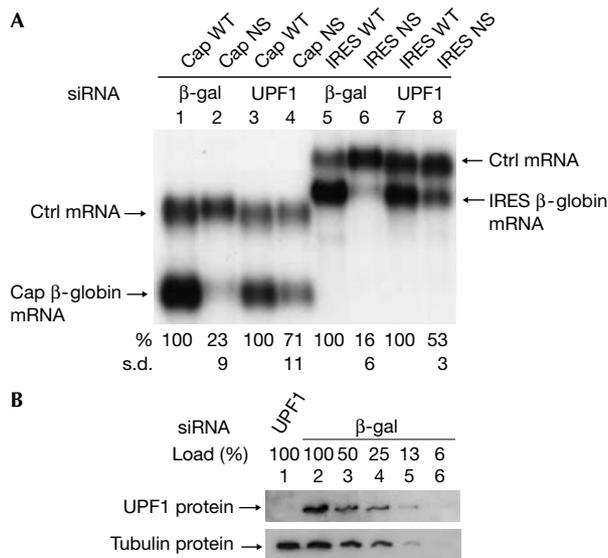
Fig 2 | NMD is supported by cap-dependent and IRES-dependent translation. (**A**) Representative northern blot of cap-dependent β-globin WT and NS constructs (lanes 1–4) and IRES WT and NS constructs (lanes 4–8). NS-containing constructs in both cases were present at 10–30% of the level of the WT transcript (lanes 2 and 6) in control cells treated with siRNA against β-gal. siRNA directed against central NMD factor UPF1 increased levels of NS messenger RNA three- to fourfold (lanes 4 and 8). The control mRNAs are extended β-globin forms (see Methods). The percentages shown are mean values and standard deviations (s.d.) derived from the results of at least three independent experiments. (**B**) Immunoblotting demonstrates the efficacy of RNA interference against UPF1. The level of UPF1 protein in cells treated with siRNA against UPF1 (lane 1) is less than 13% of that in control cells treated with siRNA against β-gal, as shown by the dilution series in lanes 2–6. β-gal, β-galactosidase; IRES, internal ribosome entry sequence; NMD, nonsense-mediated decay; NS, nonsense mutation; siRNA, short interfering RNA; WT, wild type.

Fig 3 | IRES-dependent translation is specifically abolished by an IRES point mutation. (**A**) Immunoblotting for the β-globin protein. The upper band corresponds to the extended β-globin product from the WT + 300 + e3 transfection control and the lower band corresponds to β-globin protein produced from the cap WT construct (lane 1), the mut IRES WT construct (lane 2), the IRES WT construct (lane 3) or empty vector (lane 4). (**B**) Luminometer measurements of firefly luciferase produced from the bicistronic luciferase constructs shown in Fig 1D,E, after normalization to *Renilla* luciferase activity. Ctrl, control; IRES, internal ribosome entry sequence; mut, mutant; WT, wild type.

compared with positive control constructs in which translation was initiated from the cap (that is, transcripts lacking stem–loop and IRES; Fig 1B). Quantitative assessment of NS mRNA undergoing translation initiated from the cap, compared with NS mRNA undergoing IRES-driven translation, indicates that both constructs are expressed at about 20% of the level of the corresponding WT mRNA, and hence undergo NMD at similar efficiency (Fig 2A, compare lanes 1 and 2 with lanes 5 and 6). To demonstrate that NMD is responsible for decreased amounts of the NS transcripts, short interfering RNA (siRNA)-mediated knockdown of the central NMD factor up-frameshift protein 1 (UPF1) was performed (Fig 2B), resulting in a three- to fourfold specific increase in the levels of the NS mRNAs, both for cap- and for IRES-mediated translation initiation (Fig 2A, compare lanes 3 and 4 with lanes 7 and 8).

As translation is required for mammalian NMD, the translation ability of the EMCV IRES in HeLa cells was confirmed by western blotting for the β-globin protein produced from the IRES-containing construct (Fig 3A) and by assaying the activity of firefly luciferase produced from the EMCV IRES in a bicistronic construct
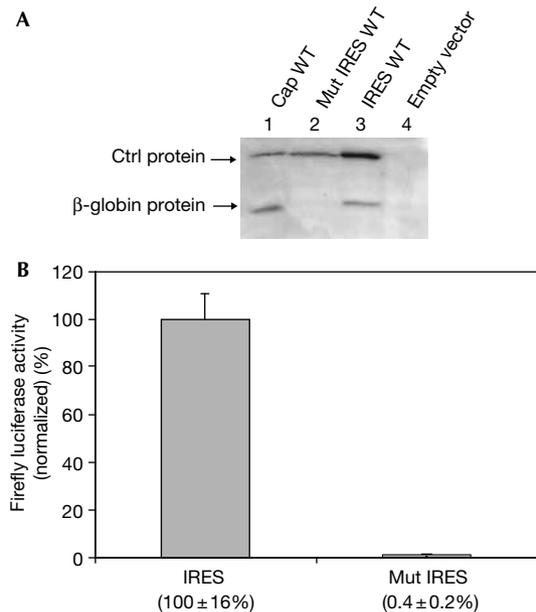
(Figs 1D,3B). To ensure further that translation of the IRES constructs truly initiated from the IRES and did not result from ribosomes reading through from the cap structure, a point mutation was introduced into the EMCV IRES, changing a crucial GNRA tetraloop (Robertson *et al*, 1999) to an inactive sequence of four cytosines (Fig 1C,E). As expected, given that both cap-dependent and IRES-driven translation should be essentially non-existent, this mutation abolished β-globin expression, as assessed by western blotting (Fig 3A), and also virtually eliminated firefly luciferase activity in the bicistronic construct, reducing it to less than 1% of the level observed in the unmutated EMCV IRES (Fig 3B). Importantly, mutation of the IRES resulted in increased abundance of NS β-globin mRNA to levels similar to WT β-globin (Fig 4, compare lanes 7 and 8). Further, siRNA directed against UPF1 resulted in no increase in NS transcript levels under these conditions (Fig 4, compare lanes 5 and 6). These data demonstrate that a functional IRES is required for the downmodulation of NS transcripts under conditions in which translation initiation from the cap is blocked. Furthermore, IRES-mediated translation initiation is sufficient to result in NMD of an NS construct.

In summary, these experiments show that translation initiation from the cap is not essential for NMD. Although we expect our reporter mRNAs to be capped and presumably to be bound first by the nuclear CBC during transcript maturation and then subsequently by eIF4E, these experiments provide strong evidence
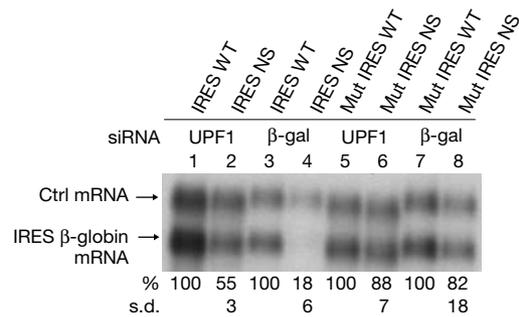
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Fig 4 | The mut IRES construct does not support NMD. A representative northern blot of IRES WT and NS constructs (lanes 1–4) and mut IRES WT and NS constructs (lanes 4–8) is shown. For the intact IRES, in control cells treated with β-gal siRNA, levels of the NS construct were 10–25% of levels of the WT construct (lanes 3 and 4). The NS construct increased three- to fourfold in cells treated with UPF1 siRNA (compare lanes 2 and 4). In control cells treated with β-gal siRNA, in contrast, the mutant IRES NS construct was present at 65–100% of the level of the mutant IRES WT construct (lanes 7 and 8), and in cells treated with UPF1 siRNA, the abundance of the mutant IRES NS construct failed to increase (compare lanes 6 and 8). The percentages shown are mean values and standard deviations (s.d.) derived from the results of at least three independent experiments. β-gal, β-galactosidase; IRES, internal ribosome entry sequence; mut, mutant; NMD, nonsense-mediated decay; NS, nonsense mutation; siRNA, short interfering RNA; WT, wild type.

that ribosomal recruitment by these proteins is dispensable for NMD. These results confirm and extend a previous report by Wang *et al* (2002) in which it was shown that translation driven from a poliovirus IRES allowed some reduction in the levels of a PTC-containing transcript. However, the extent of degradation was only one-third of that seen with translation initiated from the cap. As the study by Wang *et al* (2002) was not designed to compare directly IRES with cap-dependent translation in NMD, rigorous controls for ribosomal read-through and translation of the IRES-containing transcript were not included. Consequently, firm conclusions about requirements for a particular mode of translation initiation for NMD could not be drawn.

Our data suggest that recruitment of the ribosome by cap-binding proteins is not required for NMD. However, this conclusion does not exclude the possibility that cap-binding proteins might have a role in NMD that is unrelated to the mode of translation initiation. Our experiments are not designed to investigate whether the nuclear CBC is the cap-binding protein present on most transcripts as they undergo NMD, as has been reported on the basis of co-immunoprecipitation data (Ishigaki *et al*, 2001; Lejeune *et al*, 2002). However, our experiments directly address the requirement of translation mediated by the cap structure through its binding proteins. They support a model of NMD in which translation itself—regardless of how it is initiated—determines whether NMD occurs. As EMCV IRES- and cap-mediated translation initiation share the requirement for eIF4G sequences, it would be interesting to test NMD substrate mRNAs that initiate through the hepatitis C virus or cricket paralysis virus IRESs. These IRESs do not bind to eIF4G and, in the latter case, do not require any initiation factor for translation. However, these IRESs are less active in transfected

HeLa cells, which limits their use for studies of strictly translation-dependent pathways such as NMD.

METHODS

Constructs. The sequences of all oligonucleotides used for cloning and mutagenesis are available on request from the authors. EMCV IRES WT and EMCV IRES NS were created by inserting DNA coding for a 42 nt stem–loop (De Gregorio *et al*, 1999) into an *Apa*I site created by site-directed mutagenesis (GeneTailor Site-Directed Mutagenesis System; Invitrogen, Carlsbad, CA, USA) at base pair 754 of the plasmids pCI-neo β-globin WT and pCI-neo β-globin NS 39, described previously (Neu-Yilik *et al*, 2001). The amplified 611 base pair EMCV IRES from plasmid pT3Luc was inserted into the *Xho*I site of the resultant plasmids, described previously (Bergamini *et al*, 2000). For a transfection control, the EMCV IRES was inserted into the *Xho*I site of pCI-neo WT + 300 + e3, described previously (Gehring *et al*, 2003), to create IRES WT + 300 + e3. The dual luciferase plasmid pCRREL was created from the dual luciferase plasmid pREL (Huez *et al*, 1998). The amplified EMCV IRES from pT3luc was inserted between the *Bam*HI cleavage site at the end of the *Renilla* luciferase open reading frame and a *Sac*I site created by site-directed mutagenesis upstream of the firefly luciferase open reading frame. The corresponding group of mutant IRES plasmids was created by using site-directed mutagenesis to change the GNRA tetraloop of the EMCV IRES to four cytosines in all of the above constructs. DNA sequencing was used to confirm all modifications. To assay cap-dependent translation, constructs pBluescript β-globin WT and β-globin NS 39, described previously (Enssle *et al*, 1993), were used. The transfection control was pCI-neo WT + 300 + e3 (Gehring *et al*, 2003).

RNA interference, cell culture and DNA transfections. HeLa cells were grown in Dulbecco's modified Eagle's medium under standard conditions. One million cells were initially transfected with 1 nmol siRNA directed against either UPF1 (Gehring *et al*, 2003) or β-galactosidase (Hayes & Bruggink, 2001), using Oligofectamine (Invitrogen, Carlsbad, CA, USA) as the transfection reagent and following the manufacturer's instructions. After 24 h, 2×10^5 cells were split into six-well plates. DNA transfection by the calcium phosphate method followed 24 h later. In general, for constructs undergoing cap-dependent translation, 0.8 μg of transfection control WT + 300 + e3, 4.2 μg of reporter pBluescript β-globin WT or NS 39 and 0.2 μg of a green fluorescent protein (GFP) expression vector were transfected. For EMCV IRES constructs, 3.3 μg of transfection control IRES WT + 300 + e3, 1.7 μg of reporter pCI-neo β-globin WT or NS 39 and 0.2 μg of a GFP expression vector were transfected. The appropriate siRNA was also included at 0.1 nmol/well. Cells were incubated in 3% CO_2 for 20 h, then in 5% CO_2 for another 24 h before collection.

RNA analysis. Total cytoplasmic RNA was isolated and northern blotting was performed by methods described previously (Enssle *et al*, 1993). Northern blotting was performed according to standard protocols, using 1.5–5 μg of total cytoplasmic RNA.

Signal quantification. Radioactive signals were quantified in an FLA-3000 fluorescent image analyzer (Raytest, Fujifilm). Mean values and standard deviations of all experiments shown were calculated from at least three independent experiments.

Protein analysis. Immunoblot analysis of UPF1 was carried out using 10–30 µg protein from whole-cell extracts loaded on an 8% polyacrylamide gel electrophoresis gel, and for β-globin, 100–375 µg protein was loaded on a 15% polyacrylamide gel electrophoresis gel. Proteins were then transferred onto a polyvinylidene difluoride membrane using a semi-dry electroblotting system (1 mA/cm^2, 70 min). Membranes were blocked with 5% non-fat milk powder in TBS–Tween (0.1%). Primary antibody against UPF1 was used at 1:2,000 dilution and horseradish peroxidase-conjugated rabbit secondary antibody was used at 1:10,000 dilution. Immunoblotting against β-globin was performed as described (Thermann *et al*, 1998). Signals were detected using the ECL or ECL-plus reagents (GE Healthcare–Amersham, Piscataway, NJ, USA).

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