Replication-dependent histone (RDH) mRNAs have a non-polyadenylated 3′-UTR that ends in a highly conserved stem-loop structure. Nonetheless, a subset of RDH mRNAs has a poly(A) tail under physiological conditions. The biological meaning of poly(A)-containing (+) RDH mRNAs and details of their biosynthesis remain elusive. Here, using HeLa cells and Western blotting, qRT-PCR, and biotinylated RNA pulldown assays, we show that poly(A)+ RDH mRNAs are post-transcriptionally regulated via adenylation- and uridylylation-element-mediated mRNA decay (AMD). We observed that the rapid degradation of poly(A)+ RDH mRNA is driven by butyrate response factor 1 (BRF1; also known as ZFP36 ring finger protein–like 1) under normal conditions. Conversely, cellular stresses such as UV C irradiation promoted BRF1 degradation, increased the association of Hu antigen R (HuR; also known as ELAV-like RNA-binding protein 1) with the 3′-UTR of poly(A)+ RDH mRNAs, and eventually stabilized the poly(A)+ RDH mRNAs. Collectively, our results provide evidence that AMD surveils poly(A)+ RDH mRNAs via BRF1-mediated degradation under physiological conditions.

Histone proteins regulate gene expression (1, 2). Metazoan histone genes are classified into two types: (i) replication-dependent histone (RDH)1 genes, which express mRNAs with a highly conserved histone stem-loop (HSL) structure in the 3′-UTR, and (ii) replication-independent histone (RIH) genes, whose mRNAs contain a poly(A) tail. In general, RDH mRNAs are tightly regulated at the transcriptional and post-transcriptional levels during the cell cycle to ensure that an adequate amount of histone proteins is produced to pack the newly synthesized DNA in the S phase (1, 3, 4). At the onset of the S phase, stem loop–binding protein is cotranscriptionally recruited to nascent RDH pre-mRNAs via its direct interaction with the HSL located in the 3′-UTR. Then, U7 small nuclear ribonucleoprotein–protein complex is loaded onto a histone downstream element (HDE) located downstream of the HSL. Next, the sequence between HSL and HDE is cleaved and then trimmed. Finally, mature RDH mRNAs are exported from the nucleus into the cytoplasm to synthesize histone proteins. At the end of the S phase or under certain types of stress, actively translated RDH mRNAs undergo drastic messenger ribonucleoprotein remodeling for rapid degradation (3, 5).

Although the majority of RDH mRNAs have no poly(A) tail at the 3′-end, a subset of RDH mRNAs contains a poly(A) tail possibly because of the improper or inefficient 3′-end processing of the nascent RDH pre-mRNAs (6–11). In addition, some physiological processes such as differentiation and stress responses lead to the accumulation of poly(A)+ RDH mRNAs within the cell (6, 12–15). Such an improperly or inefficiently processed alternative form of RDH pre-mRNAs has a greater chance to make use of a putative polyadenylation signal downstream of the HSL. As a consequence, a conventional polyadenylation complex may be recruited for the 3′-end processing of the RDH pre-mRNAs, leading to formation of an extended 3′-UTR harboring a novel cis-acting element(s). Indeed, the 3′-end formation accompanying alternative polyadenylation or alternative splicing is known to induce a new 3′-UTR with a novel cis-acting element(s) (16, 17).

cis-Acting elements in 3′-UTRs of mRNAs affect the fate of mRNAs in multiple ways such as degradation, translation, and localization because these elements can serve as a binding platform for trans-acting factors such as RNA-binding proteins and noncoding RNAs (18). One of the best-characterized cis-acting elements is the adenylation- and uridylylation-rich element (ARE) found in the 3′-UTR of many unstable transcripts encoding proto-oncogenes, growth factors, or cytokines (19–22). Rapid degradation of ARE-containing mRNAs, referred to as ARE-mediated mRNA decay (AMD), is regulated by trans-acting ARE-binding proteins, which are either stabilizing factors (e.g. Hu antigen R (HuR)) or destabilizing factors (e.g. tristetraprolin, butyrate response factors (BRFs) 1 and 2 (also known as...
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ZFP36 ring finger protein—like 1 and 2), KH-type splicing-regulatory protein (KSRP), and AU-rich element RNA-binding protein 1 (AUF1). In this study, we provide molecular evidence that poly(A)$^+$ RDH mRNAs are post-transcriptionally regulated via BRF1.

Results

Inhibition of proteasomes abrogates stress-mediated stabilization of poly(A)$^+$ RDH mRNAs

Recent data suggest that under stressful conditions such as UVC irradiation and treatment with puromycin (Puro; a potent inhibitor of translation), poly(A)$^+$ RDH mRNAs are stabilized by HuR in a translation-independent manner (23). In contrast, under normal conditions, poly(A)$^+$ RDH mRNAs are rapidly degraded. These previous observations imply the presence of a putative labile cellular factor(s) responsible for rapid degradation of poly(A)$^+$ RDH mRNAs under normal conditions. We also hypothesized that the putative labile cellular factor(s) is degraded under stressful conditions.

To test the above hypothesis, we determined whether treatment with MG132, a potent inhibitor of the proteasome, can inhibit UVC-mediated up-regulation of poly(A)$^+$ RDH mRNAs (Fig. 1A). Consistent with another report (23), the levels of all tested poly(A)$^+$ RH mRNAs (poly(A)$^+$ forms of HIST1H1E mRNA, HIST1H3A mRNA, HIST1H4E mRNA, HIST1H4K mRNA, and HIST2H3D mRNA) were found to be up-regulated 29.0–97.5-fold upon UVC irradiation. In contrast, the levels of total RH mRNAs (poly(A)$^+$ RH mRNAs and HSL$^+$ RDH mRNAs) and H3F3A mRNA, one of the RIH mRNAs, were only marginally affected by UVC irradiation. Notably, treatment with MG132 drastically inhibited the UVC-mediated up-regulation of all the tested poly(A)$^+$ RDH mRNAs. Similarly, treatment with Puro up-regulated the poly(A)$^+$ RDH mRNAs 10.1–125.3-fold. This up-regulation was almost completely reversed by MG132 treatment (Fig. 1B).

In the 3’-UTR, the poly(A)$^+$ RDH mRNAs harbor a novel cis-acting element called the PRSE, which is critical for stabilization of these mRNAs under various types of stress (23). To test whether the effect of MG132 on the stability of poly(A)$^+$ RDH mRNAs is related to the PRSE, a plasmid expressing HIST1H4K-MT10 reporter mRNA, which harbors the minimal PRSE of the HIST1H4K gene in the 3’-UTR, was transiently transfected into HeLa cells. The amount (Fig. 1C) and half-life (Fig. 1E) of poly(A)$^+$ HIST1H4K-MT10 mRNA were found to be significantly increased upon UVC irradiation, in agreement with another report (23). Of note, the observed increase by UVC irradiation was completely blocked by MG132 treatment (Fig. 1C and E). In addition, the up-regulation of HIST1H4K-MT10 mRNA upon Puro treatment was reversed by MG132 treatment (Fig. 1D and F). All these data suggest that stabilization of poly(A)$^+$ RDH mRNA via a PRSE under stressful conditions depends on a proteasomal activity and that a putative labile cellular factor(s) is responsible for rapid degradation of poly(A)$^+$ RDH mRNAs under normal conditions.

AMD participates in the regulation of poly(A)$^+$ RDH mRNAs

HuR binds to an ARE or a U-rich sequence (24). The PRSE serves as the binding platform for HuR (23), implying the presence of a putative ARE(s) in the PRSE. Indeed, the PRSE sequence contains several putative binding sites for HuR that are similar to the typical ARE consensus motif (A/U)UUU(A/U) (Fig. 2A). To determine whether the stability of poly(A)$^+$ RDH mRNAs is influenced by AMD, we constructed several HIST1H4K-MT10 variants that contain mutations in putative AREs of the PRSE (Fig. 2A). Then, we tested whether the steady-state level of HIST1H4K-MT10 mRNA is affected by the mutations (Fig. 2B). Compared with the intact HIST1H4K-MT10 mRNA, the levels of all the variants increased 3.6–4.6-fold, indicating that poly(A)$^+$ RDH mRNAs are AMD substrates. Of note, among the tested mutants, only HIST1H4K-MT10-A2 mRNA manifested a weak response to UVC irradiation (Fig. 2C), indicating that the second ARE within the PRSE is largely responsible for the stabilization of poly(A)$^+$ RDH mRNAs under stressful conditions.

BRF1 and BRF2 are down-regulated under stressful conditions

According to our findings that (i) MG132 treatment inhibits the up-regulation of poly(A)$^+$ RDH mRNAs upon exposure to cellular stresses (Fig. 1) and (ii) the AREs in the PRSE are involved in the regulation of poly(A)$^+$ RDH mRNAs, we speculated that a labile decay-promoting ARE-binding protein(s) can facilitate the degradation of poly(A)$^+$ RDH mRNAs. Indeed, it has been reported that some ARE-binding proteins are targeted by proteasomal activity (25, 26). To identify the factors that are susceptible to stresses, the expression of previously known ARE-binding proteins was analyzed by Western blotting in total-cell extracts prepared from HeLa cells either pretreated or not pretreated with MG132 and either irradiated with UVC or not (Fig. 2D). The expression of KSRP, AUF1, and HuR was not significantly affected by UVC irradiation. In contrast, the levels of BRF1 and -2 diminished 2.5- and 3.3-fold, respectively, upon UVC irradiation. Furthermore, the expression of BRF1, but not BRF2, was restored by MG132 treatment. Puro treatment yielded a similar pattern except that BRF2 expression was partially restored by MG132 treatment (Fig. 2E). All these data suggest that BRF1 (and possibly BRF2) plays a role in the control of stability of poly(A)$^+$ RDH mRNA.

BRF1 associates with poly(A)$^+$ RDH mRNAs via the PRSE

Cellular stresses stabilize poly(A)$^+$ RDH mRNAs probably by invoking degradation of BRFs. Accordingly, cellular stresses may affect an interaction of BRFs with poly(A)$^+$ RDH mRNAs. Therefore, we determined whether BRFs bind to poly(A)$^+$ RDH mRNA and whether their interaction is affected by cellular stresses. To this end, we performed a pulldown assay using in vitro-synthesized biotinylated RNA, either PRSE RNA or PRSE-Rev RNA, and the extracts obtained from UVC-irradiated or nonirradiated HeLa cells (Fig. 3A). The PRSE-Rev RNA served as a negative control because it contains reverse-complementary sequences of the PRSE. Under nonirradiation conditions, HuR specifically associated with the PRSE RNA. This association was gradually augmented by UVC irradiation in a time-dependent manner (3.7-fold at 2 h and 4.9-fold at 4 h). Intriguingly, BRF1, but not BRF2, specifically associated with the PRSE RNA under nonirradiation conditions. In contrast,
when the cells were irradiated with UVC, the BRF1 association was reduced 3-fold. Of note, the association of nucleolin (an ARE-binding protein) with the PRSE was marginally affected by UVC irradiation.

Next, we investigated whether MG132 can block the UVC-mediated dissociation of BRF1 from the poly(A)$^+$ RDH mRNAs (Fig. 3B). In line with MG132-dependent inhibition of BRF1 degradation (Fig. 2), MG132 treatment blocked the UVC-mediated dissociation of BRF1 from the PRSE. Conversely, the augmented association of HuR with the PRSE upon UVC irradiation was inhibited by MG132, suggesting that HuR competes with BRF1 for binding to the PRSE of poly(A)$^+$ RDH mRNAs.

We next tested the possible competition between BRF1 and HuR for binding to the cell (27, 28). HeLa cells were transfected with in vitro-synthesized biotinylated PRSE or PRSE-Rev RNAs. After that, the cells were subjected to in vivo RNA pulldown experiments.

Figure 1. Inhibition of proteasomes abrogates stress-mediated stabilization of poly(A)$^+$ RDH mRNAs. A, the effect of MG132 treatment on poly(A)$^+$ RDH mRNAs under UVC irradiation conditions. HeLa cells either pretreated or not pretreated with MG132 for 6 h were either not irradiated or irradiated with UVC for 4 h. Then, total-RNA samples were purified and analyzed by qRT-PCR. The levels of poly(A)$^+$ RDH mRNAs, RH mRNAs, and total RDH mRNAs were normalized to the amounts of β-actin mRNA. The normalized levels of each mRNA in the nonirradiated and MG132-untreated cells were arbitrarily set to 1.0. Mean values ± S.D. (error bars) are shown in each bar graph. Two-tailed equal-variance Student’s t test was carried out for statistical analysis: *, p < 0.05; **, p < 0.01. All data were obtained from three independent biological replicates (n = 3). B, effects of MG132 treatment on poly(A)$^+$ RDH mRNAs during Puro treatment, as performed in A except that HeLa cells were either treated with Puro or not for 3 h. n = 3; **, p < 0.01. C, the effect of MG132 treatment on exogenously expressed poly(A)$^+$ HIST1H4K-MT10 reporter mRNAs under UVC irradiation. Cells transfected with a plasmid expressing HIST1H4K-MT10 mRNAs and phCMV-MUP as a reference plasmid were either pretreated or not pretreated with MG132 for 6 h. After that, the cells were either not irradiated or irradiated with UVC for 4 h. The level of HIST1H4K-MT10 mRNA was normalized to that of MUP mRNA. The normalized levels of the reporter mRNA in the nonirradiated and MG132-untreated cells were arbitrarily set to 1.0. n = 3; *, p < 0.05. D, as performed in C except that the cells either pretreated or not pretreated with MG132 were either treated with Puro or not for 6 h. n = 3; **, p < 0.01. E, half-life of HIST1H4K-MT10 mRNA under UVC irradiation conditions. HeLa cells either pretreated or not pretreated with MG132 for 6 h were either irradiated with UVC or not. Two hours later, the cells were treated with DRB and harvested at the indicated time points. The levels normalized to MUP mRNAs at 0 min were arbitrarily set to 100%. Percent remaining is presented on a log2 scale. Calculated half-life values are given in each panel. Error bars represent S.D. n = 3. F, half-life of HIST1H4K-MT10 mRNA under Puro-treated conditions. Error bars represent S.D. n = 4.
The results showed that although the amounts of biotinylated RNAs obtained after in vivo RNA pulldown were comparable before and after UVC irradiation (Fig. 3C), PRSE RNAs, but not PRSE-Rev RNAs, associated with BRF1 and HuR under nonirradiation conditions (Fig. 3D). Notably, UVC irradiation triggered dissociation of BRF1 from PRSE and an association of HuR with PRSE (Fig. 3D), indicating that BRF1 competes with HuR for binding to PRSE in poly(A)/H11001 RDH mRNAs.

**BRF1 destabilizes poly(A)/H11001 RDH mRNAs**

We next investigated whether BRF1 can trigger rapid degradation of poly(A)/H11001 RDH mRNAs via targeting the PRSE under normal conditions. First, down-regulation of BRF1, but not BRF2, increased the amount of poly(A)/H11001 HIST1H4K-MT10 mRNAs (Figs. 4 and S1), indicating that BRF1 rather than BRF2 functions as the main destabilizing factor for poly(A)/H11001 RDH mRNAs under normal conditions. Second, in line with our observations that HIST1H4K-MT10 variants lacking one of the AREs are up-regulated under normal conditions (Fig. 3), the amount of those variants was not affected by BRF1 down-regulation (Fig. 4, A and B). Third, our half-life analysis revealed that under nonirradiated conditions, all the tested endogenous poly(A)/H11001 RDH mRNAs were rapidly degraded with half-lives ranging from 40.8 to 72.3 min (Fig. 4D). Conversely, UVC irradiation increased the half-lives of poly(A)/H11001 RDH mRNAs 1.6–4.7-fold. Intriguingly, the observed increase in half-life was reversed by overexpression of MYC-BRF1. Of note, neither UVC irradiation nor BRF1 overexpression affected the stability of H3F3A mRNA. Overexpression of BRF1 was confirmed by Western blotting (Fig. 4C). Taken together, all these data indi-
BRF1 regulates poly(A)$^+$ RDH mRNAs

**Discussion**

Alteration of 3′-UTRs of mRNAs is critical for the regulation of gene expression. This event can generate multiple variants of mRNA containing a new repertoire of cis-acting elements, which recruit distinct families of trans-acting factors onto the 3′-UTR of the mRNAs to produce diverse physiological outcomes (16–18). Likewise, the extended 3′-UTR of poly(A)$^+$ RDH mRNAs may contain a new cis-acting element(s), which can serve as a platform for a different regulatory mode of gene expression, as compared with HSL$^+$ RDH mRNAs. In this study, we provide evidence that BRF1 promotes rapid degradation of poly(A)$^+$ RDH mRNAs via an association between BRF1 and AREs within the PRSE in the extended 3′-UTRs. On the basis of our observations, we propose a scenario for post-transcriptional regulation of RDH mRNAs (Fig. 4E).

Stem loop–binding protein is cotranscriptionally recruited to the HSL in the 3′-UTR of newly synthesized nascent RDH pre-mRNAs in the nucleus. Then, the histone 3′-end–processing complex is loaded onto the HDE located downstream of the HSL and cleaves a site between HSL and HDE for formation of typical HSL$^+$ RDH mRNAs. If the 3′-end processing becomes inefficient or inappropriate because of intrinsic or extrinsic stresses, a putative polyadenylation signal down-
stream of the HDE will be recognized, thus leading to the formation of poly(A)⁺ RDH mRNA (6, 12–15). Even under normal conditions, poly(A)⁺ RDH mRNAs can be generated by misprocessing of RDH pre-mRNAs (23). The newly generated aberrant poly(A)⁺ RDH mRNAs will be harmful because they may serve as a template for the synthesis of histone proteins, thereby upsetting the balance between the amounts of histone protein and DNA (9, 13, 14). Therefore, the aberrant poly(A)⁺ RDH mRNAs should be cleared to maintain a proper ratio of histone protein to DNA. In this regard, the PRSE of poly(A)⁺ RDH mRNAs can serve as a mediator linking poly(A)⁺ RDH mRNAs to a surveillance pathway managed by AMD, particularly by BRF1.

In conclusion, the present study suggests that the fate of poly(A)⁺ RDH mRNAs can be modulated via AMD. Although the functional role of poly(A)⁺ RDH mRNAs under stressful conditions is currently unknown, further attempts to identify their biological function and relation with AMD will help to expand our knowledge on the biology of regulation of histone gene expression.

**Experimental procedures**

**Plasmid construction**

The following plasmids were described previously: phCMV-MUP (29) and pcDNA3.1-HIST1H4K-MT10, pSK-PRSE,
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and pSK-PRSE-Rev (23). Plasmids pcDNA3.1-HIST1H4K-MT10-A1, -A2, and -A3 were generated by polymerase chain reaction (PCR) using pcDNA3.1-HIST1H4K-MT10 as a template. The resulting plasmids contained the following nucleotide substitutions. For MT10-A1, the first ARE was changed from 5'-AATTATTATTCCAA-3' to 5'-AATTACCCCTCCAA-3'. For MT10-A2, the second ARE was changed from 5'-TGAGGTTTTAGGG-3' to 5'-TGAGGCCCCAGGGT-3'. For MT10-A3, the third ARE was changed from 5'-TTACATTATTAGGG-3' to 5'-TTACACCCAAGGG-3'. pCMV-MYC-BRF1 was generated by inserting a DNA fragment containing the human BRF1 coding sequences (GenBank™ accession number NM_004926) from the pCMV-SPORT6-BRF1 (hMU001454; the Korean Human Gene Bank) into pCMV-MYC (Takara Bio).

Cell culture and transfection

HeLa cell culture and transfection have been described elsewhere (23, 27, 30). When indicated, the cells were either treated with 400 μg/ml Puro (MilliporeSigma) for 3 or 6 h before harvesting or irradiated with UVC light (50 J/m^2; 254 nm) and harvested 4 h later. To inhibit proteasomal activity, the cells were incubated with 100 μM MG132 (MilliporeSigma) for 6 h. For evaluation of mRNA half-life, the cells were incubated with 100 μg/ml 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole (DRB; MilliporeSigma) and then harvested at various time points.

siRNA sequences

The following siRNAs were utilized for specific down-regulation: 5'-r(AGGGUAACAGAUGCUACACU)d(TT)-3' for BRF1 and 5'-r(GGAAGUAUGUUAGGAGAAC)d(TT)-3' for BRF2. The sequence for nonspecific control siRNA has been described previously (31).

Quantitative RT-PCR (qRT-PCR) analysis

Total or poly(A)-specific complementary DNAs (cDNAs) were prepared using oligo(dT) (19-mer) or a random hexamer, respectively. qRT-PCR analysis was performed with gene-specific oligonucleotides (Table S1) as described previously (23).

Western blotting

Primary antibodies against the following proteins were employed: HuR (2A3; Santa Cruz Biotechnology), BRF1 (Cell Signaling Technology), BRF2 (Santa Cruz Biotechnology), KSRP (Cell Signaling Technology), nucleolin (Bio-Techne), AUF1 (MilliporeSigma), β-actin (MilliporeSigma), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (AbFrontier). Band intensities were quantitated in Multi Gauge software (version 3.0, FujiFilm).

Pulldown assay using biotinylated RNAs

This assay involving in vitro–synthesized RNAs has been described elsewhere (23, 27). Briefly, cells were lysed with NET-2 buffer (50 mM Tris, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine hydrochloride, and 0.05% Nonidet P-40, pH 7.4) to obtain total-cell extracts, which were then incubated with streptavidin-agarose (Thermo Fisher Scientific) and in vitro–synthesized biotinylated RNAs. The bound proteins were examined by Western blotting.

In vivo cross-linking followed by RNA pulldown experiments

This assay was carried out as described previously (27, 28). Briefly, in vitro–synthesized biotinylated RNAs were transfected into HeLa cells. After 6 h, the cells were either irradiated with UVC or not. An additional 4 h later, the cells were subjected to UV cross-linking (254 nm; 400 ml/cm^2) before harvesting. To normalize the level of biotinylated RNAs in the pulldown sample, in vitro–synthesized firefly luciferase RNA was added as a spike-in before purification.


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References


