Cell, Vol. 106, 607–617, September 7, 2001, Copyright ©2001 by Cell Press

Evidence for a Pioneer Round of mRNA Translation: mRNAs Subject to Nonsense-Mediated Decay in Mammalian Cells Are Bound by CBP80 and CBP20

Yasuhito Ishigaki,[2] Xiaojie Li, Guillaume Serin, and Lynne E. Maquat[1]
Department of Biochemistry and Biophysics
School of Medicine and Dentistry
University of Rochester
601 Elmwood Avenue, Box 712
Rochester, New York 14642

Summary

Nonsense-mediated decay (NMD) eliminates mRNAs that prematurely terminate translation. We used antibody to the nuclear cap binding protein CBP80 or its cytoplasmic counterpart eIF4E to immunopurify RNP containing nonsense-free or nonsense-containing transcripts. Data indicate that NMD takes place in association with CBP80. We defined other components of NMD-susceptible mRNP as CBP20, PABP2, eIF4G, and the NMD factors Upf2 and Upf3. Consistent with the dependence of NMD on translation, the NMD of CBP80-bound mRNA is blocked by cycloheximide or suppressor tRNA. These findings provide evidence that translation can take place in association with CBP80. They also indicate that CBP80-bound mRNA undergoes a "pioneer" round of translation, before CBP80-CBP20 are replaced by eIF4E, and Upf2 and Upf3 proteins dissociate from upstream of exon-exon junctions.

Introduction

Mammalian cells have evolved numerous ways to ensure the quality of mRNA and, by so doing, the accuracy of gene expression (reviewed in Maquat and Carmichael, 2001). One such way surveys all translated mRNAs in order to distinguish those that prematurely terminate translation from those that do not. If translation termination is sufficiently premature, then the mRNA will be subject to nonsense-mediated decay (NMD) (reviewed in Maquat, 1995, 2000; Li and Wilkinson, 1998; Hentze and Kulozik, 1999; Frischmeyer and Dietz, 1999). Generally, NMD is elicited if translation terminates more than 50–55 nucleotides upstream of the 3′-most exon-exon junction of an mRNA, and most probably requires the splicing-dependent deposition of proteins known to occur 20–24 nucleotides upstream of exon-exon junctions (Le Hir et al., 2000a, 2000b). According to one scenario, one or more of these proteins recruits Upf3 protein, a nucleocytoplasmic shuttling protein involved in NMD (Lykke-Andersen et al., 2000; Serin et al., 2001). Upf3 protein then interacts directly with Upf2 protein, which in turn interacts with Upf1 protein (Serin et al., 2001). Several observations support this scenario. (1) Tethering any of the three Upf proteins to the 3′-untranslated region of β-globin mRNA converts the normal termination codon to the type that elicits NMD (Lykke-Andersen et al., 2000). (2) Upf3 protein is predominantly localized to nuclei, while Upf1 and Upf2 proteins are detected only in the cytoplasm, suggesting that Upf3 protein associates with spliced mRNA before Upf1 and Upf2 proteins (Lykke-Andersen et al., 2000; Serin et al., 2001). (3) Upf3 protein can be detected as a component of spliced mRNP in HeLa cells (Lykke-Andersen et al., 2000; notably, Upf1 and Upf2 proteins were not assayed). (4) Upf3 protein can not be detected as a component of the splicing-dependent complex formed in HeLa cell nuclear extract (data not shown), suggesting that it may be recruited to mRNP after complex formation.

Most mammalian mRNAs are subject to NMD before they are released from an association with nuclei into the cytoplasm (see, e.g., Belgrader et al., 1993; Carter et al., 1996; Zhang et al., 1998a, 1998b; Thermann et al., 1998). However, some mRNAs are degraded after they are released into the cytoplasm (see, e.g., Sun et al., 2000; Rajavel and Neufeld, 2001). NMD depends on translation regardless of its cellular site, which is readily conceivable for cytoplasmic NMD but less so for nucleus-associated NMD. As one possibility, nucleus-associated NMD takes place during mRNA export at a point when mRNA copurifies with nuclei but is translated by cytoplasmic ribosomes. A precedent for this possibility is provided by the Balbiani ring granule of the insect *Chironomus tentans*. This granule, a 35–40 kb mRNP, is invariably exported from nuclei to the cytoplasm 5′-end first so that cytoplasmic ribosomes associate with the 5′ end before the 3′ end has transited the nuclear pore complex (Mehlin et al., 1992). Nevertheless, the possibility that nucleus-associated NMD reflects a translation-like mechanism in the nucleoplasm can not be ruled out.

Much can be learned about the mechanism of NMD at either cellular site by continuing to identify proteins associated with the process. One way to do so is to identify mRNP proteins. We were particularly interested in determining if there is a difference in the cap binding protein associated with mRNA that is a substrate for nucleus-associated NMD and mRNA that is a substrate for cytoplasmic NMD. CBP80 is a component of the nuclear cap binding complex that is added cotranscriptionally and functions in nuclear RNA processes such as pre-mRNA splicing (Izaurralde et al., 1994; Lewis and Izaurralde, 1997). Mammalian CBP80 is likely to be a nucleocytoplasmic shuttling protein based on studies of its ortholog in *C. tentans* (Visa et al., 1996) or *S. cerevisiae* (Shen et al., 2000). eIF4E is the 24 kDa cytoplasmic cap binding protein that replaces CBP80 at an undefined point after mRNA export to the cytoplasm and functions in translation initiation (reviewed in Gingras et al., 1999). The finding that a fraction of eIF4E localizes to the nucleus suggests that eIF4E may also manifest a nuclear function (Lejbkowicz et al., 1992; Dostie et al., 2000). Recent studies of *S. cerevisiae* suggest that CBP80, together with the translation initiation factor

[1]Correspondence: lynne_maquat@urmc.rochester.edu
[2]Present address: Division of Radiation Biology, Faculty of Pharmaceutical Science, Kanazawa University, Takana-machi 13-1, Kanazawa 920-0934 Japan.

eIF4G, mediates translation initiation. First, CBP80 was found to bind eIF4G in vitro (Fortes et al., 2000). Second, TAP-tagged CBP20, which forms the heteromeric nuclear cap binding complex with CBP80, was found to associate with eIF4G in extracts (Fortes et al., 2000). Furthermore, CBP80 added to extracts was found to stimulate translation 2.5-fold, provided that extracts were derived from strains harboring a mutated form of eIF4G that interacted only weakly with eIF4E and the poly(A) binding protein Pab1p (Fortes et al., 2000). Very recently, CBP80 was also shown to interact with eIF4G in mammalian cells (McKendrick et al., 2001). Considering that nucleus-associated NMD is restricted to newly synthesized mRNA, CBP80 could be involved. In contrast, eIF4E was the logical cap binding protein to be involved in cytoplasmic NMD.

Here, we use Western blotting and RT-PCR to analyze, respectively, the protein and RNA components of mammalian cell RNP immunopurified using antibody to either CBP80 or eIF4E. Remarkably, our results indicate that both nucleus-associated and cytoplasmic NMD take place in association with CBP80. Therefore, a first round of translation in mammalian cells can take place in association with CBP80. We identify other proteins associated with CBP80-bound mRNA as CBP20, poly(A) binding protein II (PABP2), and Upf3 protein, which are primarily nuclear, and eIF4G, Upf2 protein, and ribosomal protein L10, which are primarily cytoplasmic. CBP20 forms the nuclear cap binding complex with CBP80 (Izaurralde et al., 1994), PABP2 binds nascent poly(A) tails to stimulate their extension and control their length (Wahle, 1991; Bienroth et al., 1993), eIF4G mediates translation initiation in association with eIF4E (reviewed in Gingras et al., 1999) and, presumably, CBP80 (Fortes et al., 2000; McKendrick et al., 2001), and Upf2 and Upf3 proteins function in NMD (Lykke-Andersen et al., 2000; Mendell et al., 2000; Serin et al., 2001). The presence of ribosomal protein, together with our finding that the NMD of mRNA immunopurified using antibody to CBP80 is abrogated by cycloheximide or suppressor tRNA, is consistent with the well-established dependence of NMD on translation (reviewed in Maquat, 2000). We suggest the possibility of a "pioneer" round of translation involving CBP80 that, depending on the mRNA, takes place either in association with nuclei or in the cytoplasm.

Results

Nucleus-Associated NMD of β-Globin mRNA Takes Place In Association with CBP80, CBP20, eIF4G, PABP2, and Upf2 and Upf3 Proteins

Considering that β-globin mRNA is subject to nucleus-associated NMD in nonerythroid cells such as HeLa and Cos cells (Kugler et al., 1995; Zhang et al., 1998a), we first aimed to determine if NMD is evident for β-globin mRNP immunopurified using anti-CBP80 antibody. We assayed endogenous CBP80 in order to avoid artifactual interactions that could be due to abnormally high levels of CBP80 produced from transiently introduced expression vectors. Therefore, only RNA substrates—either nonsense-free or nonsense-containing—derived from transiently introduced vectors. Notably, the detection of mRNP proteins in immunopurified complexes does not depend on the presence of these RNAs since they represent only a small fraction of cellular RNA. However, quantitative analysis of these RNAs in the complexes allows for identification of those proteins associated with NMD.

Cos cells were transiently transfected with two plasmids: a test *pmCMV-Gl* plasmid, which expressed β-globin (*Gl*) mRNA that was either nonsense-free (Norm) or harbored a premature termination codon at position 39 within exon 2 (Ter) (Zhang et al., 1998a; Figure 1A), and the reference *phCMV-MUP* plasmid (Belgrader and Maquat, 1994), which expressed mRNA for the mouse major urinary protein (MUP) and served to control for variations in the efficiencies of cell transfection and RNA recovery. After 40 hr, cells were lysed. Lysates were first cleared by incubation with protein A-agarose beads and subsequently incubated with either anti-CBP80 antibody or, as a control, normal rabbit serum (NRS). After 90 min at 4°C, tRNA, RNase inhibitor and protein A-agarose beads were added, and the incubation was continued for another 60 min. The beads were washed extensively, bound material was eluted with SDS-containing buffer, and protein and RNA were purified for analysis by Western blotting and RT-PCR, respectively.

The results of Western blotting revealed that CBP80 was immunopurified using anti-CBP80 antibody but not NRS (Figure 1B, left), indicating that immunopurification was specific for CBP80-containing complexes. The results of RT-PCR revealed that *Gl* and *MUP* mRNAs were also immunopurified using anti-CBP80 antibody but not NRS (Figure 1B, right), offering additional evidence for the specificity of immunopurification. Quantitation of each CBP80-associated mRNA demonstrated that the level of nonsense-containing (Ter) *Gl* mRNA was reduced to 19% ± 4% the level of nonsense-free (Norm) *Gl* mRNA (Figure 1B, right). This level of reduction represented the full extent of NMD, since the level of nonsense-containing *Gl* mRNA in total-cell mRNA (i.e., mRNA that had not been immunopurified) was reduced to essentially the same extent (i.e., 23% ± 4% of nonsense-free *Gl* mRNA) (Figure 1B, right). These data suggest that the NMD of *Gl* mRNA takes place in association with CBP80.

In order to gain additional insight into the nature of mRNP that is a target for NMD, the experiment was scaled up and repeated using anti-CBP80 antibody and, in parallel, anti-eIF4E antibody. Results of Western blotting confirmed the specificity of each immunopurification by demonstrating that CBP80 but not eIF4E was immunopurified using anti-CBP80 antibody, eIF4E but not CBP80 was immunopurified using anti-eIF4E antibody, and neither protein was immunopurified using NRS (Figure 1C, upper). Calculations indicated that 34% ± 2% of cellular CBP80 was immunopurified using anti-CBP80 antibody, and 15% ± 1% of cellular eIF4E was immunopurified using anti-eIF4E antibody. The finding that the NMD of *Gl* mRNA takes place in association with CBP80 was corroborated using RT-PCR: the level of nonsense-containing *Gl* mRNA was 17% ± 4% and 20% ± 4% of normal in complexes immunopurified using anti-CBP80 antibody and in total-cell RNA, respectively (Figure 1C, lower). The level of nonsense-containing *Gl* mRNA was comparably reduced to 12% ± 3% of normal in complexes immunopurified using anti-

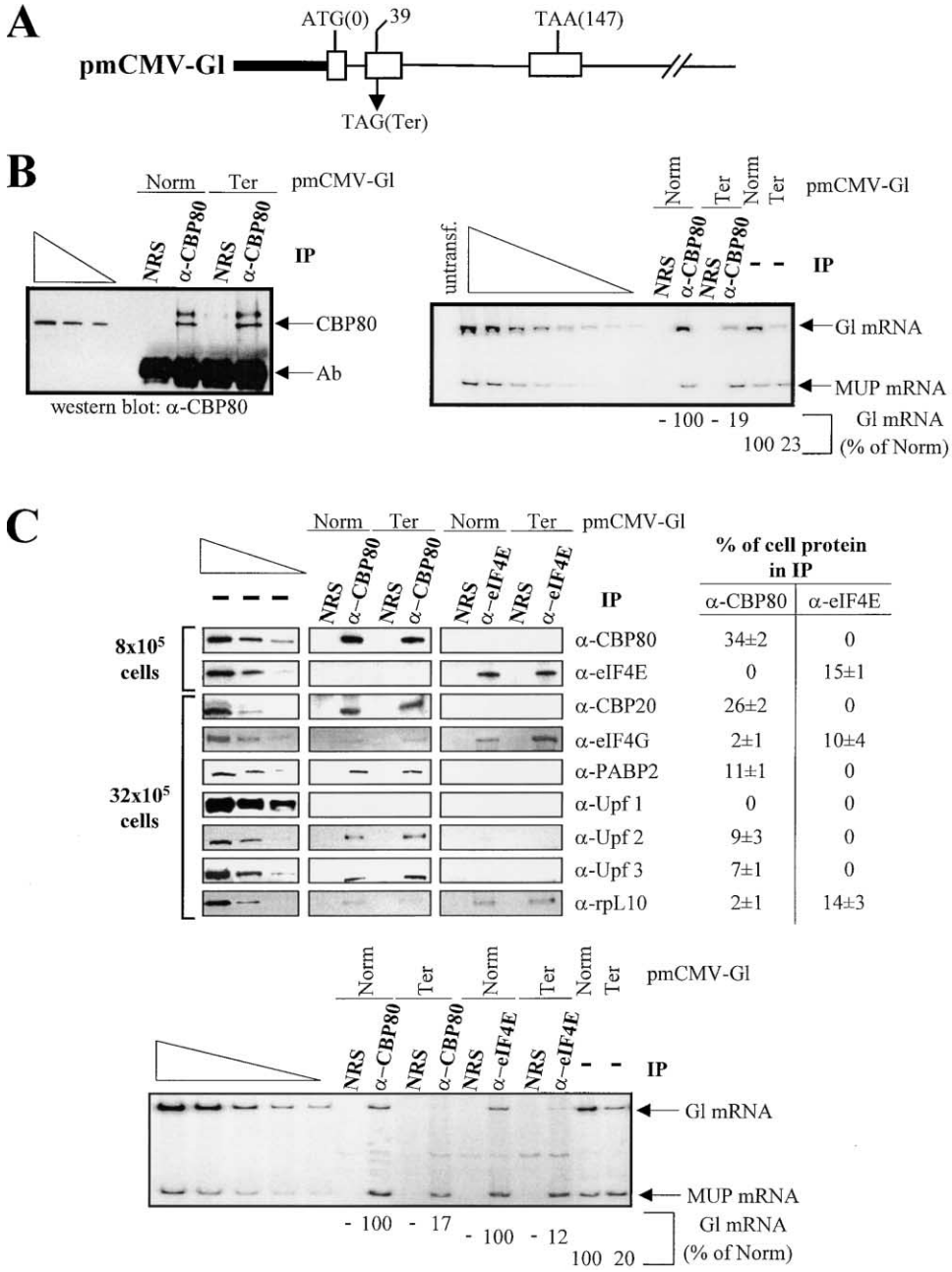

Figure 1. Nonsense-Containing β-Globin (*Gl*) mRNP Immunopurified Using Anti-CBP80 Antibody Is a Target for NMD

(A) Structure of the *pmCMV-Gl* test plasmids, which harbored either a nonsense free (Norm) *Gl* allele or a TAG nonsense codon at position 39 (Ter). Cos cells were transiently transfected with a test *pmCMV-Gl* plasmid and the reference plasmid *phCMV-MUP*.

(B, left) Total-cell RNP was then immunopurified using either normal rabbit serum (NRS) or anti-CBP80 antibody (α-CBP80) and subjected to Western blotting using anti-CBP80 antibody. The three leftmost lanes analyzed decreasing amounts of protein, demonstrating that the conditions used for Western blotting were quantitative.

(B, right) RNA was purified from total-cell RNP either before (-) immunopurification (IP) or after immunopurification (NRS or α-CBP80). The levels of *Gl* and *MUP* mRNAs were then analyzed by RT-PCR. The leftmost lane demonstrates the absence of either mRNA in untransfected cells, and the adjacent eight lanes analyzed decreasing amounts of RNA, demonstrating that the conditions of RT-PCR were quantitative.

(C, upper) As in B (left) except that immunopurifications were scaled up (each IP lane derives from either 8×10^5 or 32×10^5 cells) and also performed with anti-eIF4E antibody, and Western blotting also utilized anti-eIF4E, anti-CBP20, anti-eIF4G, anti-PABP2, anti-human Upf1, anti-human Upf2, anti-human Upf3/3X, and antiribosomal protein (rp) L10 antibodies. Furthermore, the percent (%) of each protein in each IP (tabulated to the right) was determined by comparing the Western blot intensity of each IP lane to the intensities of 3-fold dilutions of extract from either 8×10^5 or 32×10^5 cells before IP (three leftmost lanes). For easy visualization, bands corresponding to antibody (e.g., see Ab in B, left) are not shown. Protein detectably reacting with anti-Upf3/3X antibody is likely to be Upf3 protein rather than Upf3X protein (which derives from the X-linked *UPF3* gene that is related to the *UPF3* gene) since it comigrated with the HeLa cell Upf3 protein. The Upf3X isoform likely comigrated with antibody, making it undetectable using electrophoresis in either one or two dimensions (data not shown).

(C, lower) As in B (right) except that RNA analysis was extended to immunopurifications using anti-eIF4E antibody. Each panel of results is representative of five independently performed experiments where the efficiency of NMD varied by no more than 4% (see text for specific standard deviations).

eIF4E antibody (Figure 1C, lower). These results demonstrate that more than 80% of CBP80-bound *Gl* mRNA is degraded when the mRNA harbors the nonsense codon. Since CBP80 is thought to be replaced by eIF4E in the cytoplasm (Visa et al., 1996; Shen et al., 2000), it is likely that only the 17% of nonsense-containing *Gl* mRNA that escapes NMD is available for binding to eIF4E. Considering that the difference between 17% and 12% is within experimental variations, the finding that 12% of nonsense-containing *Gl* mRNA is bound by eIF4E may indicate that eIF4E-bound *Gl* mRNA is essentially immune to NMD. Alternatively, the difference may indicate that NMD also involves eIF4E-bound mRNA.

Additional evidence for specificity of the immunopurifications and the idea that CBP80-bound mRNA is susceptible to NMD was provided by the findings that complexes immunopurified using anti-CBP80 antibody but not anti-eIF4E antibody contained four other proteins detectable by Western blotting: CBP20, which together with CBP80 forms the heterodimeric nuclear cap binding complex; poly(A) binding protein II (PABP2), which binds newly synthesized poly(A) tails of pre-mRNA and mRNA; and Upf2 and Upf3 proteins (Figure 1C, upper). Since Upf2 and Upf3 proteins are known to be involved in NMD, their presence in CBP80-containing but not eIF4E-containing complexes offers strong evidence that *Gl* mRNA is a substrate for NMD when bound by CBP80 and is not detectably a substrate when bound by eIF4E. The presence of Upf2 and Upf3 proteins as well as PABP2 in CBP80-containing complexes was dependent on RNA as evidenced by their absence in immunopurified complexes that were treated with RNase A prior to dissociation from anti-CBP80 antibody (data not shown). In contrast, the presence of CBP20 in CBP80-containing complexes was not dependent on RNA (data not shown), as would be expected considering the direct interaction between CBP20 and CBP80. Interestingly, CBP20, PABP2, and Upf3 protein are mostly nuclear, consistent with the newly synthesized nature of both CBP80-containing mRNP and nucleus-associated NMD. However, PABP2 and Upf3 proteins shuttle, opening up the possibility that nucleus-associated NMD could take place on the cytoplasmic side of the nuclear envelope. In support of this possibility, Upf2p is not detected in nuclei (Lykke-Andersen et al., 2000; Serin et al., 2001). Upf1 protein, which is known to interact with Upf2 and Upf3 proteins in HeLa cells, was not detected in either CBP80-bound or eIF4E-bound *Gl* mRNA (Figure 1C, upper). Its absence from CBP80-bound mRNA is consistent with its putative role in NMD only after translation termination (see Discussion). Both CBP80 and eIF4E were associated with eIF4G and ribosomal protein L10 (Figure 1C, upper), consistent with the translational dependence of NMD (reviewed in Maquat, 2000). Notably, the amounts of eIF4G and L10 associated with CBP80 were small but reasonable considering the amounts associated with eIF4E, which supports the bulk of cellular translation.

In search of additional indications that the CBP80-bound *Gl* mRNP complexes under evaluation realistically represent complexes formed in vivo, mRNP immunopurified using anti-CBP80 antibody was isolated from nuclear and cytoplasmic fractions of transfected cells. Nuclear fractions were not contaminated by cytoplasm, as indicated by the absence of detectable eIF4A (data not shown), which has been shown to be undetectable in nuclei (Lejbkowicz et al., 1992). CBP80-bound *Gl* mRNA was detected by RT-PCR in both nuclear and cytoplasmic fractions (Figure 2), consistent with CBP80 being a shuttling protein. Furthermore, the extent of NMD for CBP80-bound *Gl* mRNA in both cellular fractions was comparable (Figure 2). This result is expected given that *Gl* mRNA is subject to nucleus-associated NMD (Zhang et al., 1998a; Thermann et al., 1998), and is consistent with the idea that NMD takes place in association with CBP80.

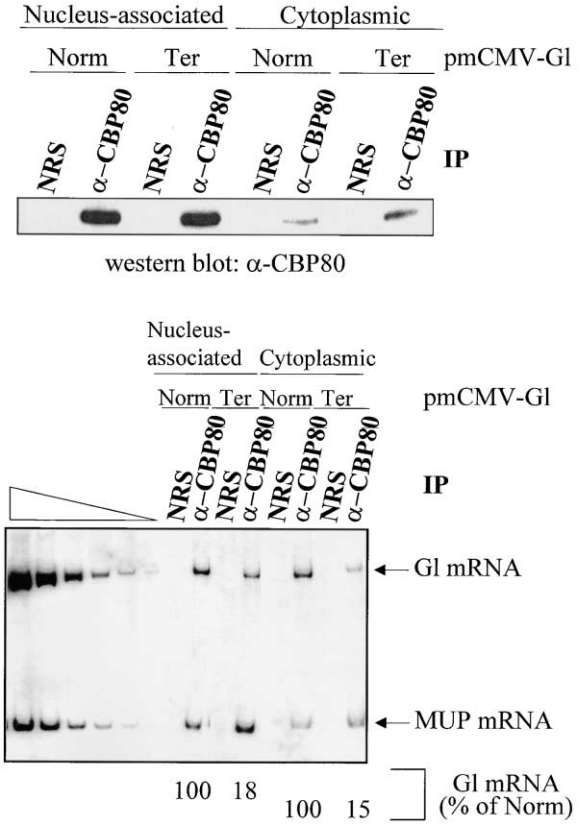

Figure 2. The NMD of CBP80-Bound *Gl* mRNA Is Nucleus-Associated

As in Figure 1B except that nuclear and cytoplasmic fractions were immunopurified with NRS and anti-CBP80 antibody. Nuclear fractions were free of cytoplasm as evidenced by the absence of detectable reactivity with anti-eIF4A antibody (data not shown). The amount of reactivity obtained with anti-CBP80 antibody after immunopurification of nuclear and cytoplasmic fractions can be quantitatively compared, as evidenced by analyzing decreasing amounts of protein, demonstrating that the conditions used for Western blotting were quantitative (data not shown). The efficiency of NMD varied by no more than 2% in two independently performed transfections.

As another means of confirming that the CBP80-containing complex is a substrate for NMD, mRNP was immunopurified from untransfected cells using anti-Upf3/3X antibody. This antibody reacts with Upf3 and Upf3X proteins, which derive from non-X-linked and X-linked *UPF3* genes, respectively (Lykke-Andersen et al., 2000; Serin et al., 2001; data not shown). Notably, both Upf3 and Upf3X proteins are mostly nuclear and shuttle between nuclei and cytoplasm (Lykke-Andersen et al., 2000; Serin et al., 2001). Consistent with findings

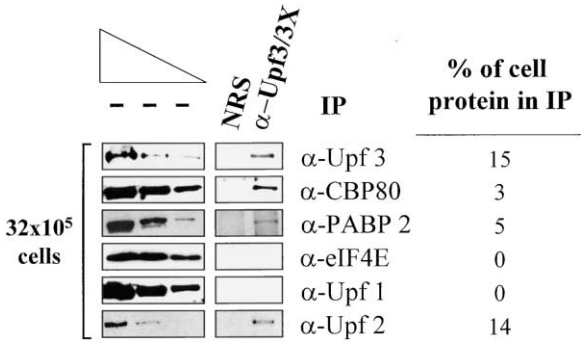

Figure 3. RNP Immunopurified Using Anti-Upf3/3X Antibody Contains CBP80, PABP2, and Upf2 Protein But Not eIF4E or Upf1 Protein

As in Figure 1C, upper, except that cells were not transfected and immunopurifications were performed with anti-Upf3/3X antibody. Results are representative of three independently performed immunopurifications.

described above, CBP80, PABP2, and Upf2 protein, but not eIF4E or Upf1 protein, were detected in complexes immunopurified using anti-Upf3/3X antibody (Figure 3). The presence of CBP80 and the absence of eIF4E in complexes immunopurified using anti-Upf3/3X antibody corroborate the idea that NMD is largely confined to CBP80-bound mRNA. The finding that there is more Upf2 protein in complexes immunopurified using anti-Upf3/3X antibody than in complexes immunopurified using anti-CBP80 antibody is consistent with data demonstrating that Upf2 and Upf3 proteins can interact independently of RNA (Lykke-Andersen et al., 2000), whereas the interaction between CBP80 and Upf3 is dependent on RNA (data not shown).

Evidence that CBP80-Bound *Gl* mRNA Is Translated

The finding that NMD takes place in association with CBP80 implies that CBP80-bound mRNA can be translated, which is consistent with the presence of eIF4G and ribosomal protein in immunopurifications using anti-CBP80 antibody (Figure 1C). To obtain more direct evidence for the translatability of CBP80-bound mRNA, the effect of a block in translation on the level of total-cell CBP80-bound mRNA was quantitated. Addition of 100 μg/ml of cycloheximide for two hr prior to cell harvesting abrogated the NMD of CBP80-bound *Gl* mRNA so that the level of nonsense-containing mRNA was increased from 15% to 42% of normal (Figure 4A). Therefore, CBP80-bound mRNA is a substrate for protein synthesis. This conclusion was corroborated using an amber suppressor tRNA (Belgrader et al., 1993) that directs the incorporation of serine at the nonsense codon of nonsense-containing *Gl* mRNA. Coexpression of a plasmid producing suppressor tRNA abrogated the NMD of CBP80-bound *Gl* mRNA: the level of nonsense-containing *Gl* mRNA was increased from 23% of normal to 37%, 46%, or 62% of normal as the ratio of suppressor tRNA expression vector to *Gl* mRNA expression vector was increased (Figure 4B). In contrast, coexpression of a plasmid harboring the suppressor tRNA gene in antisense orientation had no effect on the NMD of CBP80-bound *Gl* mRNA (Figure 4B).

Cytoplasmic NMD of *GPx1* mRNA Also Takes Place In Association with CBP80, CBP20, eIF4G, PABP2, and Upf2 and Upf3 Proteins

Unlike nucleus-associated NMD, for which data are lacking, cytoplasmic NMD has been demonstrated to take place on cytoplasmic ribosomes (Rajavel and Neufeld, 2001). In order to gain insight into the mechanistic relationship between NMD at the two cellular sites, the cap binding protein associated with mRNA that is a substrate for cytoplasmic NMD was determined. To this end, Cos cells were transiently transfected with two plasmids: a test *pmCMV-GPx1* plasmid, which expressed glutathione peroxidase1 (*GPx1*) mRNA that was either nonsense-free (Norm) or harbored a premature termination codon at position 46 within exon 1 (Ter) (Moriarty et al., 1998; Figure 5A), and the reference *phCMV-MUP* plasmid. Immunopurifications were performed using either anti-CBP80 or anti-eIF4E antibody as described above for studies of *Gl* mRNP.

The results of Western blotting confirmed the specificity of each immunopurification, i.e., the absence of detectable eIF4E in the immunopurification using anti-CBP80 antibody, the absence of detectable CBP80 in the immunopurification using anti-eIF4E antibody, and the absence of either protein in the immunopurification using NRS (Figure 5B, upper). Furthermore, quantitation of *GPx1* and *MUP* mRNAs by RT-PCR revealed the presence of each mRNA in immunopurifications using anti-CBP80 or anti-eIF4E antibody but not NRS (Figure 5B, lower). Remarkably, despite its cytoplasmic location, NMD was evident for CBP80-bound *GPx1* mRNA: the level of nonsense-containing mRNA was 25% ± 5% of normal, which was comparable to the (29% ± 4% of normal) level of nonsense-containing mRNA that had not undergone immunopurification (Figure 5B, lower). The level of nonsense-containing mRNA bound by eIF4E was 21% ± 2% of normal (Figure 5B, lower), indicating that *GPx1* mRNA is no longer appreciably susceptible to NMD once CBP80 is replaced by eIF4E, as was found for *Gl* mRNA. The difference between 25% and 21% may indicate that NMD involves eIF4E-bound mRNA. Nevertheless, as was concluded from the studies of *Gl* mRNA, NMD involves CBP80-bound mRNA.

Also consistent with findings using *Gl* mRNA, results of Western blotting revealed that CBP80-bound *GPx1* mRNA, but not eIF4E-bound *GPx1* mRNA, was associated with CBP20, PABP2, Upf2 protein, and Upf3 protein (Figure 5B, upper). Furthermore, both CBP80-bound and eIF4E-bound *GPx1* mRNAs were associated with eIF4G and ribosomal protein L10, and neither was detectably associated with Upf1 protein (Figure 5B, upper; data not shown for L10). The analysis of cell fractions was used to corroborate the biological significance of the *GPx1* mRNP under study. NMD was evident for CBP80-bound *GPx1* mRNA in the cytoplasmic but not nuclear fraction (Figure 6). This result is consistent with the fact that NMD of *GPx1* mRNA takes place only after export to the cytoplasm, and indicates that translation in the cytoplasm can take place in association with CBP80.

Discussion

We have determined that mRNA bound by the cap binding proteins CBP80 and CBP20 and the poly(A) binding

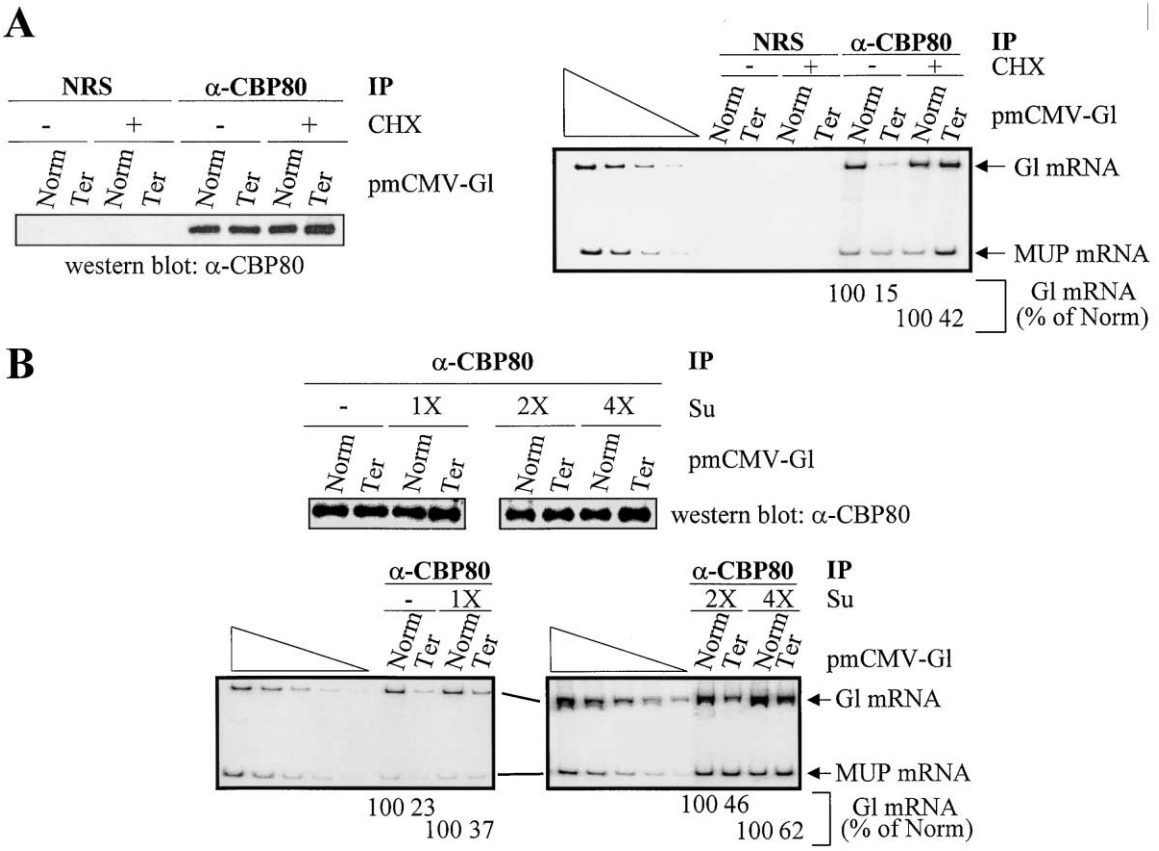

Figure 4. Cycloheximide and Suppressor tRNA Abrogate the NMD of CBP80-Bound *Gl* mRNA, Providing Evidence that CBP80-Bound mRNA Is Translated

(A) As in Figure 1B except that cycloheximide was (+CHX) or was not (−CHX) added two hr prior to cell harvest.

(B) As in Figure 1B except that a derivative of *p53tsSu*$^+$ that harbored the amber suppressor tRNA in either antisense orientation (−Su) or sense orientation at increasing concentrations (1X, 2X, and 4X Su) was included in the transfection, and immunopurifications using NRS were not performed. Results are representative of two independently performed experiments.

protein PABP2 is translated and subject to NMD regardless of whether NMD takes place in association with nuclei or in the cytoplasm. Even though CBP80, CBP20, and PABP2 are primarily nuclear, their function in cytoplasmic NMD is attributable to their ability to shuttle between nuclei and cytoplasm. The site of nucleus-associated NMD remains uncertain. For the sake of unification, it could be argued that nucleus-associated NMD also takes place in the cytoplasm, albeit during the process of mRNA export from nuclei. Consistent with this possibility, but certainly not proof, the replacement of CBP80 and CBP20 by the primarily cytoplasmic cap binding protein eIF4E is generally concomitant with the loss of susceptibility to NMD for both nucleus-associated and cytoplasmic NMD. Furthermore, Upf2 protein, which is also bound to mRNA subject to NMD at either cellular site, is detected only in the cytoplasm, albeit mostly at the nuclear periphery (Lykke-Andersen et al., 2000; Serin et al., 2001). Additionally, Upf1 protein, which is known to be required for NMD (Sun et al., 1998) and interacts with Upf2 protein (Serin et al., 2001), is also detected only in the cytoplasm (Lykke-Andersen et al., 2000; Serin et al., 2001). However, the fact that some eIF4E is present in nuclei makes the replacement of CBP80-CBP20 with eIF4E in nuclei a possibility for nucleus-associated NMD. Upf1 and Upf2 proteins could also be present in nuclei at undetectable levels. Even our finding that the nucleus-associated NMD of CBP80-bound *Gl* mRNA is abrogated by either a cycloheximide-induced block in translation or suppressor tRNA that recognizes the nonsense codon as a codon for serine does not preclude the possibility of cycloheximide-sensitive or suppressor tRNA-responsive translation in nuclei. Nevertheless, the simplest interpretation of existing data is that nucleus-associated NMD involves cytoplasmic ribosomes during mRNA export.

Our findings are important for a number of reasons. Most notably, CBP80, CBP20, and, presumably, PABP2, can now be defined as components of the translation initiation complex that typifies early rounds—possibly exclusively the first round—of translation in mammalian cells. The idea of a "pioneer" round that is distinct from subsequent rounds has recently been suggested for transcription by RNA polymerase II (reviewed in Orphanides and Reinberg, 2000). A pioneer round of translation during which NMD generally functions certainly makes sense considering that NMD surveys mRNAs to eliminate those that prematurely terminate translation as a form of quality control. Notably, our data do not eliminate the possibility that NMD takes place on eIF4E-

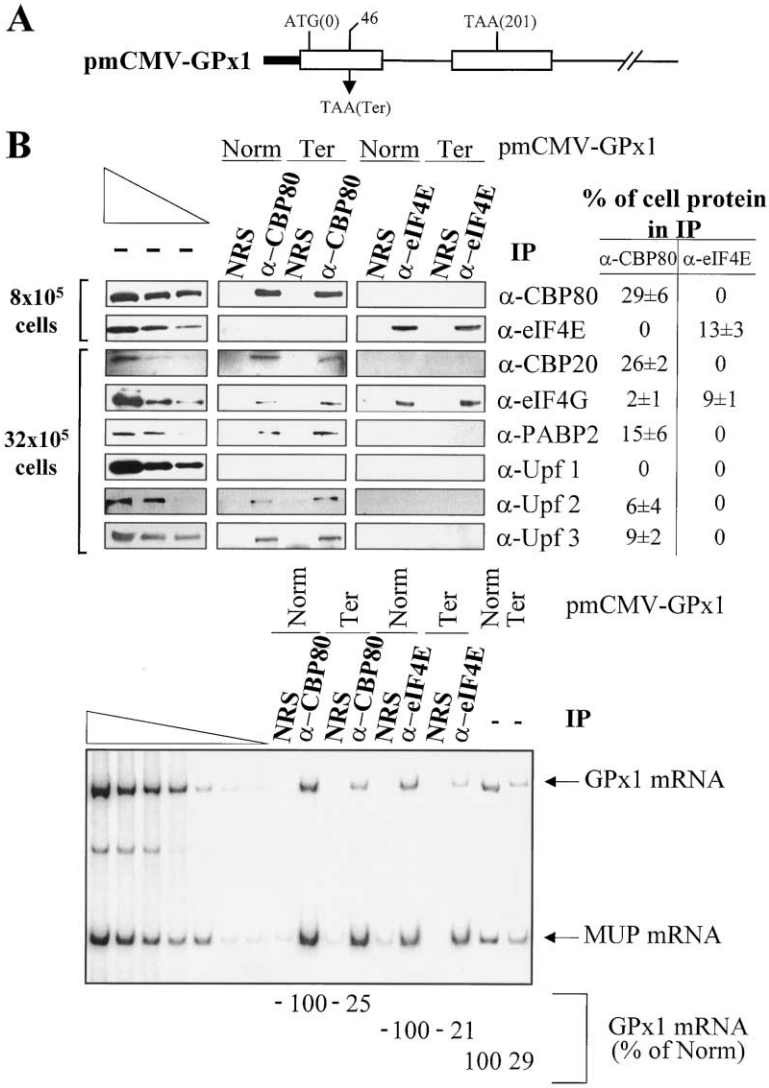

Figure 5. Nonsense-Containing Glutathione Peroxidase 1 (*GPx1*) mRNA Immunopurified Using Anti-CBP80 Antibody Is Subject to NMD

(A) Structure of the *pmCMV-GPx1* test plasmids, which harbored either a nonsense-free (Norm) *GPx1* allele or a TAA nonsense codon at position 46 (Ter).

(B) As in Figure 1C except that *GPx1* mRNA was analyzed in place of *GI* mRNA. Each panel of results is representative of three independently performed experiments, and the efficiency of NMD varied by no more than 5% (see text for specific standard deviations).

bound mRNA. The finding that the absence of CBP80 in *S. cerevisiae* does not preclude NMD (Das et al., 2000) indicates that eIF4E-bound mRNA can serve as a substrate for NMD when CBP80 is absent. Only when more is understood about the rate of and requirements for CBP80-CBP20 replacement by eIF4E will there be an understanding of whether or not a fraction of eIF4E-bound mRNA, in addition to CBP80-bound mRNA, normally serves as a substrate for NMD.

Details of the intermolecular interactions within the pioneer initiation complex, and in particular, if they relate to or are different from those of the steady-state initiation complex, have yet to be resolved. In the steady-state initiation complex, eIF4G is a multipurpose adaptor, interacting directly with eIF4E, eIF3, eIF4A, and PABP1 (reviewed in Hentze, 1997; Gingras et al., 1999; Sachs and Varani, 2000; see Figure 7). For a comparable complex to exist during the pioneer round of translation, CBP80-CBP20 would interact with eIF4G analogously to the way eIF4E interacts with eIF4G; similarly, PABP2 would functionally replace PABP1 (Figure 7).

Consistent with recent studies of mammalian cells demonstrating that CBP80 and eIF4G coimmunoprecipitate (McKendrick et al., 2001), recent studies of *S. cerevisiae* demonstrated that eIF4G1 interacts directly with CBP80 in vitro in a way that is competed by eIF4E (Fortes et al., 2000). Additionally, TAP-tagged CBP20 copurified with CBP80, eIF4G1, and a small amount of eIF4E, indicating an association in extracts. For reasons unknown, however, CBP80-CBP20 stimulated cap-dependent translation only in extracts of yeast strains expressing eIF4G1 that harbored mutations weakening its binding to eIF4E and Pab1p; in fact, addition of CBP80–CBP20 to wild-type extracts inhibited translation (Fortes et al., 2000). The CBP80-interacting region of *S. cerevisiae* eIF4G1 was found to reside between residues 490 and 592 (Fortes et al., 2000). These residues are situated immediately C-terminal to (and were suggested to interfere with) the eIF4E interacting region of eIF4G1. A database search revealed that these residues are 63% identical to those in *S. cerevisiae* eIF4G2, but they show no obvious similarity to either human eIF4G1 or human eIF4G2 (data not shown). Therefore, there is currently no indication for mammalian cells that eIF4G interacts directly with

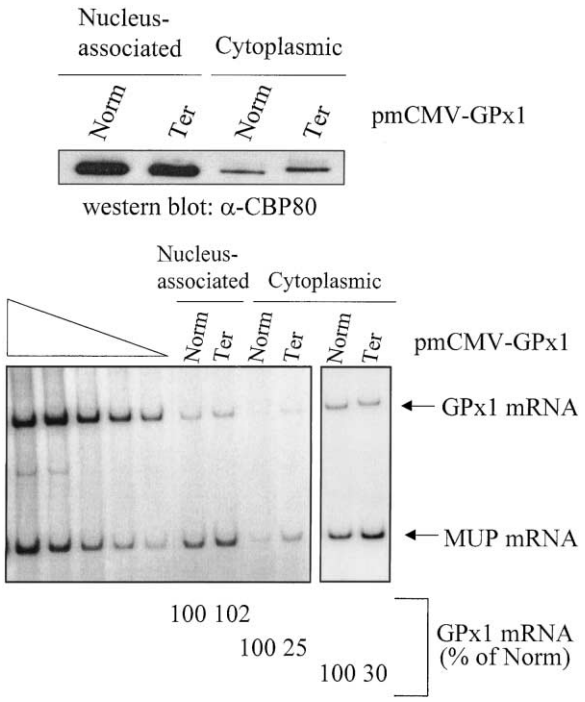

Figure 6. The NMD of CBP80-Bound *GPx1* mRNA Is Cytoplasmic

As in Figure 2 except that *GPx1* mRNA was analyzed in place of *Gl* mRNA, and the analysis of NRS was omitted. The efficiency of NMD varied by no more than 5% in two independently performed experiments. In the RNA analysis, the righthand box represents a separate and more efficient transfection relative to the leftmost box.

CBP80 as it does in *S. cerevisiae*. Exemplifying another apparent difference between yeast and mammals, eIF4E was not detected in immunopurifications containing CBP80 and eIF4G, unlike the situation in *S. cerevisiae*, where eIF4E and CBP80 can simultaneously be present in eIF4G1-containing complexes independent of RNA (Fortes et al., 2000).

There is also no evidence for mammalian cells that eIF4G interacts with CBP20. A database search revealed that CBP20 and eIF4E from *S. cerevisiae*, *D. melanogaster*, and humans likely derived from a common ancestor. For example, human CBP20 and human eIF4E share 26% amino acid similarity throughout their lengths (data not shown). Mutation of Trp73 in human eIF4E or the corresponding Trp75 in *S. cerevisiae* eIF4E has been shown to abolish the interaction with eIF4G (Pyronnet et al., 1999; Marcotrigiano et al., 1999; Ptushkina et al., 1998). While human CBP20 can be aligned with human and *S. cerevisiae* eIF4E, human CBP20 lacks a Trp residue that would be required if it were to interact with eIF4G as does eIF4E (data not shown).

With regard to the possibility that PABP2 interacts with eIF4G in mammalian cells, PABP1 contains four RRMs, the two N-terminal of which in human and *S. cerevisiae* are critical for the interaction with eIF4G (Imataka et al., 1998; Kessler and Sachs, 1998). Since PABP2 contains only a single RRM (Wahle et al., 1993; Nemeth et al., 1995), and since a single RRM either does not interact, or in the case of RRM2 of human PABP1 interacts only weakly, with eIF4G (Imataka et al., 1998), it is not likely that PABP2 interacts significantly with eIF4G in mammalian cells.

Upf2 protein has been proposed to interact with eIF4A because it, like its *S. cerevisiae* counterpart, harbors regions similar to those of eIF4G that interact with eIF4A (Mendell et al., 2000; Lykke-Andersen et al., 2000; Serin et al., 2001) and has been shown using two-hybrid analysis to interact directly with eIF4AI (Mendell et al., 2000). We were not able to assay for the presence of eIF4A in

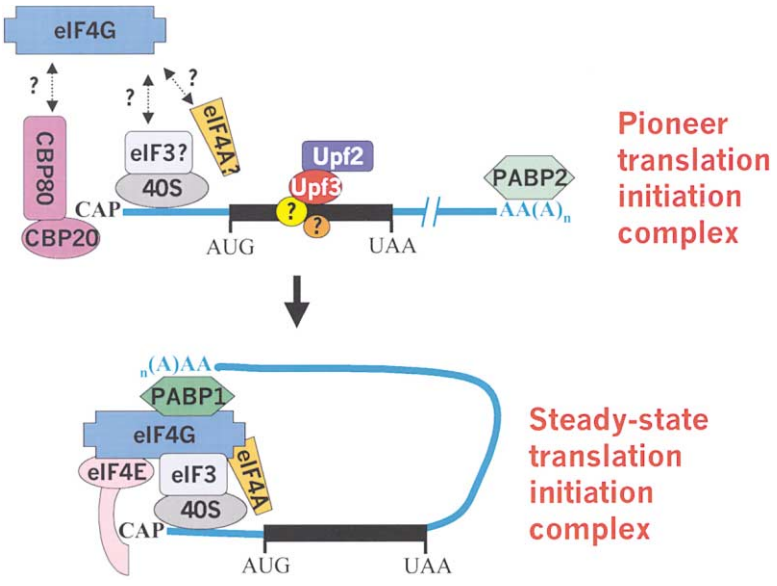

Figure 7. Model for the Pioneer Translation Initiation Complex, Shown Relative to the Steady-State Translation Initiation Complex

The pioneer translation initiation complex, which is the substrate for NMD (as well as the first round of translation for both nonsense-free and nonsense-containing mRNAs), contains CBP80-CBP20 bound to the mRNA cap structure, PABP2 bound to the poly(A) tail, and Upf2 and Upf3 proteins probably bound close to, but upstream of, exon-exon junctions. Notably, the number of Upf2 and Upf3 molecules per cell is adequate for binding to the majority of exon-exon junctions (L.E.M. and G.S, in press). At least for mRNAs subject to cytoplasmic NMD, this complex functions in association with cytoplasmic ribosomes. It is uncertain if eIF4G is a multipurpose adaptor as it is in the steady-state translation initiation complex (Hentze, 1997; Gingras et al., 1999; Sachs and Varani, 2000), although it is present. Yellow and dark orange representations of proteins with question marks signify the possible presence of components of the complex that binds mRNA as a consequence of splicing in the nucleus (Le Hir et al., 2000a, 2000b) and is thought to recruit Upf3 protein. Alternatively, or additionally, Upf3 protein may interact with RNA directly, since it contains an RRM-like domain (Lykke-Andersen et al., 2000). Upf1 protein is thought to associate after translation initiation, possibly as a consequence of translation termination (see Discussion). The presence of eIF3 has yet to be tested, and the low specificity of available anti-eIF4A antibody precluded eIF4A detection (data not shown). The bulk of cellular translation involves the steady-state translation initiation complex, which derives from remodeling of the pioneer translation initiation complex.

immunopurifications using either anti-CBP80 or anti-Upf3/3X antibody because available anti-eIF4A antibody had insufficient reactivity (data not shown). Neither were we able to detect a complex of Upf2 protein and either eIF4AI or eIF4AIII in immunopurifications of epitope-tagged versions of each protein that were transiently produced in HeLa cells (Serin et al., 2001). Nevertheless, it is still possible that Upf2 protein functions to accelerate NMD by competing with eIF4G binding proteins (Mendell et al., 2000).

Interestingly, Upf1 protein was not detected in anti-CBP80 immunopurifications. This protein, a group 1 RNA helicase known to function in NMD (Sun et al., 1998; Bhattacharya et al., 2000; Pal et al., 2001), has been shown to coimmunoprecipitate with Upf2 and Upf3 proteins in RNase-treated HeLa cell extract (Lykke-Andersen et al., 2000) as well as in cells that have been transiently transfected with Upf protein expression plasmids (Serin et al., 2001). It may be that the interaction of Upf1 protein with mRNP is too transient to detect. A number of observations support this possibility. (1) A mutated Upf1 protein that functions in a dominant-negative manner impairs NMD in HeLa cells when wild-type Upf3 or Upf2 protein but not wild-type Upf1 protein is tethered downstream of a nonsense codon, arguing that Upf1 protein acts downstream of the Upf3 and Upf2 proteins (Lykke-Andersen et al., 2000). (2) Upf3 protein is nuclear, Upf2 protein is cytoplasmic but perinuclear, and Upf1 protein is cytoplasmic (Lykke-Andersen et al., 2000; Serin et al., 2001), possibly reflecting the order of assembly on spliced mRNA. (3) The finding that Upf3 protein interacts directly with Upf2 protein, which in turn interacts directly with Upf1 protein (Serin et al., 2001), also suggests that Upf1 protein may be the last Upf protein to bind to spliced mRNA. Similarly to what has been recently proposed (Lykke-Andersen et al., 2000), ribosomes translating mRNA during the pioneer round of translation may displace Upf2 and Upf3 proteins bound at or near exon-exon junctions located either upstream or less than 50–55 nucleotides downstream of a translation termination codon. However, if translation terminates more than 50–55 nucleotides upstream of an exon-exon junction, then Upf1 protein, which in *S. cerevisiae* functions in translation termination and binds to release factors (Czaplinski et al., 1998; Wang et al., 2001), may interact with the downstream complex of bound Upf2 and Upf3 proteins after translation termination and elicit NMD.

All data previous to this study indicated that nucleus-associated NMD is restricted to newly synthesized mRNA given that mRNA released into the cytoplasm is immune to NMD (for review, see Maquat, 1995, 2000; Li and Wilkinson, 1998; Hentze and Kulozik, 1999). However, our finding for *GPx1* mRNA, which is subject to cytoplasmic NMD, that the level of cytoplasmic nonsense-containing mRNA becomes undetectable 12 hr after promoter inactivation, while the level of cytoplasmic nonsense-free mRNA was only slightly reduced, led us to propose that cytoplasmic NMD is not limited to newly synthesized mRNA (Sun et al., 2000). This conclusion must be revised with our findings here that the cytoplasmic NMD of *GPx1* mRNA, like nucleus-associated NMD of *Gl* mRNA, takes place in association with CBP80, and that a population of eIF4E-bound *GPx1* mRNA exists that is immune to NMD. These findings indicate that the complete disappearance of nonsense-containing *GPx1* mRNA 12 hr after promoter inactivation must reflect not only NMD but also decay of the fraction of mRNA that escaped NMD. Future studies aim to determine the requirements for CBP80 replacement by eIF4E.

Experimental Procedures

Cell Culture, Plasmids, and Transfection

Monkey kidney Cos-7 and mouse NIH3T3 cells were cultured in MEM-α (Gibco BRL) supplemented with 10% FCS. Cells were transfected with test plasmids *pmCMV-Gl* (Norm or 39Ter; Sun et al., 1998) or *pmCMV-GPx1* (Norm or 46Ter, also called 46 TAA; Moriarty et al., 1998) and reference plasmid *phCMV-MUP* (Belgrader and Maquat, 1994) by using calcium phosphate (Wigler et al., 1979). Where indicated, cycloheximide (100 μg/ml; Sigma) was added 2 hr before harvesting. Alternatively, either *p53tsSu*$^+$, which expresses an amber suppressor tRNA (Belgrader et al., 1993), or a derivative of *p53tsSu*$^+$ that harbored the amber suppressor tRNA in antisense orientation was included in the transfection.

Antibodies to Human Upf2 and Upf3 Proteins

Rabbit polyclonal antisera were raised (Rockland, Inc.) against His6-tagged human Upf2 protein (Serin et al., 2001; amino acids 441–761) and His6-tagged human Upf3 protein (Serin et al., 2001; amino acids 1–177). Each antibody was purified using protein A agarose (Sigma) following the protocol described in Harlow and Lane (1999).

Cell Lysis and Immunopurifications

Immunopurification and RT-PCR were according to Chu et al. (1999), with modification. Briefly, two days after transfection, 3.2×10^7 Cos-7 cells, or where indicated, NIH3T3 cells were washed three times with ice-cold phosphate-buffered saline (PBS). Cells were harvested and suspended in PBS. After centrifugation, cell pellets were resuspended in 0.5 ml of NET-2 buffer (50 mM Tris-HCl, [pH 7.4], 300 mM NaCl, 0.05% NP-40) containing 100 U of RNase-inhibitor (Promega). Cells were sonicated with three 10 s bursts using a Branson Sonifier. After sonication, homogenates were centrifuged at 10,000 $\times$ g for 10 min at 4°C. The supernatants were used as the source of antigen in the immunopurifications. They were first cleared by incubation with end-over-end rotation in the presence of 100 μl of protein A-agarose beads (Boehringer Mannheim) for 30 min at 4°C followed by centrifugation at 10,000 $\times$ g. Cleared supernatants were then rotated in the presence of a specific anti-serum for 90 min at 4°C, after which 0.5 mg of yeast tRNA (Sigma), 100 U of RNase-inhibitor, and protein A-agarose beads was added. The incubation was continued for another 60 min at 4°C. The beads were washed six times with NET-2 buffer, suspended in 50 μl of SDS sample buffer (0.1 M Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 12% 2-mercaptoethanol), and split into two portions. In some experiments, RNase A (20 μg; Sigma) or, as a control, BSA (20 μg; New England Biolabs) was added to the beads immediately prior to splitting. One portion (six-tenths) was used as a source of protein, which was analyzed by SDS-PAGE and Western blotting. The other portion (four-tenths) was extracted with phenol, chloroform, and isoamyl alcohol, and precipitated using ethanol. Precipitates were dissolved in 50 μl of NET-2 buffer, treated with 10 U of DNase I (Promega) for 15 min at 37°C, extracted, precipitated, dissolved in 30 μl of water, and used as a source of RNA for RT-PCR (Sun et al., 1998). Notably, this protocol was scaled up to include as many as eight individual immunopurifications, which were pooled as a source of protein and RNA. In cases where nuclear and cytoplasmic fractions were analyzed, nuclei and cytoplasm were separated from 1–2 $\times$ 10^8 transfected cells as previously described (Belgrader et al., 1994).

Western Blot Analysis

Immunopurified protein or protein prior to immunopurification (5–10 μl) was electrophoresed in 8% (eIF4G, Upf1 protein or Upf2 protein), 10% (CBP80), 12% (eIF4E or CBP20), 15% (rpL10), or a gradient of 4%–15% polyacrylamide (Bio-Rad) either with or without (PABP2) 12% 2-mercaptoethanol. Protein was transferred to Hy-

bond ECL nitrocellulose (Amersham), and probed with antibody against CBP80 (Izaurralde et al., 1994), CBP20 (Izaurralde et al., 1995), eIF4E (Morley and McKendrick, 1997), eIF4G (Morley and McKendrick, 1997), PABP2 (Krause et al., 1994), Upf1 protein (Pal et al., 2001), Upf2 protein, Upf3 protein, or ribosomal protein (rp) L10 (QM rabbit polyclonal IgG; Santa Cruz Biotechnology). In the case of cellular fractionations, nuclear fractions were deemed free of cytoplasm by the absence of detectable reactivity with antibody against eIF4A (Lejbkowicz et al., 1992). Reactivity to each primary antibody was detected using a 1:5000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit antibody (Amersham). Reactivity of the secondary antibody was visualized by SuperSignal West Pico or West Femto Solution (Pierce).

RT-PCR

Immunopurified *Gl*, *GPx1*, or *MUP* transcripts (10 μl) or transcripts prior to immunopurification were analyzed by RT-PCR as described (Sun et al., 1998). *GPx1* RNA was amplified using the primers 5′ ACCACCGTAGAACGCAGATCG 3′ (sense) and 5′ CTTCTCACCATT CACCTCGCACTT 3′ (antisense). *Gl* RNA was amplified using the same sense primer that was used to amplify *Gl* RNA and 5′ GGGTTTAGTG GTACTTGTGAGC 3′ (antisense). *MUP* RNA was amplified using 5′ CTGATGGGGCTCTATG 3′ (sense) and 5′ TCCTGGTGAGAAGT CTCC 3′ (antisense). Half of each RT-PCR reaction was electrophoresed in 5% polyacrylamide. The simultaneous analysis of serial dilutions of RNA ensured that the RT-PCR was quantitative. RT-PCR products were quantitated by PhosphorImaging (Molecular Dynamics).

Acknowledgments

We thank Linda McKendrick, Serafín Piñol-Roma, Nahum Sonenberg, and Matthias Hentze for helpful conversations, Elisa Izaurralde and Joe Lewis for providing anti-CBP80 antibody, Simon Morley for anti-4E, anti-4A, and anti-4G antibodies, and Elmar Wahle for anti-PABP2 antibody. This work was supported by Public Health Service Grants DK 33938 and GM 59614 from the National Institutes of Health.

Received March 22, 2001; revised July 25, 2001

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