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Inhibition of Nonsense-mediated mRNA Decay by the Natural Product Pateamine A through Eukaryotic Initiation Factor 4AIII*[S]

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Nonsense-mediated mRNA decay (NMD) in mammalian cells is a key mechanism for the removal of mRNA containing premature stop codons and is mediated by the coordinated function of numerous proteins that dynamically associate with the exon junction complex. The information communicated by these interactions and the functional consequences from a mechanistic perspective, however, are not completely documented. Herein, we report that the natural product pateamine A (PatA) is capable of inhibiting NMD through direct interaction with eIF4AIII, which is independent of its inhibition of translation initiation. Furthermore, we have characterized the mechanisms by which PatA and cycloheximide modulate NMD. Unlike CHX, PatA was found to inhibit NMD by a novel mechanism that is independent of the phosphorylation of Up-frameshift protein 1.

In mammalian cells, nonsense-mediated mRNA decay (NMD)[2] is one of the key RNA surveillance mechanisms to specifically degrade mRNA with premature stop codons (PTCs) located more than 50–55 nucleotides upstream of the final exon-exon junction. PTCs can be formed in genes containing a nonsense mutation or frameshift mutation or as a result of errors that occur during transcription or RNA splicing (1–4). After splicing, the exon junction complex (EJC) imprints mature mRNAs 20–24 nucleotides upstream of the exon-exon junction (5). The EJC is a dynamic multiprotein complex that plays an essential role in NMD. The core EJC proteins eIF4AIII, Y14, Magoh, and MLN51 form a platform to interact with several other proteins in a dynamic fashion to regulate NMD (6). The spatial-temporal regulation of NMD by the EJC and its partner proteins has been extensively investigated, leading to the proposition of the "linear interaction model" (4, 7). According to this model, deposition of the EJC onto mRNA causes the Y14-Magoh and eIF4AIII complex to effectively recruit Upf3 that interacts with Upf2. Although the mechanism of loading Upf1 onto EJC is poorly understood, it has been shown that phosphorylation and dephosphorylation of Upf1 by SMG-1, -5, -6, and -7 affect the interaction with the EJC complex through Upf2 or Upf3, causing dissociation from ribosome. Association of Upf1 with the EJC remodels the EJC to expose the mRNA for degradation (8–13). Most recently, SMG6 was identified as an endonuclease to cleave the aberrant mRNA near the PTC (14).

It has been demonstrated that NMD occurs in the cytoplasm and is coupled with translation to inspect mRNA for PTCs through ribosome scanning (15–19). The fact that all known translation inhibitors were found to block NMD supports this model (17, 20). Some studies have suggested that ribosome scanning that displaces EJCs occurs during the "pioneer round" of translation mediated by the association of nuclear cap-binding proteins (CBPs) such as CBP20 and CBP80 with NMD-targeted mRNAs as opposed to the subsequent "steady-state" translation via eukaryotic translation initiation factor 4A (21). However, another study suggested that NMD can occur independently of the CBPs using encephalomyocarditis virus and hepatitis C virus (HCV) internal ribosome entry site (IRES)-initiated mRNA, which is translated independently of the cap (22, 23).

Pateamine A (PatA) was first isolated from the marine sponge *Mycale* sp. and shown to possess potent anti-proliferative and immunosuppressive activities (24–28). Recently, we and others demonstrated that PatA specifically binds to eukaryotic translation initiation factor 4A I/II to stabilize its closed conformation, leading to the inhibition of eukaryotic translation initiation by perturbing the eIF4F complex, which is composed of translation initiation factors eIF4E, eIF4G, and eIF4AI/II (29–32). The eIF4A proteins are the prototypical D-E-A-D box proteins, having RNA-dependent ATPase activity and ATP-dependent RNA helicase activity (33). In addition to eIF4AI/II, PatA was also shown to bind the closely related homolog eIF4AIII and stimulate its ATPase activity (30, 32). The main cellular function of eIF4AIII is to serve as a core component of the EJC (34–38).

Given the known cellular function of eIF4AIII in NMD, we have sought to distinguish the two effects of PatA as medi-

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[2] The abbreviations used are: NMD, nonsense-mediated mRNA decay; PatA, pateamine A; DMDA-PatA, *desmethyl*,*desamino*-pateamine A; B-PatA, biotinylated PatA; TCR, T-cell receptor; HCV, hepatitis C virus; IRES, internal ribosome entry site; CrPV, the cricket paralysis virus; EJC, the exon junction complex; PTC, premature stop codon; CHX, cycloheximide; eIF, eukaryotic initiation factor; UPF, up-frameshift; CBP, cap-binding protein.

ated through perturbation of its different targets, eIF4AI/II and eIF4AIII, and to determine if PatA has an impact on NMD through its interaction with eIF4AIII in addition to its indirect effect on NMD via inhibition of eIF4AI/II-mediated translation initiation. Using a well studied T-cell receptor β (TCR-β) reporter system, we observed inhibition of NMD by PatA and its active analog *desmethyl*,*desamino*-PatA (DMDA-PatA) (26, 31) through specific interaction with eIF4AIII. We have demonstrated that this eIF4AIII-dependent inhibition of NMD by PatA is likely mediated through stabilization of the interaction between Upf1 and the ECJ complex. Furthermore, we found that PatA affected the dynamic association of Upf1 to the EJC without affecting Upf1 phosphorylation. This is in contrast to cycloheximide (CHX), a translation elongation inhibitor that also inhibits NMD. Together, these results shed new light on the regulation of NMD by the EJC and Upf proteins and suggest that PatA may serve as a new molecular probe of the NMD process.

MATERIALS AND METHODS

Reagents—PatA, DMDA-PatA, and biotinylated PatA were synthesized as previously reported (26, 27, 31). CHX and puromycin were purchased from Sigma-Aldrich. Antibodies were purchased from various commercial sources: antibodies against eIF4AI, UPF1, UPF2, UPF3b, Y14, Magoh, PABP, eIF4AIII, horseradish peroxidase-conjugated secondary antibodies, and ExactaCruz^{TM} D and F (Santa Cruz Biotechnology, Santa Cruz, CA); anti-FLAG (Sigma-Aldrich); and antibodies against phospho-(Ser/Thr)ATM/ATX substrate (Cell Signaling Technology). Antibodies against eIF4AIII were generously provided by Dr. Jens Lykke-Anderson.

Plasmid Constructs—pTCRβ minigene reporter vectors (WT/PTC) were previously described (39). HCV IRES and CrPV IRES sequences were amplified from HCV IRES and CrPV IRES dual luciferase plasmids (generously provided by Dr. Peter Sarnow, Stanford University) by PCR with primers: D1, tctgtcgacggcgacactccaccatag; D2, ccgtcgacctggtttttctttgaggt; D3, tctgtcgacaaagcaaaaatgtgatcttgctt; and D4, cctgtcgactatcttgaaatgtagcaggtaaa. pTCRβ vectors and PCR products of HCV IRES or CrPV IRES were digested with SalI restriction enzyme and ligated. pCI-FLAG-UPF3a, UPF3b vectors were constructed as previously described (40–42).

Cell Culture, Transfection, and Screening Stable Cell Lines—HeLa and HEK293T cells were cultured in low glucose Dulbecco's modified Eagle' medium (Invitrogen) plus 10% fetal bovine serum (Invitrogen). Plasmids pTCRβ WT/PTC, pHCVIRESTCRβ WT/PTC, or pCrPVIRES WT/PTC were transfected into HeLa cells with Lipofectamine 2000 (Invitrogen). Other vectors were transfected into HEK293T cells with SuperFect transfection reagent (Qiagen). To screen for stable cell lines, HeLa cells were split into 10-cm dishes 24 h after transfection and selected with 800 μg/ml G418 for a total of 3 weeks. At the end of the selection process, positive cell clones were maintained in normal media plus 200 μg/ml G418.

Pulldown Assays and Immunoprecipitation—For B-PatA pulldown assay, HEK293T cell lysate was prepared in 50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, and a mixture of protease inhibitors (Roche Applied Science). 300-μl aliquots of lysate were treated as indicated (Fig. 3*A*), with first incubation for 1 h at 4 °C with mixing, and second incubation for 2 h, followed by addition of pre-blocked (10 mg/ml bovine serum albumin) streptavidin-Sepharose beads and incubation for another 1 h at 4 °C. The beads were washed three times, each time with 1 ml of lysis buffer, resuspended in gel loading buffer, boiled for 5 min, and finally subjected to SDS-PAGE.

For immunoprecipitation, HEK293T or HeLa stable cell lines were treated with different compounds as indicated for 2 h, and then lysed in a lysis buffer (50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100 and protease inhibitors), including 100 μg/ml RNase A for protein interaction assay or 100 unit/ml RNAseOUT (Invitrogen) for samples containing RNA. Cell lysate was subsequently incubated with respective antibodies followed by addition of Protein G plus agarose (Santa Cruz Biotechnology) with incubation for 2 h or overnight. The beads were washed 4 times with the same buffer without the protease inhibitors. For Western blotting, 2× gel loading buffer was used to elute proteins. For Northern blotting analysis, RNA was extracted with 800 μl of TRIzol (Invitrogen) from each reaction sample.

mRNA Decay Assays—HeLa cells stably expressing different reporters were treated with different concentration of PatA, DMDA-PatA, or CHX for 2 h as described in the figure legends. Total RNAs were extracted with TRIzol and subjected to Northern blotting. For mRNA decay curve, HeLa cells stably expressing different NMD reporters were treated as indicated compounds (DMSO, CHX 100 μg/ml and DMDA-PatA 0.5 μM) in the presence of 5 μg/ml Actinomycin D for 0, 30, 90, and 180 min and analyzed for reporter gene transcript levels by Northern blotting.

Northern Blotting—Total RNA was resolved on a 1.2% agarose gel, and transferred onto GeneScreen Plus Nylon membrane in 10× SSC buffer. After blotting, the membranes were cross-linked under UV, washed with 2× SSC buffer for 5 min and dried at room temperature. The membrane was pre-hybridized in hybridization solution (50% formamide, 2% 50× Denhardt's Solution, 8% diethylpyrocarbonate-water, and 1% SDS) for at least 4 h at 42 °C. Products were amplified by primers: D5, 5′-CGAGTTGGTTCAGCTGCTGC; D6, 5′-ATCAGTTCCATAGGTTGGAATC (D5 and D6 for Neomycin); D7, 5′-AACACATGGAGGCTGCAGTCACCC; and D8, 5′-CGAGAACAGTCAGTCTGGTTCCTG (D7 and D8 for TCR-β), and labeled with [α-^{32}P]dCTP (Amersham Biosciences) via the Random Primers Labeling kit from Invitrogen, and then purified by G50 spin column from Amersham Biosciences. The ^{32}P-labeled probes were denatured with 2 mg of salmon sperm DNA, and then hybridized onto the membrane while rotating overnight at 42 °C. After hybridization, the membrane was washed with 2× SSC plus 0.1% SDS and 0.1× SSC plus 0.1% SDS sequentially, followed by autoradiography. ImageJ software (downloaded from rsbweb.nih.gov/ij/) was used to quantify the bands. GraphPad Prism 4 software was used to perform Student's *t* test analysis ($n = 3$).

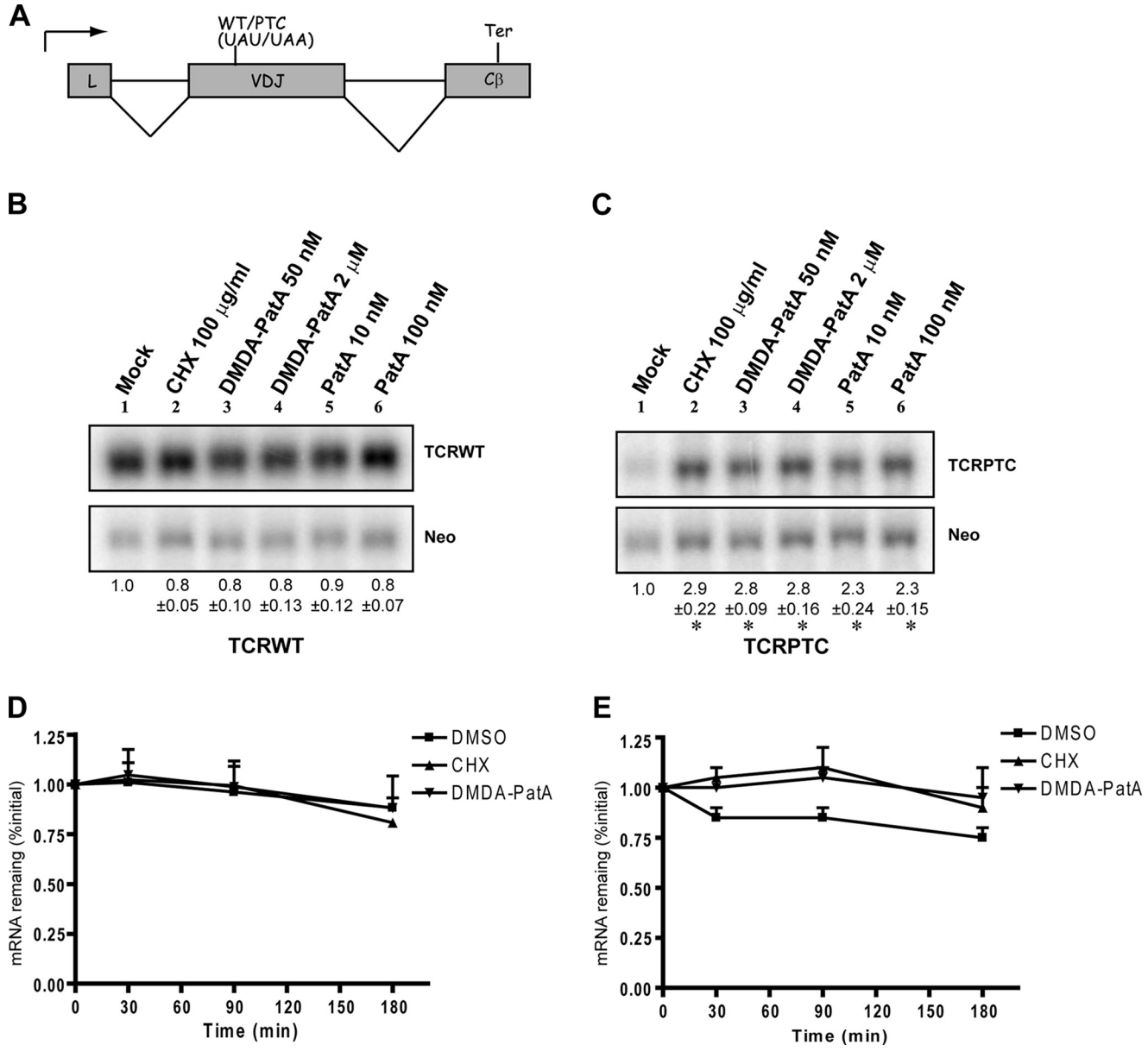

FIGURE 1. **Inhibition of NMD by PatA.** *A*, schematic depiction of the reporter vectors. *B* and *C*, Northern blot analysis of TCR-β mini gene expression from HeLa cells containing integrated mini TCR-β reporter genes with or without PTC in VDJ exon region (TCRWT/PTC). Cells were treated as indicated with different compounds for 2 h. Relative levels of mRNA are quantified, normalized to neomycin control, and expressed as the percentage of TCRWT/PTC without drug treatment as shown below the *bottom panel* ($n = 3$; $p < 0.05$). *D* and *E*, decay curves of TCRWT and TCRPTC mRNA. Cells were treated with DMSO, DMDA-PatA, or CHX in the presence of 5 μg/ml actinomycin D, respectively, for different times. Relative mRNA levels are quantified and normalized to nucleolin control. The values of relative mRNA were plotted as shown.

RESULTS

Inhibition of NMD by PatA—Several translation elongation inhibitors, including CHX and puromycin have been reported to block NMD (17, 20). Unlike CHX and puromycin, PatA specifically inhibits eukaryotic translation initiation rather than elongation (31, 32), prompting us to speculate whether PatA had any effect on NMD like CHX. Moreover, PatA also binds to eIF4AIII, a key component of the EJC complex, raising the possibility that PatA may affect NMD through its interaction with eIF4AIII. To examine if PatA affected NMD, we employed the TCR-β minigene model system (17, 39). These reporter genes were stably integrated into HeLa cells expressing transcripts without (TCRWT) or with a PTC (TCRPTC) (Fig. 1*A*), and mRNA level was monitored by Northern blot analysis after drug treatment. As shown in Fig. 1*B*, treatment with PatA (*lanes 5* and *6*) as well as other translation inhibitors did not affect wild-type TCR-β reporter transcripts in comparison with the control (Fig. 1*B*, *lane 1 versus lanes 2–6*). DMDA-PatA behaved similarly to PatA (*lanes 3* and *4 versus lanes 5* and *6*), and DMDA-PatA was employed in a majority of the subsequent experiments. However, PatA significantly increased TCR-β transcripts with a PTC similar to CHX (Fig. 1*C*, *lane 1 versus lanes 2–6*), suggesting that PatA and DMDA-PatA inhibit NMD.

Inhibition of NMD by PatA Is Independent of Its Effect on Translation Initiation—PatA and DMDA-PatA have been shown to inhibit eIF4AI (and eIF4AII)-dependent translation

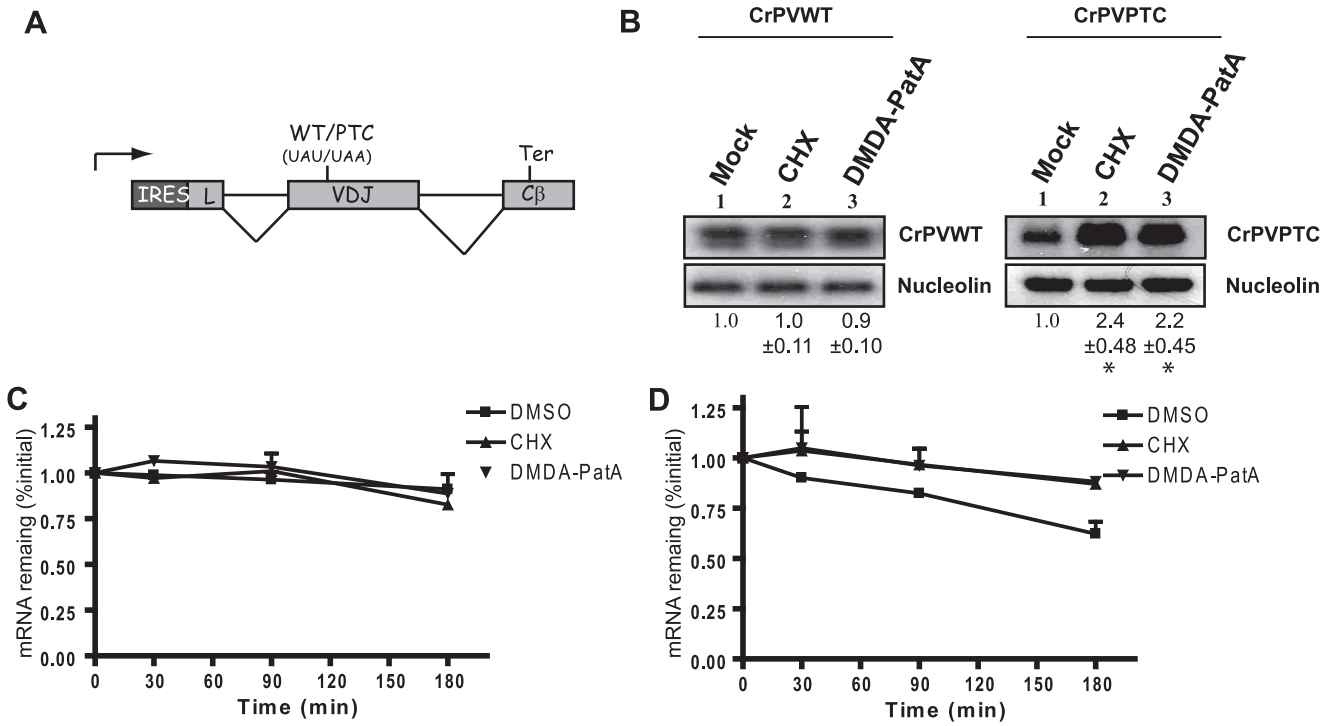

FIGURE 2. **Inhibition of NMD by PatA is independent of cap-mediated translation initiation.** *A*, schematic depiction of IRES-driven mini TCR-β gene reporter system. *B*, HeLa cells with stably integrated CrPVWT and CrPVPTC were treated as indicated for 2 h. Levels of mRNA from the reporter genes were detected by Northern blotting and were quantified, normalized against nucleolin (control), and expressed as the percentage of HCVWT/PTC or CrPVWT/PTC without drug treatment ($n = 3$; $p < 0.05$). *C* and *D*, decay curves of CrPVWT and CrPVPTC mRNA. Cells were treated with DMSO, DMDA-PatA, or CHX in the presence of 5 μg/ml actinomycin D, respectively, for different times. Relative mRNA levels are quantified, normalized to nucleolin control. The values of relative mRNA were plotted as shown.

initiation (31, 32), which may account for their inhibition of NMD. Alternatively, the inhibitory effect of PatA on NMD could also result from its interaction with eIF4AIII, thus interfering directly with the integrity of the EJC complex. The HCV and CrPV IRESes (Hepatitis C and Cricket Paralysis Virus internal ribosome entry sequences) have been demonstrated to initiate translation independent of eIF4F (43), especially eIF4AI/II, and to be resistant to PatA (supplemental Fig. S1) (31, 32). To distinguish between these possibilities, we designed and constructed new NMD reporter genes whose translation is driven by either the CrPV or the HCV IRES elements. Reporter plasmids containing both HCV IRES and CrPV IRES sequences in front of the TCRWT/PTC sequences were named HCVWT/PTC and CrPVWT/PTC, respectively (Fig. 2*A*). The reporter vectors containing PTCs were then allowed to stably integrate into HeLa cell genomic DNA. The resulting stable cell lines with integrated reporter genes gave similar results, ~2-fold increases, as TCRPTC (Fig. 1*C*) after CHX and DMDA-PatA treatments (Fig. 2*C* and supplemental Fig. S3). These results are consistent with those obtained from other transiently transfected reporters in HEK293T or HeLa cells (data not shown). In contrast, both DMDA-PatA and CHX did not affect the wild-type CrPV (Fig. 2*B*) and HCV (data not shown) reporters. Because both HCV IRES and CrPV IRES-mediated translation of the reporter is insensitive to PatA, these observations indicate PatA is capable of inhibiting NMD independent of its effects on translation initiation.

To exclude the possibility that the effect of PatA on NMD was mediated through enhancement of transcription of reporter mRNA carrying PTC, both TCRPTC/WT and CrPVPTC/WT integrated HeLa cells were pretreated with Actinomycin D (an inhibitor of general transcription) before treatment with DMDA-PatA or CHX (Figs. 1*D*, 1*E*, 2*C*, and 2*D*). The decay rates of mRNA bearing PTC were decreased in the present of DMDA-PatA or CHX when compared with control. However, we did not observe the same decrease in the rate of decay of wild-type mRNA, indicating that PatA inhibited NMD independent of transcription of the same mRNA.

PatA Does Not Affect the Integrity of the Core EJC through Binding to eIF4AIII—Having shown that the inhibition of NMD by PatA under the IRES-driven reporter system is independent of eIF4AI and eIF4AII-mediated translation initiation, we turned to eIF4AIII which has also been shown to bind to PatA (32). Using a biotinylated PatA (B-PatA) (31), eIF4AIII was efficiently captured from 293T cell lysates, and the amount of eIF4AIII captured was decreased when DMDA-PatA was preincubated as a competitor (Fig. 3*A*), confirming previous observations (32). In addition to eIF4AIII, the EJC component Y14 was also captured by B-PatA and exhibited similar sensitivity to competition by DMDA-PatA (Fig. 3*A*). Thus, PatA not only interacts with eIF4AIII, but may also indirectly affect the integrity of the EJC components.

The integrity of the EJC in the presence of DMDA-PatA was examined by co-immunoprecipitation of endogenous EJC components using eIF4AIII and Y14 antibodies. Treatment of

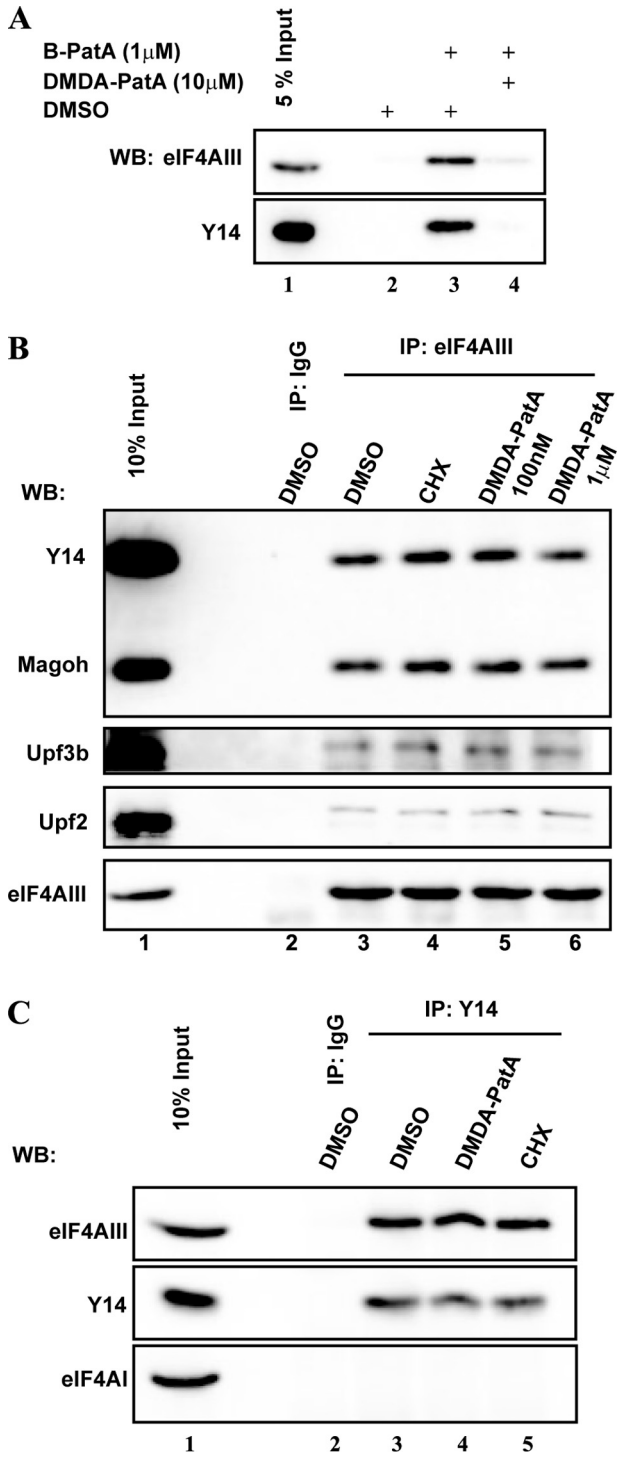

FIGURE 3. **PatA binds to eIF4AIII.** *A*, equal aliquots of HEK293T cell lysates were preincubated as indicated with either DMSO (*lanes 2* and *3*) or DMDA-PatA (*lane 4*), followed by incubation with DMSO (*lane 2*) or B-PatA (*lane 3* and *4*). Total cell lysates were loaded in *lane 1*. B-PatA-bound proteins were captured using streptavidin-agarose and resolved by SDS-PAGE. Proteins were visualized by immunoblotting with indicated primary antibodies. *B*, endogenous eIF4AIII was immunoprecipitated from HEK293T cells treated with DMSO, 100 μg/ml CHX, 100 nM and 1 μM DMDA-PatA, respectively, for 2 h. Immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted with indicated antibodies. *C*, endogenous Y14 was immunoprecipitated from lysates of HEK293T cells treated with either DMSO, 500 nM DMDA-PatA, or 100 μg/ml CHX for 2 h. Immunoprecipitated proteins were subjected to Western blot with indicated antibodies.

cells with either DMDA-PatA or CHX had no detectable effects either on the EJC complex containing Y14, eIF4AIII, and Magoh, or on the association of Upf2 and Upf3b with EJC complex (Fig. 3*B*). Furthermore, no change was seen in the interaction of endogenous Y14 and eIF4AIII under similar treatments (Fig. 3*C*).

PatA Stabilizes the Interaction of the EJC with RNA through Interaction with eIF4AIII—We and others have previously demonstrated that RNA-binding affinity of eIF4AI increases in the presence of PatA, and that the interaction between PatA and eIF4AI is dependent on the presence of RNA (30–32). Thus, the affinity of the EJC for RNA in the presence and absence of DMDA-PatA was examined by co-immunoprecipitation of reporter mRNA with eIF4AIII and Y14 in HeLa cells with integrated CrPVWT and CrPVPTC reporter genes (Fig. 4, *A* and *B*). After immunoprecipitation of eIF4AIII and Y14, the presence of TCR-β WT or PTC-containing reporter mRNA was detected by Northern blotting. For CrPVWT, treatment with both DMDA-PatA and CHX led to higher levels of eIF4AIII and Y14 bound to RNA in comparison to control (Fig. 4, *A* and *B*, *lanes 7* and *8 versus lane 6*). Neither DMDA-PatA nor CHX caused any changes in the total protein levels of eIF4AIII and Y14 as determined by immunoblotting (data not shown). Thus, the differences in the amounts of mRNA were not due to different amounts of eIF4AIII and Y14 captured. For the PTC-containing reporter, DMDA-PatA treatment dramatically enhanced TCR-β transcript bound to eIF4AIII or Y14 while CHX treatment had a smaller effect (Fig. 4, *A* and *B*, *lane 8 versus 6*). We note that for the PTC-containing reporter, treatment with DMDA-PatA or CHX caused increases in the level of reporter mRNA (Fig. 4, *A* and *B*, *lanes 3* and *4 versus lanes 1* and *2*) due to the inhibition of NMD (Fig. 2*C*). However, roughly equal amounts of eIF4AIII and Y14 were immunoprecipitated in each sample, indicating that the difference in the mRNA bound to EJC between DMDA-PatA and CHX treatments was due to enhancement in affinity of EJC to mRNA by these inhibitors. These results were also suggestive to potentially distinct mechanisms of NMD inhibition by DMDA-PatA and CHX.

PatA Enhances the Interaction of Upf1 with Other Components of the NMD Pathway Independent of Upf1 Phosphorylation—During NMD, Upf3, Upf2, and Upf1 are sequentially recruited to the EJC with Upf1 binding being a critical step for triggering mRNA degradation. Thus, the interaction of Upf1 with other proteins involved in NMD was determined upon DMDA-PatA and CHX treatments (Fig. 5). Endogenous Upf1 was immunoprecipitated, and levels of the bound Upf2, Upf3b, Y14, and eIF4AIII were all appreciably increased in the presence of either DMDA-PatA or CHX (Fig. 5*A*, *lane 3 versus lanes 4* and *5*). For control, we determined the levels of protein expression by Western blot, and no changes were observed between control and drug-treated samples (data not shown).

The interaction of Upf1 with EJC has been shown to be regulated through phosphorylation of Upf1 by SMG-1, a phosphatidylinositol 3-kinase-related protein kinase (12, 13, 44–48). Thus, the phosphorylation status of Upf1 was assessed with a phospho-(Ser) ATM/ATX substrate antibody after immunoprecipitation of Upf1 (49, 50). As shown in Fig. 5*B*, treatments of cells with CHX or puromycin (which also enhanced the

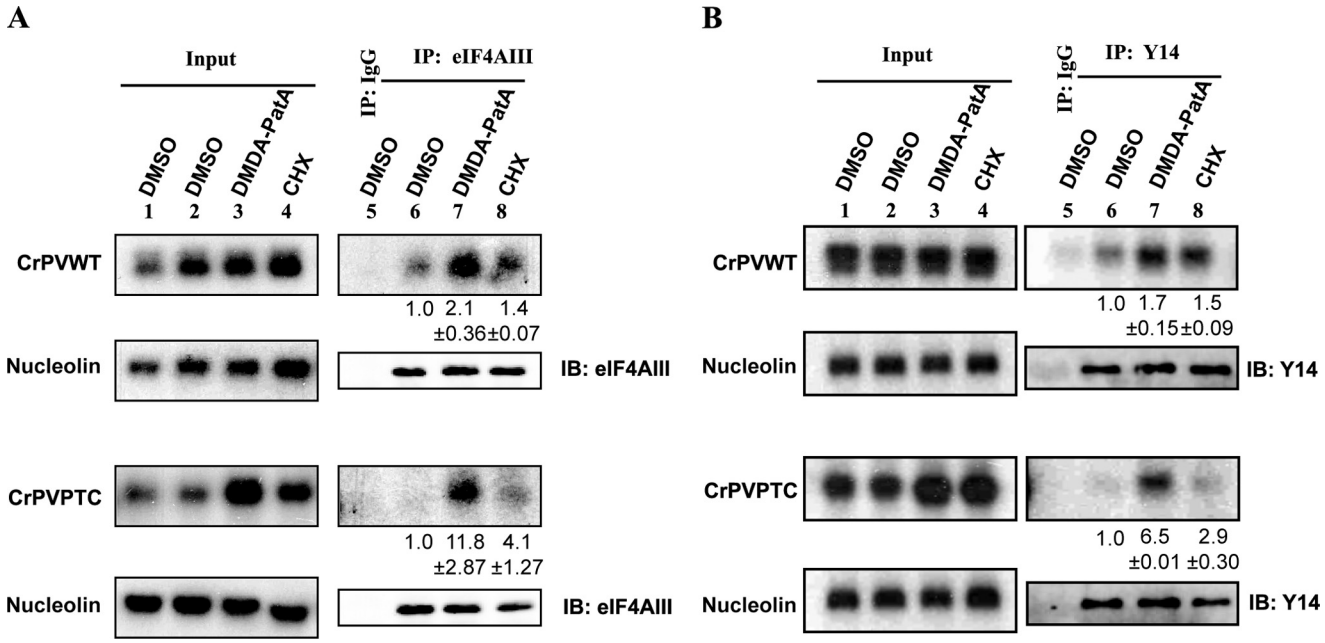

FIGURE 4. **PatA stabilizes the interaction of the EJC complex with RNA.** HeLa cells stably expressing CrPVWT/PTC were treated with either DMSO, 500 nM DMDA-PatA, or 100 μg/ml CHX for 2 h. Endogenous eIF4AIII (*A*) and Y14 (*B*) were immunoprecipitated (without RNase A) from different cell lysates. Captured RNAs were detected by Northern blotting analysis using indicated probes. *Inputs* are RNA from cell lysates before immunoprecipitation. 5% of immunoprecipitated proteins were analyzed by Western blotting with eIF4AIII and Y14 antibodies as control for Northern blotting. Bound mRNA was quantified, normalized against the corresponding control protein, and expressed as the percentage of mRNA from DMSO-treated cells.

interaction of Upf1 with the EJC, data not shown) increased the phosphorylation of Upf1 (Fig. 5*B*, *lanes 4* and *5 versus lane 2*). In contrast, DMDA-PatA did not appreciably affect Upf1 phosphorylation (Fig. 5*B*, *lane 3 versus lane 2*). Thus, the mechanism of NMD inhibition by DMDA-PatA is different from that of CHX and puromycin.

Increased Interaction between Upf1 and EJC in the Presence of PatA or CHX Is Mediated by Upf3b—It has been shown that Upf1 exerts its effect on NMD through its interaction with Upf2 or Upf3b, which are capable of mediating NMD either interdependently or independently (1, 41, 51). The interactions of Upf2 with the Upf3 and the EJC in the presence and absence of DMDA-PatA and CHX in 293T cells were examined by co-immunoprecipitation of Upf2 with other appropriate proteins. As shown in Fig. 6*A*, treatment of 293T cells with DMDA-PatA or CHX did not change the levels of Upf1, eIF4AIII, and Upf3b that were associated with Upf2. These observations thus ruled out Upf2 as a mediator of the inhibitory effect on NMD by DMDA-PatA or CHX.

We next determined whether the interaction between Upf3 and Upf1 as well as the EJC complex was affected by DMDA-PatA or CHX. Upf3 has two isoforms, Upf3a and Upf3b, alternatively known as Upf3 and Upf3x. Of the two isoforms of Upf3, Upf3b has been shown to play a critical role in mediating the interaction of Upf1 and EJC. We determined the relative amounts of Upf1 and EJC that were bound to Upf3b in the presence and absence of DMDA-PatA and CHX. FLAG-tagged Upf3a (as a control) and Upf3b were ectopically overexpressed in HEK293T cells that were treated with DMDA-PatA or CHX. Immunoprecipitation with anti-FLAG antibodies revealed that the interaction of Upf3b with Upf1 was enhanced by both DMDA-PatA and CHX (Fig. 6*B*). In contrast, there was no change in the interactions of Upf3a with Upf1 and other factors examined, except for a decrease in its interaction with Upf2 (Fig. 6*B*). These results suggest that both compounds increased the interaction of Upf1 with EJC through Upf3b rather than Upf3a or Upf2.

DISCUSSION

PatA inhibits eukaryotic translation initiation through binding to eIF4AI (31, 32) and likely disrupting the eIF4F complex (31). Herein, we have demonstrated that PatA is also capable of inhibiting NMD of mRNA through directly targeting eIF4AIII, a core component of the EJC. That is, for a specific mRNA such as the HCV or CrPV IRES-driven reporters where translation is refractory to PatA, NMD can still be inhibited by PatA. This finding is consistent with the previous observation that knockdown of both eIF4AI and eIF4AII had no effect on the NMD pathway (35). Although it is formally possible that inhibition of NMD is secondary to the induction of stress granules by PatA followed by the movement of PTC-containing mRNA to stress granules, this is unlikely as the two IRES-driven reporter mRNAs were insensitive to PatA for translation while the corresponding NMD were inhibited.

Although translation elongation inhibitors such as CHX and puromycin have been reported to inhibit NMD (17, 20), we demonstrated that PatA, a translation initiation inhibitor, is also capable of inhibiting NMD. It is thus not surprising that the molecular mechanisms of inhibition of NMD by PatA and CHX differ from each other. We observed that in the presence of DMDA-PatA, co-immunoprecipitation of PTC-containing mRNA with eIF4AIII or Y14 was increased compared with control, whereas no change or slight change was observed for the eIF4AIII or Y14-bound PTC-containing mRNA in the presence

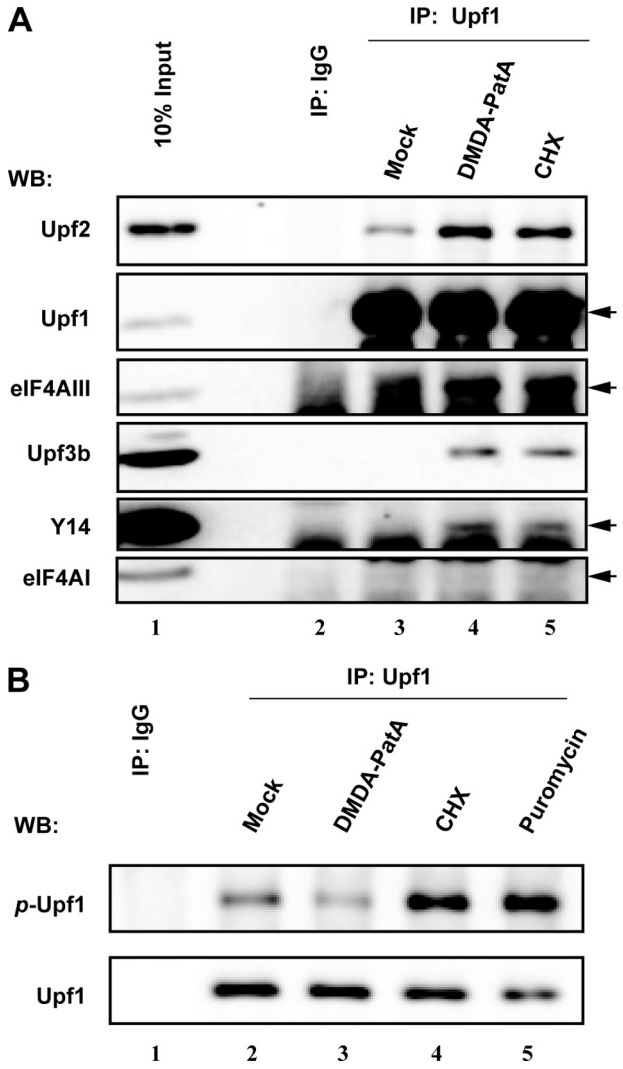

FIGURE 5. **PatA enhances the interaction of UPF1 with other components of NMD independent of phosphorylation.** *A*, HEK293T cells were treated with DMSO, 500 nM DMDA-PatA, or 100 μg/ml CHX for 2 h. Cell lysates (with RNase A) were used for immunoprecipitation with UPF1 antibody (goat normal IgG served as a control) followed by SDS-PAGE and immunoblotting using indicated antibodies. *Arrows* point to the position of the protein specifically recognized by its antibody. *B*, HEK293T cells were treated as in *A* along with 100 μg/ml puromycin for 2 h. Cell lysates (with RNase A) were subjected to immunoprecipitation with UPF1 antibody followed by Western blot using phospho-(Ser/Thr) ATM/ATR substrate antibody or UPF1 antibodies.

of CHX (Fig. 4). Furthermore, CHX induced phosphorylation of Upf1, whereas phosphorylation levels were unchanged, or slightly decreased under DMDA-PatA treatment (Fig. 5*B*). Together, these observations suggest the distinct mechanisms of inhibition of NMD by the two compounds. We surmise that these differences are a consequence of the direct action of PatA on EJC through its binding to eIF4AIII.

Activation of NMD has been proposed to occur by a series of protein-protein interactions. First, the presence of PTCs prevents the removal of EJCs from the mRNA, leading to recruitment of Upf3 through its interaction with the EJC core, which in turn recruits Upf2. Upf2 and Upf3a/b mediate the recruitment of Upf1 to EJC through their concurrent interactions with Upf1 and the binding site formed by Y14-Magoh and eIF4AIII (7), respectively, and their mutual interaction (7, 42, 52, 53). In addition, after the ribosome is stalled at the PTC, Upf1 can form SURF complex with eRF3, eRF1, and SMG-1. The dynamic association of Upf1 with two complexes plays an essential role in NMD (1, 54, 55), thus signaling the dissociation of ribosome from mRNA and the eventual degradation of the PTC-containing mRNA (1). In two independent studies, CHX has been shown to interfere with NMD and to cause phosphorylation of Upf1, respectively. In this study, we found that CHX induced phosphorylation of Upf1 led to its stable association with Upf3b and the associated EJC·mRNA complex. This is consistent with the observations made by two independent groups that the phosphorylated Upf1 significantly enhances the interaction with other components of NMD pathway (23, 51). Based on our experimental evidence, binding of PatA to eIF4AIII initiates a cascade of conformational changes by trapping eIF4AIII in an otherwise unstable conformation. This conformational change is propagated through other proteins of the EJC complex to Upf3 and subsequently Upf2 or Upf1. Thus, PatA and CHX act at different "ends" of the NMD process but achieve the same outcome: stabilization of the EJC·Upf1 interaction, perturbation of the dynamic interaction of Upf1 with the EJC complex, and the inhibition of NMD.

We have previously shown that PatA may act by "trapping" eIF4AI in a "closed" conformation, which leads to its increased enzymatic activity and prevents the necessary cycling through the different conformational states associated with its normal function (30). The increased association of RNA with the EJC in the presence of DMDA-PatA and the inhibition of NMD observed here suggest that PatA may exert similar conformational effects on eIF4AIII. The presence of PatA may trap eIF4AIII in the closed conformation as observed in structural studies of the EJC (56, 57) and does not permit its cycling through different conformations. Furthermore, this mode of action of PatA on eIF4AIII would again be consistent with proposed models for DDX proteins where a tightly regulated cycle of conformational changes and ATP hydrolysis are necessary for correct ribonucleoprotein particle remodeling (33). For both eIF4AI and eIF4AIII, PatA stimulates ATPase activity in addition to increasing RNA affinity. In fact, ATP stimulation of RNA binding to eIF4AI was no longer required in the presence of PatA (32), and we have proposed that PatA may decouple RNA release by eIF4AI during ATP hydrolysis (30). Loss of ATPase mutations in eIF4AIII have been shown to be inconsequential to NMD, whereas loss of RNA-binding mutations are necessary to disrupt NMD (38). Thus, the increased affinity of the EJC for RNA may be due to DMDA-PatA trapping eIF4AIII in a high RNA affinity conformation and not allowing for proper cycling of conformations needed for dynamic remodeling of the EJC. In addition, Magoh-Y14 inhibits the ATPase activity of eIF4AIII, which would increase eIF4AIII affinity for RNA, thus anchoring eIF4AIII onto RNA (36). Accordingly, under "normal" conditions, eIF4AIII binding to RNA is dependent on the integrity of the EJC core. However, in the presence of PatA, eIF4AIII is anchored onto RNA independent of ATP hydrolysis and trapped in a distinct conformation from its drug-free form. Given the extensive contacts between eIF4AIII and other components of the EJC complex, the alteration in the conformation of eIF4AIII inevitably causes conformational changes of other proteins within the EJC·Upf complex.

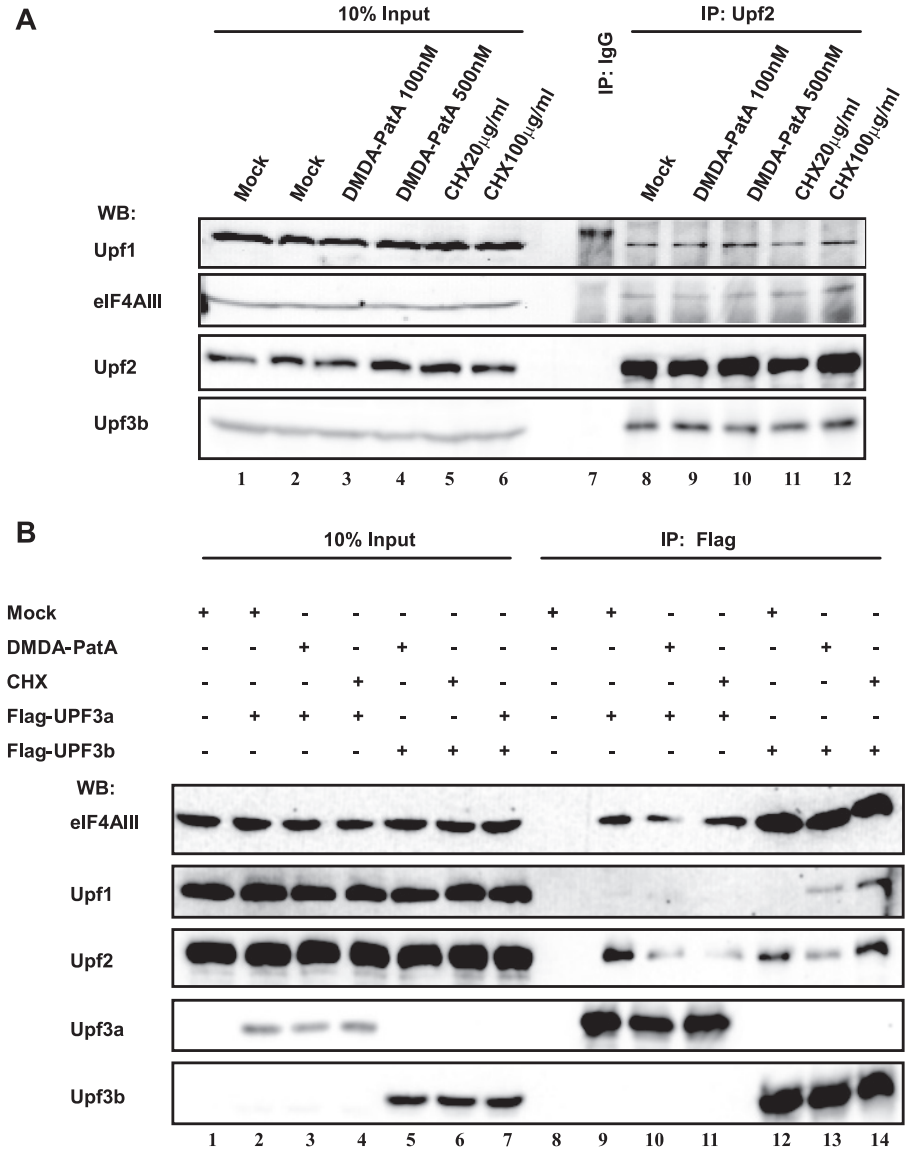

FIGURE 6. **Enhanced interaction of UPF complex with the EJC during translation inhibition is mediated by Upf3b.** *A*, HEK293T cells were treated with compounds for 2 h. Cell lysates (with RNase A) were subjected to immunoprecipitation and Western blot analysis. *B*, FLAG-tagged UPF3a or UPF3b were ectopically overexpressed in HEK293T cells, which were treated as indicated for 2 h. This was followed by immunoprecipitation using anti-FLAG antibodies conjugated to agarose and Western blot analysis using indicated antibodies.

The interaction of the EJC with mRNA is a crucial marker for targeting aberrant mRNA for NMD. We observed higher association of PTC-containing mRNA with eIF4AIII or Y14 under DMDA-PatA treatment compared with control (Fig. 4). Furthermore, co-immunoprecipitation analysis of EJC proteins demonstrated no changes in Magoh and Y14 when eIF4AIII was immunoprecipitated. Based on these observations, we conclude that the EJC is more tightly associated with the mRNA as it has been demonstrated that Y14 does not bind RNA on its own, and is associated with RNA through its binding to eIF4AIII (36). These results also support the notion that PatA directly acts on eIF4AIII and the enhanced association with PTC-containing RNA in the presence of DMDA-PatA is likely a consequence of the conformational change of eIF4AIII similar to eIF4AI (30). For CHX, the slight differences from DMSO control were observed for PTC-containing mRNA, in contrast to WT mRNA, where both DMDA-PatA and CHX treatments caused increased association of the EJC with mRNA. Our co-immunoprecipitation results also demonstrated that the interactions of the core EJC proteins were not changed from carrier control for CHX, suggesting that, in the presence of CHX, the EJC is not tightly associated with PTC-containing mRNA.

The increase in EJC association with WT reporter mRNA in the presence of CHX (Fig. 4) may be a consequence of translation inhibition where EJCs are not removed by the ribosome, whereas for DMDA-PatA the direct action of trapping eIF4AIII caused the similar end result. For CrPVPTC, unlike the situation for WT, the presence of the PTC induces NMD. Thus, lack of accumulation of EJCs under CHX treatment may indicate a normal NMD function of EJC removal is not inhibited, whereas as the global NMD process is inhibited, *i.e.* CHX inhibits a step following EJC removal. For DMDA-PatA, direct action on eIF4AIII results in increased EJC association with the RNA regardless of the presence of a PTC.

Given the multitude of proteins involved in the formation of the EJC·Upf ternary complex during NMD, we extensively examined the effects of DMDA-PatA on the association of Upf1 with the remaining complex by its co-immunoprecipitation with a number of proteins. Although an increase in interaction was seen with Upf2, eIF4AIII, and Y14 in the presence of CHX or DMDA-PatA when Upf1 was immunoprecipitated, no reciprocal increase in Upf1 was observed when Upf2 was pulled down, suggesting that Upf2 may not be a relevant mediator of the effect of DMDA-PatA on NMD. In contrast, consistent enhancement was seen for the interaction between Upf3b and Upf1 in reciprocal co-immunoprecipitation experiments. It is thus highly likely that the increase in the interaction between Upf1 and EJC is mediated by Upf3b independent of Upf2. This is also consistent with recent evidence that Upf1 is capable of independently interacting with Upf2 and Upf3b (51). Although a mutant of Upf1 (VV204–205DI) is functional in NMD and can be phosphorylated, it only interacts with Upf3b, but not Upf2 (51). Thus, the inhibitory effects of PatA and CHX on NMD are mediated by Upf3b to cause an increase in Upf1 associated with EJC and its phosphorylation in the case of CHX.

It has been reported that CHX can change the phosphorylation status of Upf1, which is also sensitive to phosphatidylinositol 3-kinase inhibitors (58). However, the mechanism of upregulation of Upf1 phosphorylation by CHX has remained unclear. Does CHX directly activate the kinase of Upf1, or is Upf1 phosphorylation indirectly caused by stabilization of the polysome? This question remains to be answered and is beyond the scope of this study. However, an important observation from our studies was that PatA enhanced the interaction of Upf1 with the EJC independent of Upf1 phosphorylation. This is consistent with our model in which the increase in Upf1 interaction with the EJC complex is mediated through conformational changes.

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