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NMD resulting from encephalomyocarditis virus IRES-directed translation initiation seems to be restricted to CBP80/20-bound mRNA

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Nonsense-mediated messenger RNA decay (NMD) generally degrades mRNAs that prematurely terminate translation as a means of quality control. NMD in mammalian cells targets newly spliced mRNA that is bound by the cap-binding protein heterodimer CBP80/20 and one or more post-splicing exon junction complexes during a pioneer round of translation. NMD targets mRNA that initiates translation using the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES), therefore NMD might target not only CBP80/20-bound mRNA but also its remodelled product, eIF4E-bound mRNA. Here, we provide evidence that NMD triggered by translation initiation at the EMCV IRES, similar to NMD triggered by translation initiation at an mRNA cap, targets CBP80/20-bound mRNA but does not detectably target eIF4E-bound mRNA. We show that EMCV IRES-initiated translation undergoes a CBP80/20-associated pioneer round of translation that results in CBP80/20-dependent and Upf factor-dependent NMD when translation terminates prematurely.

Keywords: nonsense-mediated mRNA decay; EMCV IRES; pioneer round of translation; CBP80/20; eIF4E; Upf proteins
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INTRODUCTION

Nonsense-mediated messenger RNA decay (NMD) in mammals is a translation-dependent surveillance mechanism that controls the quality of gene expression by degrading the mRNAs of mutated genes that contain premature termination codons (PTCs; reviewed by Behm-Ansmant *et al*, 2007; Chang *et al*, 2007; Isken & Maquat, 2007). NMD also downregulates the expression of natural genes, in some cases providing an autoregulatory circuit to inhibit the production of RNA-binding proteins, including splicing factors (Mendell *et al*, 2004; Wittmann *et al*, 2006; Lareau *et al*, 2007; Ni *et al*, 2007). However, data indicate that many, if not most, naturally occurring NMD targets result from nonproductive alternative splicing (Pan *et al*, 2006).

mRNA that is bound by the cap-binding protein heterodimer CBP80/20, which is mostly nuclear but also shuttles to the cytoplasm, is a precursor to mRNA that is bound at the cap by eIF4E, which is largely cytoplasmic (Lejeune *et al*, 2002). The NMD of nonsense-containing mRNAs generally occurs when translation initiates in an mRNA cap-dependent manner during a pioneer round of translation, which involves newly synthesized CBP80/20-bound mRNA (Ishigaki *et al*, 2001; Sato *et al*, 2008). NMD does not detectably target mRNA once CBP80/20 has been replaced by eIF4E, which supports the bulk of cellular protein synthesis (Ishigaki *et al*, 2001; Chiu *et al*, 2004; Hosoda *et al*, 2005). This is in contrast to the situation in *Saccharomyces cerevisiae*, in which NMD targets not only Cbc1/2-bound mRNA, which is orthologous to mammalian cell CBP80/20-bound mRNA, but also eIF4E-bound mRNA (Gao *et al*, 2005). The apparent restriction of NMD to CBP80/20-bound mRNA in mammals is due to the roles of CBP80 and post-splicing exon junction complexes (EJCs) in NMD, both of which are detectable on CBP80/20-bound mRNA but not on eIF4E-bound mRNA (Ishigaki *et al*, 2001; Lejeune *et al*, 2002; Hosoda *et al*, 2005; Kashima *et al*, 2006). EJCs are thought to be largely removed from newly synthesized mRNAs by translating ribosomes during the pioneer round of translation (Dostie & Dreyfuss, 2002).

The importance of EJCs to NMD is supported by reports that downregulating the EJC constituents Y14, MNL51/BTZ, eIF4AIII, Upf2 or Upf3X (also called Upf3b) inhibits NMD (reviewed by Isken & Maquat, 2007). Furthermore, mRNAs that are derived from intronless genes and, thus, lack EJCs are immune to NMD when they harbour PTCs (Maquat & Li, 2001; Neu-Yilik *et al*, 2001; Brocke *et al*, 2002; Matsuda *et al*, 2007). In classical NMD, EJCs are thought to function by recruiting the NMD factor Upf1 through the EJC constituent Upf2 once translation terminates sufficiently upstream from the EJC (Lykke-Andersen *et al*, 2001; Serin *et al*, 2001; Kim *et al*, 2005; Kashima *et al*, 2006). The importance of CBP80 to NMD is evident from the finding that CBP80 promotes the interaction of Upf2 with Upf1, the latter being stably associated with the CBP80/20 cap-binding complex

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(CBC; Hosoda *et al*, 2005). Consistent with this interpretation, tethering Upf1 sufficiently downstream from a nonsense codon bypasses the need for CBP80 so that mRNA decay is extended to eIF4E-bound mRNA (Hosoda *et al*, 2005). As there is evidence that Upf1 is the last of the Upf proteins to join the EJC (Lykke-Andersen *et al*, 2001; Hosoda *et al*, 2005; Kim *et al*, 2005; Singh *et al*, 2007), it follows that CBP80 is no longer required to promote NMD once Upf1 joins an EJC (Hosoda *et al*, 2005; Kim *et al*, 2005).

EJCs, and possibly CBP80/20, also function in fail-safe NMD, which does not seem to depend on a post-splicing EJC situated downstream from a nonsense codon (Zhang *et al*, 1998; Bühler *et al*, 2006; Matsuda *et al*, 2007). However, fail-safe NMD requires at least one EJC upstream from the nonsense codon and, similar to classical NMD, targets CBP80/20-bound mRNA but does not detectably target eIF4E-bound mRNA (Matsuda *et al*, 2007).

On the basis of the finding that translation initiation from the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) supports NMD, it was recently suggested that translation initiation from the mRNA cap is not essential for NMD (Holbrook *et al*, 2006). It follows that the recruitment of ribosomes to an mRNA by CBP80/20 and, possibly, the presence of CBP80/20 on mRNA would be dispensable for NMD. We set out to test this possibility by considering that, although never examined, CBP80/20 would probably constitute newly synthesized messenger ribonucleoprotein when translation initiates for the first time using the EMCV IRES. Thus, for the reasons mentioned above, NMD resulting from EMCV IRES-dependent translation initiation might also be restricted to CBP80/20-bound mRNA.

Here, we show that CBP80/20 is a constituent of EMCV IRES-containing messenger ribonucleoprotein. Furthermore, we show that NMD resulting from EMCV IRES-dependent translation initiation involves Upf1, Upf2, Upf3X and CBP80/20. We also used two complementary approaches to show that NMD resulting from EMCV IRES-initiated translation targets CBP80/20-bound mRNA but does not detectably target eIF4E-bound mRNA.

RESULTS AND DISCUSSION

EMCV IRES: CBP80/20 and Upf1 factors function in NMD

As noted above, classical NMD generally depends on CBP80/20 and the NMD factors Upf1, Upf2 and Upf3X. To determine whether CBP80/20 and Upf proteins function in NMD that results from translation initiation at the EMCV IRES, HeLa cells were transiently transfected with a short interfering RNA (siRNA) that downregulates CBP80/20, Upf1, Upf2 or Upf3X or, to control for nonspecific effects, with a control siRNA (Hosoda *et al*, 2005; Kim *et al*, 2005). After 2 days, cells were transfected with a pRLuc-Gl or php-EMCV IRES-RLuc-Gl test plasmid, either PTC-free (Norm) or PTC-containing at Gl codon 39 (Ter), and the pMUP reference plasmid (phCMV-MUP in Belgrader & Maquat, 1994). Each set of test plasmids (Fig 1A) encodes *Renilla* (R) luciferase (Luc) fused to β-globin (Gl). pRLuc-Gl encodes RLuc-Gl mRNA that initiates cap-dependent translation. php-EMCV IRES-RLuc-Gl encodes RLuc-Gl mRNA that initiates EMCV IRES-dependent translation, as it contains a stable hairpin (hp) structure 12 nucleotides downstream from the cap that inhibits cap-initiated ribosome scanning (De Gregorio *et al*, 1999). Consistent with the hairpin structure blocking ribosome scanning, php-EMCV IRES(CCCC)-RLuc-Gl Norm, in which the GCGA tetraloop was mutated to CCCC so as to inactivate the IRES (Robertson *et al*, 1999; Holbrook *et al*, 2006), did not produce an appreciable level of RLuc activity (supplementary Fig 1 online). By contrast, php-EMCV IRES-RLuc-Gl Norm was active, producing approximately 21-fold greater RLuc activity than php-EMCV IRES(CCCC)-RLuc-Gl (supplementary Fig 1 online).

By using the level of p62 or calnexin to control for variations in protein loading, it was shown by western blotting that the level of Upf1, Upf2, Upf3X or the combination of CBP80 and CBP20 (CBC) was downregulated to 5%, 10%, 12% or 5% and 12% the level in the presence of control siRNA, respectively (Fig 1B–E, upper panels). siRNA-mediated effects on processes other than NMD were controlled for by comparing PTC-containing mRNA with PTC-free mRNA. CBC siRNA seems to retard the nucleocytoplasmic export of spliced mRNA (C.F.W. and L.E.M., unpublished data), and the degree to which it inhibits NMD because of the direct effect of CBC on NMD or on mRNA export is uncertain. Semiquantitative reverse transcription–PCR (RT–PCR) showed that NMD reduced the level of RLuc-Gl Ter and hp-EMCV IRES-RLuc-Gl Ter mRNAs in the presence of control siRNA to 6–7% and 13–14% the level of the corresponding Norm mRNA, respectively (Fig 1B–E, lower panels). Furthermore, downregulating each protein inhibited the NMD of RLuc-Gl Ter and hp-EMCV IRES-RLuc-Gl Ter mRNAs about two- to sixfold, depending on the particular siRNA (Fig 1B–E, lower panels). This variation probably reflects (i) differences in siRNA efficiencies and differences in the cellular fraction of each targeted protein that functions in NMD, and (ii) less efficient translation initiation from the IRES compared with that from the 5′ cap, which results in less efficient PTC recognition and, as a consequence, NMD. Notably, the reliability of using semiquantitative RT–PCR was validated by using real-time PCR for samples involving Upf1 siRNA (supplementary Fig 2 online). We conclude that Upf1, Upf2, Upf3X and CBP80/20 function in NMD that results from translation initiation at either an mRNA cap or the EMCV IRES.

EMCV IRES: 4E-BP1 does not detectably inhibit NMD

To determine whether NMD that results from EMCV IRES-initiated translation targets CBP80/20-bound mRNA or eIF4E-bound mRNA or both, cells were transfected with the pACTAG2-HA_3-4E-BP1 effector plasmid, which produces 4E-BP1 (Gingras *et al*, 1998) or, as a control, pACTAG2-HA_3. 4E-BP1 inhibits the translation of eIF4E-bound mRNA but not CBP80/20-bound mRNA (Chiu *et al*, 2004). After 1 day, cells were transfected with (i) pRLuc-Gl or php-EMCV-IRES-RLuc-Gl, either Norm or Ter, and (ii) pMUP.

HA_3-4E-BP1 expression significantly reduced the abundance of the secreted major urinary protein (MUP; Fig 2A) and the activity of RLuc that derived from cap-dependent pRLuc-Gl expression but not cap-independent php-EMCV-IRES-RLuc-Gl expression (Fig 2B). This demonstrates that HA_3-4E-BP1 inhibited the cap-dependent translation of eIF4E-bound mRNA, which supports the bulk of cellular protein synthesis. As expected, RT–PCR showed that HA_3-4E-BP1 was of no importance to the NMD of RLuc-Gl Ter mRNA that involves the cap-dependent translation of CBP80/20-bound mRNA (Fig 2C), consistent with previous results (Chiu *et al*, 2004; Matsuda *et al*, 2007). HA_3-4E-BP1 was also of no consequence to the NMD of hp-EMCV IRES-RLuc-Gl Ter mRNA, which initiates translation at the EMCV IRES (Fig 2C). These data indicate that translation initiation from the EMCV IRES,

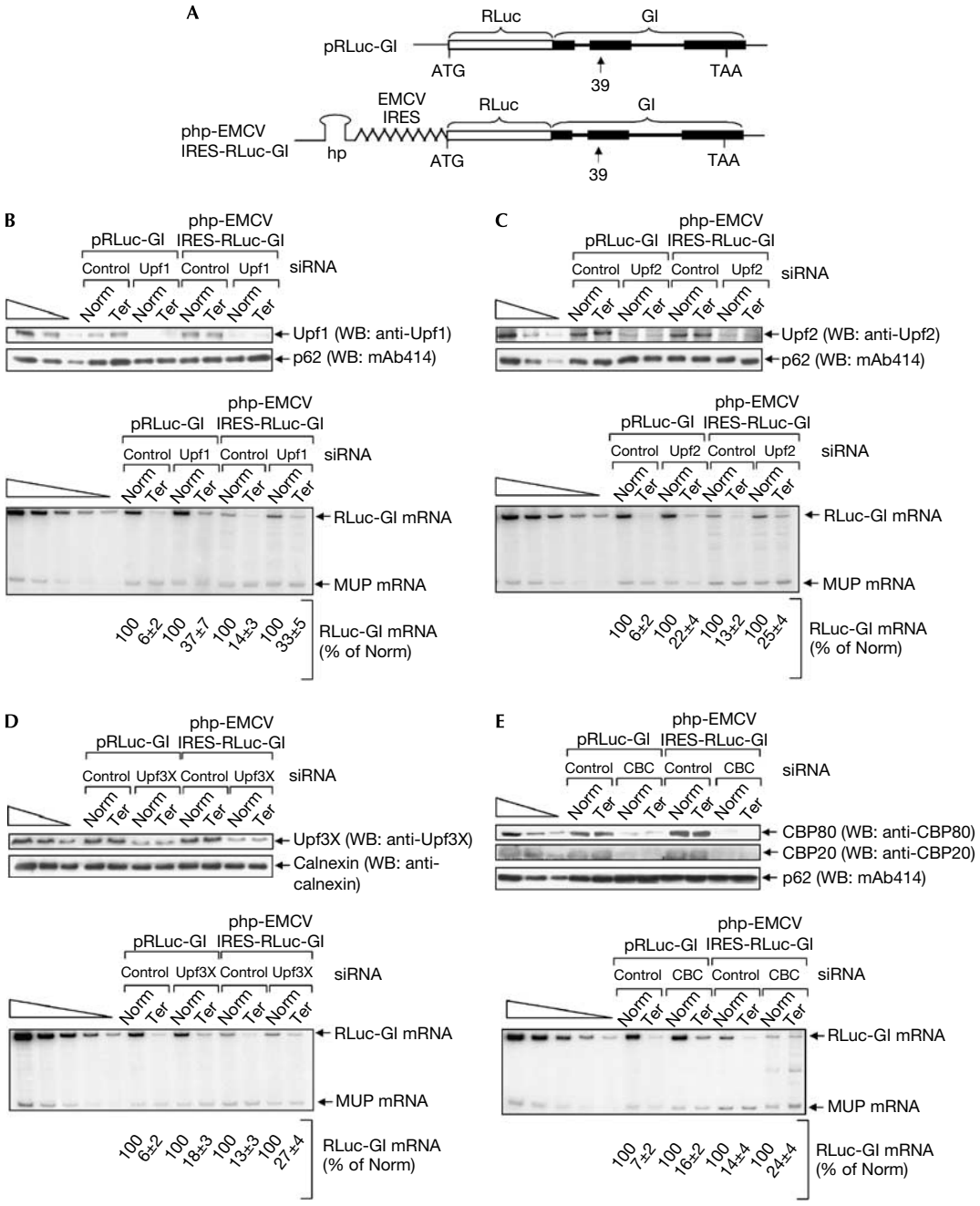

Fig 1 | Downregulating Upf1, Upf2, Upf3X or CBP80/20 inhibits nonsense-mediated messenger RNA decay that results from encephalomyocarditis virus internal ribosome entry site-initiated translation. (**A**) Diagrams of plasmid DNAs. Boxes represent exons, intervening lines signify introns and hp refers to the hairpin structure that blocks translation initiation upstream from the EMCV IRES. ATG and TAA denote translation initiation and termination codons, respectively, and 39 indicates the position of the premature termination codon when present. (**B**) (upper panel) Western blot analysis (WB) using the specified antibody (anti-) after treatment with Upf1 or control siRNA. The level of p62 was used to control for variations in protein loading. The three leftmost lanes show twofold dilutions of protein, indicating that the analysis was semiquantitative. (lower panel) Reverse transcription–PCR after treatment with Upf1 siRNA or control siRNA. The level of each RLuc-Gl test mRNA was normalized to the level of MUP mRNA. Normalized values were then calculated as a percentage of the normalized value of either RLuc-Gl Norm mRNA or hp-EMCV IRES-RLuc Norm mRNA, each of which was defined as 100%. Values are derived from three independently performed experiments. The five leftmost lanes show twofold dilutions of RNA. (**C**) As in (**B**), except that Upf2 siRNA was used instead of Upf1 siRNA. (**D**) As in (**B**), except that Upf3X siRNA was used and the level of p62 was used to control for variations in protein loading as p62 migrates with Upf3X. (**E**) As in (**B**), except that CBP80 and CBP20 (CBC) siRNAs were used. CBP20 runs as a smeared band. EMCV, encephalomyocarditis virus; Gl, β-globin; IRES, internal ribosome entry site; Luc, luciferase; MUP, major urinary protein; Norm, PTC-free; R, *Renilla*; siRNA, short interfering RNA; Ter, PTC-containing.

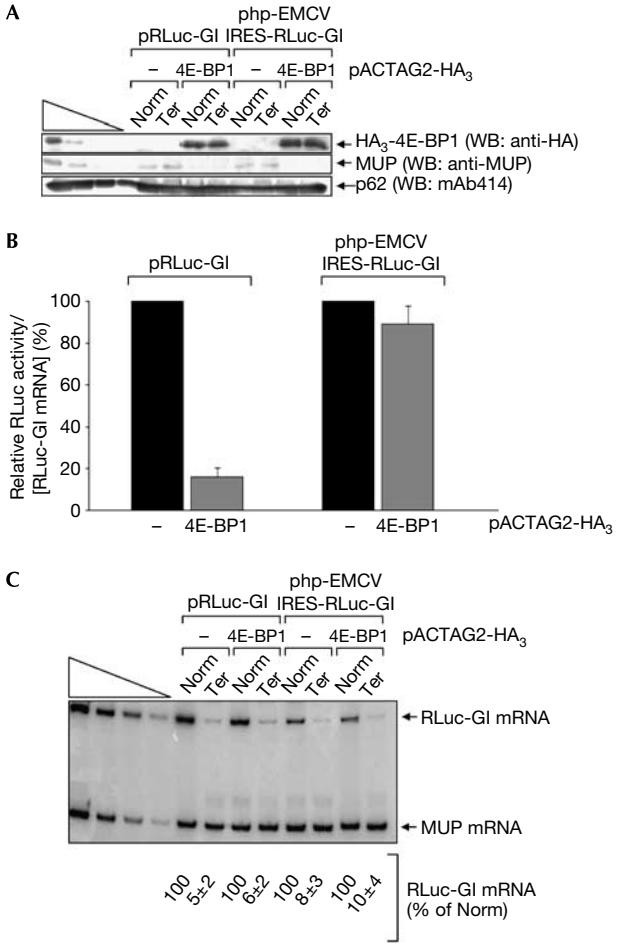

Fig 2 | 4E-BP1 expression fails to inhibit nonsense-mediated messenger RNA decay that results from encephalomyocarditis virus internal ribosome entry site-initiated translation. (**A**) Western blot analysis (WB) shows that 4E-BP1 expression inhibited the bulk of cellular translation, as measured by the reduced level of the secreted MUP. The level of p62 was used to control for variations in protein loading. The four leftmost lanes show twofold dilutions of protein. (**B**) RLuc activity assays also indicated that 4E-BP1 expression inhibited the bulk of cellular translation. RLuc activity was normalized to the level of the corresponding RLuc mRNA. (**C**) Reverse transcription–PCR as in Fig 1B (lower panel). Values are derived from three independently performed experiments. EMCV, encephalomyocarditis virus; Gl, β-globin; IRES, internal ribosome entry site; Luc, luciferase; MUP, major urinary protein; Norm, PTC-free; R, *Renilla*; Ter, PTC-containing.

similar to translation initiation from the mRNA cap, supports the NMD of newly synthesized CBP80/20-bound mRNA but not detectably its remodelled product, eIF4E-bound mRNA.

EMCV IRES: no detectable NMD of eIF4E-bound mRNA

In a second approach to determine whether NMD that is supported by EMCV IRES-initiated translation targets CBP80/20-bound mRNA or eIF4E-bound mRNA or both, the extent to which a PTC reduces the level of CBP80/20-bound hp-EMCV IRES-Gl-RLuc mRNA compared with eIF4E-bound hp-EMCV IRES-Gl-RLuc mRNA was assessed. CBP80/20-bound mRNA and eIF4E-bound mRNA are distinguishable by immunoprecipitation using anti-CBP80 and anti-eIF4E, respectively (Ishigaki *et al*, 2001; Lejeune *et al*, 2002; Chiu *et al*, 2004; Hosoda *et al*, 2005; Matsuda *et al*, 2007). If the NMD of hp-EMCV IRES-Gl-RLuc Ter mRNA is restricted to CBP80/20-bound mRNA, then a PTC should reduce the levels of CBP80/20-bound hp-EMCV IRES-RLuc-Gl Ter mRNA and eIF4E-bound hp-EMCV IRES-RLuc-Gl Ter mRNA to a comparable percentage of the corresponding Norm mRNA. If NMD targets both CBP80-bound and eIF4E-bound hp-EMCV IRES-RLuc-Gl Ter mRNA, then the level of eIF4E-bound mRNA should be reduced below the level of CBP80/20-bound mRNA when each is presented as a percentage of the corresponding Norm mRNA. Cells were transfected with pRLuc-Gl or php-EMCV IRES-RLuc-Gl test plasmid, either Norm or Ter, and pMUP. After 2 days, cells were collected, and protein and RNA were prepared before and after immunoprecipitation using anti-CBP80, anti-eIF4E or, to control for nonspecific immunoprecipitation, normal rabbit serum or mouse IgG, respectively.

Western blotting showed that CBP80 but not eIF4E was immunoprecipitated using anti-CBP80 but not normal rabbit serum (Fig 3A, upper panel), and eIF4E but not CBP80 was immunoprecipitated using anti-eIF4E but not mouse IgG (Fig 3B, upper panel). RT–PCR of samples after immunoprecipitation showed that the level of CBP80/20-bound RLuc-Gl Ter mRNA was 12 ± 4% the level of CBP80/20-bound RLuc-Gl Norm mRNA, and the level of eIF4E-bound RLuc-Gl Ter mRNA was 13 ± 4% the level of eIF4E-bound RLuc-Gl Norm mRNA, which were comparable with levels before immunoprecipitation (Fig 3A,B, lower panels). This is consistent with our previous finding that NMD resulting from cap-dependent translation initiation targets CBP80/20-bound mRNA. RT–PCR also showed that the level of CBP80/20-bound hp-EMCV IRES-RLuc-Gl Ter mRNA was 20 ± 4% the level of CBP80/20-bound hp-EMCV IRES-RLuc-Gl Norm mRNA, and the level of eIF4E-bound hp-EMCV IRES-RLuc-Gl Ter mRNA was 23 ± 5% the level of eIF4E-bound hp-EMCV IRES-RLuc-Gl Norm mRNA (Fig 3A,B, lower panels). These data, similar to the results obtained by blocking eIF4E-bound mRNA translation using 4E-BP1, indicate that NMD triggered by EMCV IRES-initiated translation is restricted to CBP80/20-bound mRNA and does not detectably target eIF4E-bound mRNA.

NMD also results when translation of a PTC-containing mRNA initiates using the type I poliovirus IRES (Wang *et al*, 2002). We suggest that NMD resulting from IRES-mediated translation initiation would generally fail to detectably target eIF4E-bound mRNA. An exception is the cricket paralysis virus IRES, for reasons unrelated to the nature of the cap-binding protein (Isken *et al*, 2008). Thus, although translation initiation from the mRNA cap is not essential for NMD, NMD seems to involve the presence of CBP80 and CBP20, the latter of which binds directly to both the 5′ cap and CBP80 (Calero *et al*, 2002; Mazza *et al*, 2002). As noted above, although eIF4E-bound mRNA can be engineered to be a target of NMD by, for example, tethering Upf1 downstream from a nonsense codon (Hosoda *et al*, 2005), the usual limitation of NMD to CBP80/20-bound mRNA might reflect (i) the ability of CBP80 to interact directly with Upf1 and promote the interaction of Upf1 with Upf2 (Hosoda *et al*, 2005) and (ii) the detectable presence of EJCs and, thus, Upf2 and Upf3 or Upf3X on CBP80-bound mRNA but not on eIF4E-bound mRNA (Lejeune *et al*, 2002; Kashima *et al*, 2006).

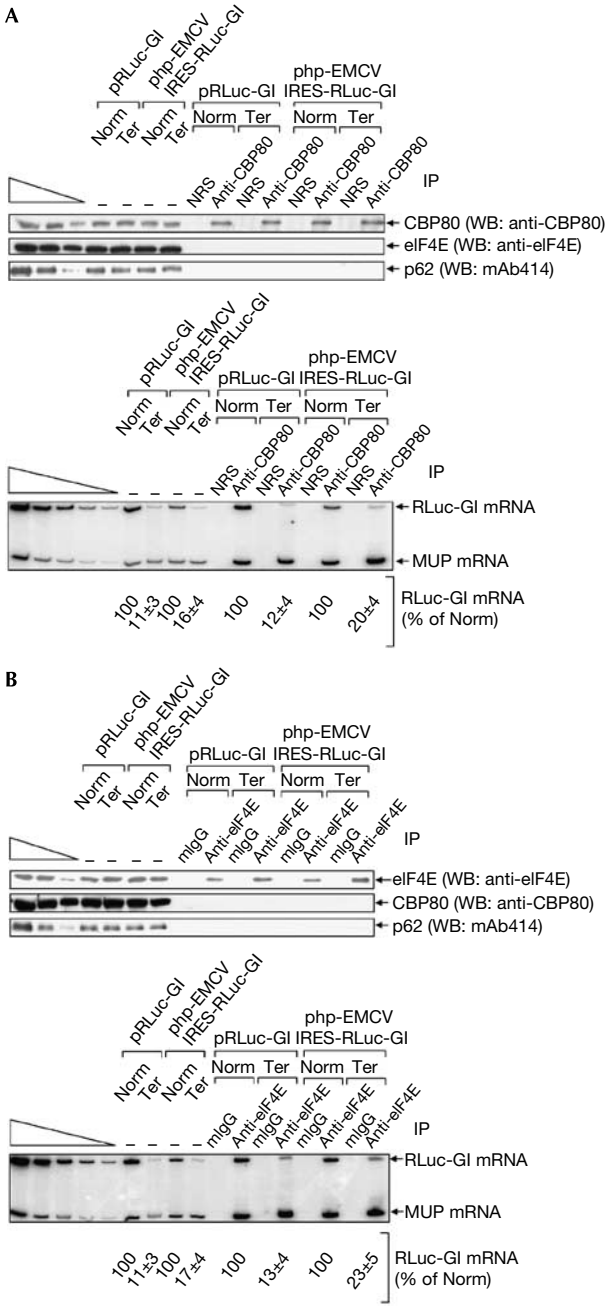

Fig 3 | Nonsense-mediated messenger RNA decay that results from encephalomyocarditis virus internal ribosome entry site-initiated translation reduces the abundance of CBP80-bound mRNA and its eIF4E-bound product to a comparable percentage of the corresponding normal messenger ribonucleoprotein. (**A**,**B**, upper panels) Western blot analysis (WB) indicated that the immunoprecipitation (IP) efficiency of CBP80 or eIF4E was 18 ± 4% or 14 ± 2%, respectively—1/100 of each sample was analysed before IP and 1/20 of each sample was analysed after IP. (**A**,**B**, lower panels) Reverse transcription–PCR of Gl and MUP mRNAs. Values are derived from two independently performed experiments. EMCV, encephalomyocarditis virus; Gl, β-globin; IRES, internal ribosome entry site; Luc, luciferase; MUP, major urinary protein; Norm, PTC-free; NRS, normal rabbit serum; R, *Renilla*; Ter, PTC-containing.

METHODS

Plasmid constructions. See the supplementary information online.

Cell transfections. HeLa CCL2 and Cos-7 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen). Cos cells (3–4 × 10^6 per 60-mm dish) were transiently transfected with 4.0 μg of pACTAG2-HA_3 or pACTAG2-HA_3-4E-BP1 together with 0.4 μg of a pRLuc-Gl test plasmid or 0.4 μg of a php-EMCV IRES-RLuc-Gl test plasmid and 0.1 μg of the pMUP reference plasmid. When immunoprecipitations were performed, HeLa cells (1 × 10^7 per 150-mm dish) were transfected with 10 μg of a pRLuc-Gl test plasmid or 10 μg of a php-EMCV IRES-RLuc-Gl-RLuc test plasmid and 2 μg of pMUP by using Lipofectamine 2000.

In experiments that used siRNA, HeLa cells (3–4 × 10^6 per 60-mm dish) were cultured as described above and transiently transfected with 100 nM of the specified *in vitro*-synthesized siRNA, 1.0 μg of a pRLuc-Gl test plasmid or 1.0 μg of a php-EMCV IRES-RLuc-Gl test plasmid and 0.3 μg of pMUP. Upf1, Upf2 and Upf3X siRNAs were as described previously (Kim *et al*, 2005). CBP80 and CBP20 siRNAs were, respectively, 5′-r(GCUGAUCUUCCUAACU ACA)d(TT)-3′ and 5′-r(GGGAACCUCUCUAAAUAAUUU)d(TT)-3′.

Immunoprecipitations. Immunoprecipitations were performed as described previously (Ishigaki *et al*, 2001).

RNA and protein isolation. Total-cell RNA and protein were isolated using Trizol reagent (Invitrogen) and passive lysis buffer (Promega, Madison, WI, USA), respectively (Ishigaki *et al*, 2001).

Western blot analyses, luciferase activity assays and RT–PCR. See the supplementary information online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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