Translation Initiation on mRNAs Bound by Nuclear Cap-binding Protein Complex CBP80/20 Requires Interaction between CBP80/20-dependent Translation Initiation Factor and Eukaryotic Translation Initiation Factor 3g*

Junho Choe1,2, Nara Oh12, Sungjin Park1, Ye Kyung Lee3, Ok-Kyu Song4, Nicolas Locker5, Sung-Gil Chi6, and Yoon Ki Kim1,3

From the 1School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Republic of Korea, the 2Panbionet Corp., Pohang, Kyungbuk 790-784, Republic of Korea, and the 3Department of Microbial and Cellular Sciences, University of Surrey, Guildford GU2 7HX, United Kingdom

Background: How the eIF3 complex and ribosomes are recruited during translation on CBP80/20-bound mRNAs remains obscure.

Results: CTIF interacts with eIF3g to recruit the eIF3 complex.

Conclusion: Translation on CBP80/20-bound mRNAs requires CTIF-eIF3g interaction.

Significance: The use of different eIF3 subunits for recruiting eIF3 complex implies that translation on CBP80/20-bound mRNAs differs mechanistically from translation on eIF4E-bound mRNAs.

In the cytoplasm of mammalian cells, either cap-binding proteins 80 and 20 (CBP80/20) or eukaryotic translation initiation factor (eIF) 4E can direct the initiation of translation. Although the recruitment of ribosomes to mRNAs during eIF4E-dependent translation (ET) is well characterized, the molecular mechanism for CBP80/20-dependent translation (CT) remains obscure. Here, we show that CBP80/20-dependent translation initiation factor (CTIF), which has been shown to be preferentially involved in CT but not ET, specifically interacts with eIF3g, a component of the eIF3 complex involved in ribosome recruitment. By interacting with eIF3g, CTIF serves as an adapter protein to bridge the CBP80/20 and the eIF3 complex, leading to efficient ribosome recruitment during CT. Accordingly, down-regulation of CTIF using a small interfering RNA causes a redistribution of CBP80 from polysome fractions to subpolysome fractions, without significant consequence to eIF4E distribution. In addition, down-regulation of eIF3g inhibits the efficiency of nonsense-mediated mRNA decay, which is tightly coupled to CT but not to ET. Moreover, the artificial tethering of CTIF to an intercistronic region of dicistronic mRNA results in translation of the downstream cistron in an eIF3-dependent manner. These findings support the idea that CT mechanistically differs from ET.

Mammalian gene expression is tightly controlled by well organized cellular systems. During mRNA transcription, pre-mRNAs undergo several modification processes in the nucleus as follows: 5’-capping, 3’-polyadenylation, and splicing (1–3). The 5’ cap structure and 3’ poly(A) tail are bound by the nuclear cap-binding protein complex, which is a heterodimer of CBP80 and CBP20 (CBP80/20), and poly(A)-binding proteins, respectively (1, 3). Properly spliced mRNAs are then exported from the nucleus to the cytoplasm through the nuclear pore complex. During mRNA export, CBP80/20 recruits ribosomes to initiate the first round of translation in what is called the pioneer round of translation. To clearly specify the cap-binding protein used, we will hereafter refer to this process as CBP80/20-dependent translation (CT)4 (4, 5). After CT, the cytoplasmic cap-binding protein, eukaryotic translation initiation factor (eIF) 4E, takes over the function of binding the cap (4, 6, 7). The ribosome is recruited by eIF4E to carry out the on-going process of polypeptide synthesis in what is called steady-state translation, responsible for the bulk protein synthesis. We will hereafter refer to this process as eIF4E-dependent translation (ET) (8, 9).

CT and ET are functionally and mechanistically different from each other (4, 10). Although ET is the step where large amounts of proteins are produced, CT is generally considered a step where the quality of mRNA is monitored and surveyed. For instance, nonsense-mediated mRNA decay (NMD), in which faulty mRNAs harboring premature termination codons (PTCs) are recognized and down-regulated (11, 12), is tightly coupled to CT but not ET (4). In addition, although CT and ET may involve common translation initiation factors (4, 10, 13, 14), they may also use distinct initiation factors to recruit ribo-

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1 Supported in part by a Seoul Science Fellowship.
2 Both authors contributed equally to this work.
3 To whom correspondence should be addressed: School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Republic of Korea. Tel.: 82-2-3290-3410; E-mail: yk-kim@korea.ac.kr.
4 The abbreviations used are: CT, CBP80/20-dependent translation; ET, eIF4E-dependent translation; CTIF, CBP80/20-dependent translation initiation factor; NMD, nonsense-mediated mRNA decay; PTC, premature termination codon; BD, binding domain; AD, activation domain; IP, immunoprecipitation; Gl, β-globin; Norm, normal; MUP, mouse urinary protein; Ter, termination; RLuc, Renilla luciferase.
CTIF-eIF3g Interaction Is Required for Ribosome Recruitment

somes, as suggested by our recent identification of CBP80/20-dependent translation initiation factor (CTIF) (5). CTIF, which contains a middle domain of eIF4GI (MIF4G), directly interacts with CBP80, is complexed with eIF3 and eIF4AIII, and associates with the active form of the CT initiation complex (5). In addition, depletion of endogenous CTIF from in vitro translation reactions or down-regulation of CTIF using small interfering RNA (siRNA) from cultured cells inhibits the efficiencies of CT and consequently NMD (5). Based on our previous findings, we proposed that CT requires a series of protein interactions of CBP80/20-CTIF-eIF3, analogous to eIF4E-eIF4GII/eIF3 interactions for ET (5).

Mammalian eIF3 consists of at least 13 different polypeptides that are designated eIF3α to eIF3m depending on their protein mass (8, 9). The eIF3 complex plays multiple roles in translation by (i) stabilizing the 40S ribosomal subunit; (ii) promoting the formation of the 43S pre-initiation complex; (iii) enhancing ribosome recruitment to mRNA via interactions with other eIFs; and (iv) helping ribosome scanning for re-initiation (8, 9, 15). In particular, eIF3g, one of the core subunits of eIF3 in yeast (8, 9) and of a stable eIF3 subcomplex in mammals (16), interacts with poly(A)-binding protein-interacting protein 1 (Paip1), helping mRNAs circulate (17).

Whereas diverse roles of eIF3 in ET have been characterized in detail as described above, the underlying molecular mechanism by which eIF3 complex is recruited to mRNA during CT remains elusive. Here, we show that CTIF directly interacts with eIF3g and demonstrate that the CTIF-eIF3g interaction is important for the formation of the CT initiation complex and efficient CT.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Plasmids pCMV-Myc-CBP80, pcDNA3-FLAG-CTIF, pmCMV-Globin (Gl), Norm, or Ter, pmCMV-glutathione peroxidase 1 (GPx1), Norm, or Ter, and pcCMV-MUP mRNA were previously described (4, 5). pRL-CMV was purchased from Promega. pcDNA-F/BoxB/R, pAN-EGFP, and pAN-EGFP-CTIF were kindly provided by Dr. Sung Key Jang.

To generate pCMV-Myc-eIF4E, which encodes Myc-tagged full-length human eIF4E, a Klenow filled-Notl fragment of pCMV-Myc (Clontech) was ligated to a Klenow filled-BamHI/HindIII fragment of pcDNA3-FLAG-eIF4E (5).

For bacterial production of glutathione S-transferase (GST)-fused human eIF3g, pGEX-eIF3g was constructed by ligating an Xhol/NotI fragment of pGEX-6p-1+Ndel (18) to an Xhol/NotI fragment of PCR-amplified eIF3g cDNA. Human full-length eIF3g cDNA was amplified from pcDNA3-FLAG-eIF3g (kindly provided by Prof. J. W. Hersey) and cloned as an XhoI/NotI fragment into the BamHI site of the vector pQE9 (Qiagen) (19) and expresses the active (His6)-tagged eIF3g construct as a 30 kDa protein in inclusion bodies. To express eIF3g in E. coli, the His-tagged eIF3g was purified using Ni-affinity chromatography from the inclusion bodies.

For bacterial production of His-eIF36g, the CTIF fragment (365–598), which encodes His6-tagged CTIF(365–598) with a cleavable tobacco etch virus protease site at the N terminus (19), a BamHI/HindIII fragment of pRSF-His vector (kindly provided by Dr. H. K. Song) was ligated to a BamHI/HindIII fragment of a PCR product amplified from pcDNA3-FLAG-CTIF using two oligonucleotides, 5′-AAGGGATCCACACTCCCAGGACAC-3′ (sense) and 5′-GAAGGCACGTCGAGCTGTACAGGG-3′ (antisense), where the underlined nucleotides specify the BamHI site.

Yeast Two-hybrid System—Yeast two-hybrid system analysis was performed with a GAL4 DNA-binding domain (BD)-fused CTIFAN (BD-CTIFΔN) and a GAL4 activation domain (AD)-fused eIF3 subunit, as described previously (20). The CTIFΔN expressed an N-terminally truncated version of CTIF spanning amino acids from 306 to 598. All plasmids that encoded GAL4 AD-fused eIF3 subunit were generously provided by J. W. Hersey. The BD-CTIFΔN protein and each of the AD-eIF3 subunits were co-expressed in the yeast strain PBN204 (MATa, pGAL1-lacZ, pGAL1-URA3, pGAL2-ADE2, trp1-901, leu2-3, gal1Δ, gal80d). PBN204 contains URA3, ADE2, and β-galactosidase reporter genes and trp1 and leu2 as selection marker genes (Panbionet Inc.). Co-transformation of pBCT-polypyrrolidiminate tract-binding protein (PTB) and pACT2-PTB and that of pBCT (Panbionet, Inc.) and pACT2 (Clontech) served as a positive and negative control, respectively, for the protein-protein interaction (21).

Cell Culture and Transfections—HeLa, HEK293T, and HEK293FT cells were grown in DMEM (Hyclone) containing 10% fetal bovine serum (Hyclone). Cells were transiently transfected with 100 nm in vitro-synthesized siRNA (Invitrogen) or indicated plasmids using either Oligofectamine (Invitrogen) or TurboFect (Fermentas), respectively. Control siRNA, Upf1 siRNA, and CTIF siRNA sequences have been reported previously (5). Endogenous eIF3b, eIF3c, and eIF3g were down-regulated using 5′-r(UCAAACCUCUUAACGGAUUU)d(TT)-3′, 5′-r(UGACCUAGAGGACUACUU)d(TT)-3′, and 5′-r(CUGGAGACUUAUGCGA)d(TT)-3′, respectively.

Protein Expression, Purification, and GST Pulldown Assay—Plasmids expressing GST, GST-eIF3g, or GST-AMSH were transformed into Escherichia coli BL21(DE3)pLysS strain. Isopropyl β-D-thio-galactoside (1 mm) was added to the culture to induce GST-fused protein expression, when the absorbance at 600 nm (A600) reached 0.5. The cultures were then incubated for an additional 3 h at 37 °C.

Recombinant His-CTIF(365–598) was overexpressed in E. coli BL21(DE3)pRIL by the addition of 1 mm isopropyl β-D-thio-galactoside when the A600 value was 0.5. After additional cultivation at 18 °C for 24 h, cells were harvested, resuspended in lysis buffer (50 mm Tris-HCl (pH 8.0), 100 mm NaCl, 5% (w/v) glycerol, and 1 mm phenylmethanesulfonyl fluoride (PMSF)), and sonicated. Total cell extracts were loaded onto a Hi-trap nickel nitriotropic acid column (GE Healthcare), and then the column was washed with the same buffer. The bound His-CTIF(365–598) was eluted at ~250 mm imidazole with buffer A (50 mm Tris-HCl (pH 8.0) and 5% (w/v) glycerol). Subsequent purification was conducted on a Hi-trap Q FF anion exchange column (GE Healthcare), and the protein was eluted at ~350 mm NaCl with buffer A.

In vitro GST pulldown assays were performed using recombinant GST, GST-eIF3g, and GST-AMSH. E. coli extracts expressing GST-tagged proteins and purified recombinant His-
CTIF(365–598) were mixed and incubated in 500 μl of binding buffer (10 mM Tris-HCl (pH 8.0), 10% (v/v) glycerol, 150 mM NaCl, 0.1% Triton X-100, 2 mM benzamidine, 1 mM PMSF, and 0.001% bovine serum albumin (BSA)) at 4 °C for 1 h. Reactions were incubated with Glutathione-Sepharose 4B resin (GE Healthcare) at 4 °C for 1 h. The resin was washed five times with binding buffer. The resin-bound proteins were resolved by SDS-PAGE and subjected to Western blotting.

Immunoprecipitation and Western Blotting—Immunoprecipitation and Western blotting were performed as described previously (5, 20, 22). The following antibodies were used: FLAG (Sigma), Myc (Calbiochem), His6 (GE Healthcare), GST (Amersham Biosciences), CTIF (5), eIF3a (Cell Signaling), eIF3b (Santa Cruz Biotechnology), eIF3c (Santa Cruz Biotechnology), eIF3g (Biologend), eIF4E (Cell Signaling), GFP (Santa Cruz Biotechnology), Upf1 (gift from L. E. Maquat), eIF4GI (gift from S. K. Jang), and β-actin (Sigma). Antibody against human CBP80 was raised in rabbits using the synthetic peptide RQH-KRRSDDDDRSSDRKD (Peptron).

Far Western Blotting—Far Western blotting was performed using purified rabbit eIF3 (23, 24), recombinant His-CTIF(365–598), and recombinant GST-eIF3g, GST-AMSH, and BSA (New England Biolabs). Briefly, the purified eIF3 was resolved by SDS-PAGE and transferred to a Hybond ECL nitrocellulose.
membrane (Amersham Biosciences). Each membrane was incubated for 24 h at 4 °C in blocking buffer (100 mM Tris (pH 7.5), 100 mM potassium acetate, 2 mM magnesium acetate, 0.1 mM EDTA, 10% glycerol, 1 mM PMSF, 1 mM benzamidine, and 0.05% Tween 20) containing 5% skimmed milk. The membranes were then further incubated for 24 h at 4 °C in blocking buffer containing 5 μg of purified His-CTIF(365–598). The membrane was then analyzed by Western blotting using α-FLAG antibody.

Polysome Fractionation—HEK293FT cells (two 150-mm culture dishes) were transiently transfected with the indicated siRNA. Three days after transfection, cytoplasmic extracts of the cells were prepared and then subjected to polysome fractionation as described previously (5).

Reverse Transcription (RT)-PCR—RT-PCRs of Gl, GPx1, MUP, RLuc, and SMG7 mRNAs using single-stranded cDNA, specific oligonucleotides, and [α-32P]dATP (PerkinElmer Life Sciences) have been described previously (5, 20, 22, 25).

F/BoxB/R mRNAs were amplified using two oligonucleotides: 5‘-CAACACCCCCA ATCTTG-3’ (sense) and 5‘-CTTTCCG CCGCACTTGGCC-3’ (antisense).

Dual Luciferase Assay—RLuc and firefly luciferase activities were measured as described previously (22, 26).

RESULTS

C-terminal Half of Human CTIF Directly Interacts with eIF3g—Our previous findings showed that CTIF interacts with CBP80 via its N-terminal half and associates with eIF3, likely via the MIF4G domain in its C-terminal half (5). However, whereas the direct interaction between CBP80 and CTIF has been demonstrated by the GST pulldown assay, the CTIF-eIF3 association was only suggested by immunoprecipitations (IPs) (5). Therefore, we aimed to clearly determine whether CTIF interacts with eIF3 directly or indirectly, and, if directly, which subunit of eIF3 interacts with CTIF. To this end, we first conducted yeast
two-hybrid analysis using GAL4 DNA-binding domain (BD)-fused human CTIF/H9004N, which contains the C-terminal half of CTIF, and a GAL4 transcription AD-fused eIF3 subunit (Fig. 1A). Specific interactions between BD-CTIF/H9004N and AD-eIF3 would direct the expression of LacZ, Ura3, and Ade2 in yeast cells. The results showed that yeast cells expressing both BD-CTIF/H9004N and AD-eIF3g exhibited the blue color in the filter assay and growth on selective media (Fig. 1A). Although we could not observe the expression of each AD-fused eIF3 subunit in yeast by Western blotting because of weak promoter (data not shown), our results suggest that CTIF/H9004N and eIF3g specifically interact in vivo.

The interaction between CTIF and eIF3g was further demonstrated by two independent approaches: in vitro GST pull-down assay (Fig. 1B) and far Western blotting (Fig. 1, C and D). The results of the GST pulldown assay showed that GST-eIF3g, but neither GST only nor GST-AMSH, which served as a negative control, interacted with purified recombinant His-CTIF(365–598) (Fig. 1B), thus indicating the direct interaction between CTIF(365–598) and eIF3g. In addition, the results of far Western blotting using purified rabbit eIF3 complex and, as a probe, purified recombinant His-CTIF(365–598) showed that His-CTIF(365–598) specifically reacted with a single protein that corresponded to eIF3g or eIF3h in size (Fig. 1C, right panel). The amount and integrity of purified eIF3 and BSA, which served as a negative control, were determined by Coomassie Blue staining (Fig. 1C, left panel). As observed in other reports (23, 24), the largest eIF3 subunit, eIF3a, was proteolyzed during the purification of the eIF3 complex from rabbit reticulocyte lysate. Furthermore, the results of far Western blotting using BSA, recombinant GST-AMSH, GST-eIF3g, and, as a probe, recombinant His-CTIF(365–598) showed that His-CTIF(365–598) specifically reacted with GST-eIF3g (Fig. 1D).

Altogether, these results clearly indicate that CTIF(365–598) directly interacts with eIF3g.

CTIF Serves as an Adaptor Protein to Link CBP80 and eIF3—Based on the CTIF-eIF3g interaction, we next aimed at characterizing the CTIF- or eIF3g-containing protein complex. To this end, we performed IPs using extracts of cells expressing FLAG-CTIF (Fig. 2A) or FLAG-eIF3g (Fig. 2B). Previous studies showed that although the CT complex contains CBP80/20, CTIF, and eIF3, the ET complex contains eIF4E, eIF4GI/II, and eIF3 (5). The IP results revealed that the CBP80 and the eIF3 subunits, eIF3a, eIF3b, eIF3c, and eIF3g, but not eIF4E, co-immunopurified with FLAG-CTIF in an RNase A-resistant manner (Fig. 2A, upper panel), suggesting that CTIF and eIF3g co-exist in the CT complex. The sufficient removal of endogenous mRNA by RNase A treatment was demonstrated by measuring the level of endogenous GAPDH mRNA (Fig. 2A, lower panel). The reciprocal IP to immunopurify FLAG-eIF3g revealed that
all tested translation factors co-immunopurified with FLAG-eIF3g in an RNase A-resistant manner (Fig. 2B). Because both CT and ET use eIF3 as a common factor to recruit ribosomes (1, 4, 5, 14), it is reasonable that eIF3g associates with both CT and ET initiation complexes.

Based on a previous report that the N-terminal half of CTIF interacts with CBP80 (5) and our findings in this study that (i) the C-terminal half of CTIF interacts with eIF3g (Fig. 1) and (ii) the CTIF-immunoprecipitated complex contains CBP80 and eIF3 (Fig. 2A), we speculated that CTIF may function to link CBP80 and eIF3 during CT. Thus, the down-regulation of CTIF would abolish an interaction between CBP80 and eIF3, without affecting the association between eIF4E and eIF3. To test this hypothesis, we performed IPs using extracts of cells expressing either Myc-CBP80 or Myc-eIF4E and depleted of endogenous CTIF by siRNA. The results showed that CTIF, eIF3a, eIF3b, and eIF3c, but not eIF4GI, co-immunopurified with Myc-CBP80 upon treatment with a nonspecific “Control” siRNA (Fig. 2C). However, a reduced or undetectable amount of co-immunoprecipitated eIF3 subunits was observed upon CTIF down-regulation (Fig. 2C). On the contrary, eIF4GI, eIF3a, eIF3b, and eIF3c co-immunopurified with Myc-eIF4E in a CTIF-independent manner (Fig. 2D). All these results strongly support the idea that CTIF serves as an adaptor protein to link CBP80 and eIF3 within the CT complex.

**Down-regulation of CTIF Triggers Redistribution of CBP80 from Polysome Fractions to Subpolysome Fractions**—The functional relevance of the CBP80/20-CTIF-eIF3 protein interac-

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**FIGURE 4. Down-regulation of eIF3g inhibits NMD.** HeLa cells were depleted of eIF3b, eIF3c, eIF3g, or Upf1 using specific siRNAs. Two days later, cells were retransfected with (i) pmCMV-Gl either Norm or Ter, (ii) pmCMV-GPx1 Norm or Ter, (iii) phCMV-MUP, and (iv) pRL-CMV. A, Western blotting demonstrating the efficient down-regulation of each indicated protein. B, RT-PCR of Gl mRNAs. The levels of Gl mRNAs were normalized to the levels of MUP mRNAs. The normalized levels of Gl Norm mRNA in the presence of each siRNA were set to 100%. C, RT-PCR of GPx1 mRNAs. As in B, except that the GPx1 mRNAs were analyzed by RT-PCR. D, RT-PCR of RLuc mRNAs. The levels of RLuc mRNAs were normalized to the levels of endogenous SMG7 mRNAs. The normalized level of RLuc mRNA in the cells co-transfected with pmCMV-Gl Norm, pmCMV-GFx1 Norm, and Control siRNA was set to 100%. E, translational efficiency of RLuc mRNA in the cells co-transfected with pmCMV-Gl Norm, pmCMV-GFx1 Norm, and Control siRNA was set to 100%.
tions was assessed using polysome fractionation (Fig. 3). Because CTIF down-regulation abolishes the association between CBP80 and eIF3 (Fig. 2C), CTIF down-regulation may lead to the inhibition of CT, consequently reducing the level of polysome-associated CBP80. To test this hypothesis, HEK293FT cells were either treated with CTIF siRNA to known down endogenous CTIF or treated with a nonspecific control siRNA. The cytoplasmic extracts of cells were separated by polysome fractionation, and the relative distribution of endogenous CBP80 and eIF4E was analyzed by Western blotting (Fig. 3).

The results showed that the level of endogenous CTIF was down-regulated to 15% of normal (Fig. 3, A and B, compare the intensity of CTIF protein). Intriguingly, although both CBP80 and eIF4E were detected in most fractions upon treatment with a nonspecific control siRNA, CBP80 but not eIF4E was redistributed from the polysome fractions to subpolysome fractions upon CTIF down-regulation (Fig. 3A and B). The intensities of endogenous CBP80 and eIF4E in each fraction were quantitated, and the relative distributions of endogenous CBP80 (Fig. 3C) and endogenous eIF4E (Fig. 3D) in each fraction are shown graphically. Therefore, taking our findings in Fig. 2 into account, it is most likely that CTIF recruits ribosomes into the CT complex via its interaction with eIF3.

Down-regulation of Endogenous eIF3g Abolishes NMD of PTC-containing Globin mRNA and GPx1 mRNA—It is well established that NMD of PTC-containing mRNAs takes place during CT but not ET (4, 10). Because recruitment of the eIF3 complex via the CTIF-eIF3g interaction is important for CT (Figs. 2 and 3), an efficient NMD might require CTIF-mediated eIF3 recruitment. We therefore analyzed the change of NMD efficiency upon down-regulation of endogenous eIF3 subunits. To this end, HeLa cells were transfected with siRNA against one of the endogenous eIF3 subunits, siRNA against Upf1, a key factor for NMD (11, 12), or a nonspecific Control siRNA. Two days later, cells were transiently re-transfected with plasmids expressing the following: (i) /H9252 globin (Gl) mRNA, either normal (Norm) or PTC-containing at the 39th amino acid position (Ter); (ii) glutathione peroxidase 1 (GPx1) either normal (Norm) or PTC-containing at the 46th amino acid position (Ter); (iii) mouse urinary protein (MUP) mRNA, which served to control for variations of transfection and RNA harvest; and
(iv) *Renilla* luciferase (RLuc) mRNA, which allowed for quantitative measurement of the overall translation efficiency (Fig. 4) (4–6, 10, 20, 22).

Western blotting results revealed that the levels of endogenous eIF3b, eIF3c, eIF3g, and Upf1 were down-regulated to 21, 25, 32, and 31% of normal, respectively (Fig. 4A). The RT-PCRs using specific oligonucleotides in the presence of [\(\alpha\-^{32}\text{P}\)]dATP showed that down-regulation of eIF3b, eIF3c, eIF3g, and Upf1 abrogated the NMD of PTC-containing Gl mRNAs by 3.0-, 4.9-, 2.6-, and 5.1-fold, respectively (Fig. 4B), and the NMD of PTC-containing GPx1 mRNAs by 2.7-, 3.1-, 2.4-, and 3.5-fold (Fig. 4C), respectively. These results suggest that eIF3 is involved in CT and, as a consequence, NMD.

Under the same conditions, we also analyzed the overall translation efficiencies by measuring the ratio of RLuc activity to RLuc mRNA. It is generally considered that overall translation efficiency mirrors the ET efficiency, as CT efficiency is very weak by comparison (1, 4, 5). The results showed that, although down-regulation of eIF3b or Upf1 had no significant influence on the abundance of RLuc mRNA, down-regulation of eIF3c and eIF3g showed an increase of the level of RLuc mRNAs by 3- and 1.5-fold, respectively, for unknown reason(s). The ratio of RLuc activity to RLuc mRNA, overall translation efficiency, was not affected by the down-regulation of eIF3b, eIF3c, and eIF3g. However, down-regulation of Upf1 slightly increased the level of overall translation of RLuc mRNAs, consistent with the previous findings (27). All these results suggest that, under our conditions, transient and temporal down-regulation of eIF3 subunits by siRNA is not sufficient for inhibition of overall translation. On the contrary, the level of down-regulation of eIF3 subunits in this study was sufficient for inhibition of CT and consequently NMD, which suggests that CT is more sensitive to the level or activity of eIF3 than ET.

**Artificially Tethered CTIF to an Intercistronic Region Directs the Translation of a Downstream Cistron in an eIF3-dependent Way**—The results in Fig. 4 suggest that CT is inhibited by the down-regulation of eIF3 subunits under the conditions where ET was minimally affected. However, we cannot completely rule out the possibility of an indirect effect by eIF3 down-regulation. Therefore to clearly address the direct role of CTIF-eIF3 interaction in CT, we employed a tethering assay using the λN/BoxB derived from λ bacteriophage (28). Indeed, it has previously been shown that when the middle region of eIF4GI is tethered to the intercistronic region of a dicistronic mRNA it can recruit ribosomes to activate the translation of the downstream cistron (29).

HEK293T cells depleted of eIF3b, eIF3c, or eIF3g were transiently co-transfected with tethering dicistronic reporter plasmid expressing dicistronic F/BoxB/R mRNAs (Fig. 5A) and effector plasmid expressing either AN-EGFP or AN-EGFP-CTIF. Western blotting results showed that the levels of endogenous eIF3b, eIF3c, and eIF3g were reduced to 39, 41, and 36% of normal, respectively, and that comparable levels of AN-EGFP and AN-EGFP-CTIF were expressed (Fig. 5B). RT-PCRs using
specific oligonucleotides and [α-32P]dATP revealed that tethering of δN-EGFP-CTIF increased the F/Box/B mRNA in abundance by 1.7- to 2.4-fold (Fig. 5C) in an eIF3-independent manner, suggesting that CTIF may contribute to another step of post-transcriptional gene regulation. More intriguingly, tethering of δN-EGFP-CTIF increased the relative RLuc activity (the ratio of RLuc activity to firefly luciferase activity) by 4-fold, compared with tethering of δN-EGFP. The increase of the relative RLuc activity was abolished by down-regulation of eIF3 subunits (Fig. 5D). Considering that eIF3 down-regulation had a marginal influence on ET efficiency under our conditions (Fig. 4), all these results suggest that a tethered CTIF recruits ribosomes via its interaction with eIF3, consequently triggering translation of the downstream cistron.

**DISCUSSION**

Here, we propose that CTIF serves as an adaptor protein to bridge CBP80/20 at the 5′-end of mRNA and eIF3 during CT. More specifically, the N-terminal half and C-terminal half of CTIF directly interact with CBP80/20 (5) and eIF3g (in this study), respectively. The CTIF-eIF3g interaction mediates the entire (or functional) eIF3 complex recruitment, which in turn leads to the ribosome loading onto mRNAs for efficient CT (Fig. 6). To support this idea, recent studies on the assembly of the eIF3 complex (16, 30) showed that eIF3g is not part of a reconstituted functional core of mammalian eIF3. Indeed, eIF3g is located on the periphery of the eIF3 complex and is not involved in interactions among three stable subcomplexes of eIF3. Therefore, during CT, eIF3g might provide a docking platform for the CTIF-eIF3 interaction.

Although we could not observe any significant interaction between CBP80 and eIF4G/II in this study (Fig. 2C) and in a previous report (5), we cannot rule out the possibility that certain environmental changes or stresses may trigger the formation of an alternative complex, in which eIF4G/II replaces CTIF during CT (Fig. 6), as proposed by others (4, 13).

After CT, CBP80/20 is replaced by eIF4E (1, 4, 6, 7). Subsequently, eIF4E interacts with eIF4G, which in turn recruits eIF3 via a direct interaction between eIF4G and eIF3e, another eIF3 subunit (31). The use of a different eIF3 subunit for recruiting a functional eIF3 complex during CT and ET via CTIF-eIF3g interaction and eIF4G/II-eIF3e interaction, respectively, may contribute to the differences in the regulation, efficiency, and biological roles of CT and ET. This view is supported by the fact that ET is able to respond quickly to changes in physiological conditions or to cellular stresses, whereas CT is relatively resistant to these changes (32–35). Further studies will be required to fully dissect the mechanistic differences between CT and ET.

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