Analysis of Turnover and Translation Regulatory RNA-Binding Protein Expression through Binding to Cognate mRNAs[▽]

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RNA-binding proteins (RBPs) that associate with specific mRNA sequences and function as mRNA turnover and translation regulatory (TTR) RBPs are emerging as pivotal posttranscriptional regulators of gene expression. However, little is known about the mechanisms that govern the expression of TTR-RBPs. Here, we employed human cervical carcinoma HeLa cells to test the hypothesis that TTR-RBP expression is influenced posttranscriptionally by TTR-RBPs themselves. Systematic testing of the TTR-RBPs AUF1, HuR, KSRP, NF90, TIA-1, and TIAR led to three key discoveries. First, each TTR-RBP was found to associate with its cognate mRNA and with several other TTR-RBP-encoding mRNAs, as determined by testing both endogenous and biotinylated transcripts. Second, silencing of individual TTR-RBPs influenced the expression of other TTR-RBPs at the mRNA and/or protein level. Third, further analysis of two specific ribonucleoprotein (RNP) complexes revealed that TIA-1 expression was controlled via HuR-enhanced mRNA stabilization and TIAR-repressed translation. Together, our findings underscore the notion that TTR-RBP expression is controlled, at least in part, at the posttranscriptional level through a complex circuitry of self- and cross-regulatory RNP interactions.

In mammalian cells, RNA-binding proteins (RBPs) are major regulators of mRNA stability and translation. Some RBPs associate with RNA sequences that are widely present in mRNAs, such as the 5' cap structure (7-methyl-guanosine) or the 3' poly(A) tail. However, a distinct, albeit heterogeneous, group of mammalian RBPs associate with specific mRNA sequences (*cis* elements) frequently present in the 5' and 3' untranslated regions (UTRs) and effect changes in mRNA stability and translation rates (16, 46, 54). Traditionally, these mRNA stability and translation determinants have been termed AU-rich elements (AREs) because several early examples of stability sequences were prominently AU rich and often included the pentamer AUUUA (13, 48, 59). However, with growing numbers of mRNA stability and translation determinants elucidated over the past decade, many examples of regulatory sequences have been described which lack AUUUA pentamers and do not exhibit a particular AU richness (15, 30, 38, 40, 41, 61, 64). Thus, while the term "ARE-RBP" is deeply ingrained in the literature of RBPs implicated in stabilizing/destabilizing mRNAs and in promoting/suppressing translation, we propose the alternative nomenclature TTR-RBPs (for turnover and/or translation regulatory RBPs) to refer to this subset of RBPs.

The group of TTR-RBPs comprises RBPs implicated primarily in mRNA decay, such as tristetraprolin (TTP), AU-binding factor 1 (AUF1; also termed heterogeneous nuclear ribonucleoprotein [hnRNP] D), the K homology splicing regulatory protein (KSRP), and butyrate response factor 1

(BRF1) (11, 39, 45, 49, 63). Other TTR-RBPs, such as the Hu proteins (HuR, HuB, HuC, and HuD) and nuclear factor 90 (NF90), can enhance the stability of target mRNAs (4, 9, 47) and in some cases influence their translation (5, 14, 26, 31, 32, 42, 44, 60). Yet another group of TTR-RBPs have been shown to inhibit protein translation, including T-cell-restricted intracellular antigen 1 (TIA-1) and the TIA-1-related protein (TIAR) (2, 3, 20, 40, 43). In some instances, these multifunctional TTR-RBPs have also been implicated in pre-mRNA splicing (18, 20, 36, 45) and can bind DNA and influence transcription (25).

Additionally, almost all of the aforementioned TTR-RBPs are known to be shuttling proteins, and their functions are closely linked to their transit between the nucleus and the cytoplasm. For example, the stabilizing and translational regulatory functions of HuR are linked to its presence in the cytoplasm (17, 29). Likewise, AUF1, TIAR, TIA-1 (48, 62), and possibly KSRP (22) continuously translocate between the nucleus and the cytoplasm. In some instances, the functions of several TTR-RBPs have been linked to their specific subcytoplasmic distributions. In this regard, there is evidence that TIAR and TIA-1 colocalization with stress granules correlates with their ability to inhibit the translation of certain target mRNAs (2, 3, 40), that the AUF1 decay-promoting function is linked to its association with the proteasome (33), and that the recruitment of AUF1, TTP, BRF1, and KSRP to the exosome or processing bodies (P bodies) is involved in target mRNA decay (12, 28). A number of posttranslational modifications, such as phosphorylation and methylation, as well as association with specific proteins, have also been shown to regulate the localization and functions of TTR-RBPs (1, 10, 34, 37, 51, 55, 56).

However, little is known about the specific mechanisms that

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FIG. 1. Detection of TTR-RBP mRNAs in immunoprecipitated TTR-RBP RNP complexes. Whole-cell lysates prepared from untreated HeLa cells were used for IP analysis using antibodies that recognized either AUF1 (A), HuR (B), NF90 (C), TIA-1 (D), TIAR (E), or species-specific control IgG in each case. In the graphs, RNA was extracted from the RNP complexes present in each IP sample, and the levels of the TTR-RBP mRNAs shown (those encoding AUF1, HuR, KSRP, NF90, TIA-1, and TIAR), mRNAs that were known targets of each TTR-RBP and were included as positive controls (those encoding Gadd45 [GADD], prothymosin α [ProT α], thymidylate synthase [TS], cytochrome *c* [CytoC], and c-Myc [MYC]) (hatched bars), as well as those of housekeeping GAPDH and UBC mRNAs (low-level contaminating transcripts that served to monitor the equality of sample input), were detected after RT followed by real-time qPCR amplification. The resulting data are represented as

regulate TTR-RBP expression levels. The polypyrimidine tract-binding protein (PTB) influences its own expression through alterations in PTB pre-mRNA splicing that trigger PTB transcript decay (58). Similar self-regulatory schemes have been reported for several other RBPs, such as hnRNP M, the sex-lethal protein Sxl, and tra2- β 1; these RBPs bind to their respective cognate pre-mRNAs and influence their splicing (8, 21, 50). Examples other than splicing factors include the RBP annexin A2, which associates with its cognate mRNA and was proposed to influence its translation (23). Among conventional TTR-RBPs, TTP was shown to bind to and promote the decay of the TTP mRNA, providing a distinct example of a posttranscriptional negative-feedback loop in this protein family (52). More recently, elevated HuR expression levels were linked to enhanced TIA-1 abundance, while TIA-1 levels negatively affected HuR abundance, although the underlying mechanisms were not investigated directly (27). In another instance of posttranscriptional control of TTR-RBP abundance, AUF1 expression was also found to be subjected to accelerated AUF1 mRNA decay (6, 57).

These earlier reports, along with the observation that TTR-RBPs are encoded by mRNAs with long 3' UTRs bearing motifs that would predictably render them targets of other TTR-RBPs prompted us to systematically study whether TTR-RBP expression might be subject to posttranscriptional regulation elicited by TTR-RBPs themselves. Here, we investigated six TTR-RBPs: AUF1, HuR, KSRP, NF90, TIA-1, and TIAR. Our results indicated that indeed each of these TTR-RBPs binds the very mRNA that encodes its own gene product. In several cases, they also bind to transcripts encoding other TTR-RBPs tested, both the endogenous mRNA and biotinylated partial RNAs. Two specific RNP associations, the HuR-TIA-1 mRNA and TIAR-TIA-1 mRNA complexes, were studied in greater depth, revealing that HuR positively controls TIA-1 expression by enhancing TIA-1 mRNA abundance and that TIAR suppresses the translation of TIA-1.

MATERIALS AND METHODS

Cell culture, transfections, RNA interference, and plasmid constructs. Human cervical carcinoma HeLa cells were cultured in minimal essential medium, and human uterine sarcoma MES-SA/Dx5 cells were grown in McCoy's 5a medium; the media were supplemented with 1% glutamine, 10% fetal bovine serum, and antibiotics. For RNA interference, the transfections were carried out using Oligofectamine (Invitrogen) and 20 nM of control small interfering RNA (siRNA) or gene-specific siRNA (targeting AUF, HuR, KSRP, NF90, TIA-1, or TIAR siRNA [see below]). The sequences of the siRNAs used in silencing experiments were as follows: AAGAGGCAATTACCAAGTTTCA (HuR siRNA, targeting the HuR coding region [CR]), AATCTTAAGTTTCGTAAGTTA (HuR3 siRNA, targeting the HuR 3' UTR), GGATCCTATCACAGGGCGAT (AUF1 siRNA), AAGATCAACCGGAGAGCAAGA (KSRP siRNA), GCCCACCCTTGCTTTTAT (NF90 siRNA), AACACAACAAATGGCCAGTA (TIA-1 siRNA), AAGGGCTATTTCATTTGTCAGA (TIAR siRNA, targeting the TIAR CR), TACCATAATGGTCATCTATTA (TIAR3 siRNA, targeting the TIAR 3' UTR), and TTCTCCGAACGTGTACAGT (control siRNA). For

reporter analyses, pEGFP-TIA-1(3'UTR) was constructed by cloning the 3' UTR of TIA-1 (1,034-bp insert) after the CR of enhanced green fluorescent protein (EGFP) in the pEGFP-N1 vector (Clontech); pEGFP-N1 was used in control transfections. Lipofectamine 2000 (Invitrogen) was used in transfection experiments to overexpress HuR (pHuR-TAP, side by side with the control vector, pTAP) (32) or to overexpress TIAR (pMT2-TIAR, side by side with the control vector, pMT2 [generously provided by P. Anderson and N. Kedersha]). Lipofectamine 2000 was also used in experiments to cotransfect siRNAs and plasmid DNA. Cells were harvested 48 h after transfection, and lysates were prepared for further analysis.

Western blotting. For Western blot analysis, whole-cell lysates were size fractionated by electrophoresis in sodium dodecyl sulfate (SDS)-containing polyacrylamide gels (SDS-polyacrylamide gel electrophoresis) and transferred onto polyvinylidene difluoride membranes. The membranes were incubated with antibodies recognizing either HuR, TIA-1, TIAR (Santa Cruz Biotechnology), AUF1 (Upstate Biotechnology), NF90 (BD Biosciences), or KSRP (a kind gift from Ching-Yi Chen, University of Alabama). Following secondary-antibody incubations, signals were detected by enhanced chemiluminescence (Amersham Biosciences). Band intensities were measured by densitometry using the ImageJ 1.36b program (NIH).

IP. For immunoprecipitation (IP) of endogenous mRNA-protein complexes from cytoplasmic extracts (500 μ g), lysates were incubated for 1 h at 4°C with protein A- and protein G-Sepharose beads (Sigma) that had been precoated with 30 μ g of either immunoglobulin G1 (IgG1) (BD Biosciences) or antibodies recognizing AUF1 (Upstate Biotechnology), NF90 (BD Biosciences), HuR, TIA-1, or TIAR (Santa Cruz Biotechnology). The beads were washed and the IP products were processed as described previously (32). RNA was extracted and employed in subsequent reverse transcription (RT), followed by real-time quantitative PCR (qPCR) analysis; the qPCR primers used are listed below.

RNA isolation, RT, and RT-qPCR. Whole-cell RNA was isolated using Trizol (Invitrogen). For qPCR analysis of either whole-cell or immunoprecipitated RNA, RNA was reverse transcribed using SSII-RT (Invitrogen) and random hexamers (U.S. Biologicals), and the resulting products were used in PCR amplification reactions employing the SYBR Green PCR master mix (Applied Biosystems). RNA extracted from whole-cell lysates was treated with DNase I (Ambion).

The primer pairs (sense and antisense in each case) for measuring the levels of mRNAs encoding TTR-RBPs in total RNA and in RNA from RNP IP were GATCCTAAAAGGGCCAAAGC and GTTGTCCATGGGGAGCTCTA for AUF1, CGCAGAGATTCAGGTTCTCC and CCAAAACCTTTGCACTTGTT for HuR, CTGGTGCTGCTGTGTAAGGA and AGGGACAATGGAGGCTCTTT for NF90, GACAGCAGGCCGCTTACTAC and GCTCTCTCGCCAAACAAAAC for KSRP, CATGGAACCAAGGATTT and CACTCCCTGTAGCCTCAAGC for TIA-1, and GCCAATGGAGCCAAAGTGTAT and CATATCCGGCTTGGTTAGGA for TIAR. The primer pair TGCACCACCACTGCTTAGC and GGCATGGACTGTGGTCATGAG was used to detect GAPDH (glyceraldehyde-3-phosphate dehydrogenase), ATTTGGGTGCGGGTCTTTG and TGCCTTGACATTCTCGATGGT for UBC, CAACTTTTACACAAGATGGTGAGTG and GAGGCCAAATGAACATGAACACAA for cytochrome c, CCAACCCCAACCATGAGAA and GGTACACACCAAGTAAAGTCAG for prothymosin α , GATGCCCTGGAGGAAGTGCT and AGCAGGCACAACACCACGTT for Gadd45, TTCGGGTAGTGGAACACAG and CAGCAGCTCGAATTTCTTCC for c-Myc, TCTGGAAGGGTGTTTGGAG and CTCCACTGGGAAGCCATAAA for thymidylate synthase, ACGTAAACGGCCACAAGTTC and AAGTCGTGCTGCTTCATGTG for EGFP, and CCCTATCAACTTTTCATGGTAGTTCG and CCAATGGATCCTCGTTAAAGGATTT for 18S rRNA.

Biotin pull-down assay. For in vitro synthesis of biotinylated transcripts, reverse-transcribed total RNA was used as a template for PCRs. All 5' oligonucleotides contained the T7 RNA polymerase promoter sequence, CCAAGCTTCTAATACGACTCACTATAGGGAGA (T7). The primers used for the amplification of sequences 623 to 1245 (CR) and 1461 to 2024 (3' UTR) of

enrichment of each mRNA in the specific TTR-RBP IP samples compared with its abundance in control IgG IP samples. The TTR-RBP mRNA enrichment levels in each IP were adjusted to the levels of GAPDH mRNA enrichment in each IP (the latter values were routinely close to 1). TTR-RBP mRNAs showing twofold or higher enrichment in TTR-RBP IPs relative to IgG IPs are shown as black bars; all others (no enrichment detected) are represented by gray bars. The data shown are the means and standard errors of the mean of four to six independent experiments. HC, heavy immunoglobulin chain; LC, light immunoglobulin chain. In the IP plus Western blots, for each TTR-RBP, the quality of the IP was monitored by subsequent Western blot (WB) analysis of the IP samples.

FIG. 2. Binding of biotinylated TTR-RBP RNAs to TTR-RBPs. (A) Schematic of TTR-RBP mRNAs, depicting in black rectangles the CRs and in white flanking rectangles the 5' UTR and 3' UTR of each TTR-RBP mRNA. The specific fragments used as templates for the synthesis of biotinylated RNAs are indicated by underlining, and the nucleotide positions amplified by PCR are shown. (B) Biotin pull-down analysis of complexes formed in vitro using biotinylated TTR-RBP mRNA segments (shown in panel A) and endogenous TTR-RBPs present in HeLa whole-cell lysates, as detected by Western blot analysis. A biotinylated RNA spanning the GAPDH 3' UTR (which is not a target of TTR-RBPs) was included as a negative control. The data are representative of three to six independent experiments.

AUF1 (NM_031370.2), 211 to 749 (CR) and 4990 to 5553 (3' UTR) of HuR (NM_001419), 951 to 1403 (CR) and 2232 to 2971 (3' UTR) of KSRP (NM_003685), 778 to 1312 (CR) and 2678 to 3196 (3' UTR) of NF90 (NM_004516), 483 to 939 (CR) and 1506 to 2030 (3' UTR) of TIA-1, 1137 to 1635 (CR) and 2369 to 2937 (3' UTR) of TIAR (NM_001033925), and 1008 to 1310 (3' UTR) of GAPDH (NM_002046) are listed below. The PCR-amplified products were resolved on agarose gels and used as templates for the synthesis of the corresponding biotinylated RNAs using T7 RNA polymerase and biotin-CTP; the biotinylated transcripts were purified before use.

The primers used for the synthesis of PCR templates bearing the T7 RNA polymerase promoter sequence (T7) were as follows (each sense and antisense): AUF1 3'UTR, (T7)GCTCCTGCCACCTGCTAATA and GCAAAGGGGTAC TTTTGAC; AUF1 CR, (T7)GAGGCCTTAGCTGGGACACT and ACGAG CTCTTCTGCAAATC; HuR 3' UTR, (T7)CCTGACCTCTAATGGCTGGA and AATGGGCTGATGGAAAACCTG; HuR CR, (T7)GTGACATCGGGAG AACGAAT and GCCACGTTTTTGTCTCTGTT; KSRP 3' UTR, (T7)CGAA TGAATGTGAACCTTCTTCATC and TGTGAAGTTAAAAACGAGCGATA; KSRP CR, (T7)TGGGGATCCTTACAAAGTGC and CACACTTGTGAGTG

GGGATG; NF90 3' UTR, (T7)AAAGCCCACCTTTGCTTTTT and CAGGG AACATCACGGTCTT; NF90 CR, (T7)TGACATCCCCGTTGTGACAGA and TCTTTGGTTTCTTGGGCATC; TIA-1 CR, (T7)GGTGATCTCAGCCAC AAAT and TGCACATTTCATGGAATTGA; TIA1 3' UTR, (T7)CTGCCAA TTTTGCCTTCAT and GGAAATTCCTTGAGGCACCTTC; TIAR 3' UTR, (T7)TGGTGGTCCACGTTAAGACA and TTTTAAAAACACCCAAGATT TTT; TIAR CR, (T7)TCGTAAACCACCTGCACCTA and GCAGCAGAAG GTGATTGATCT; and GAPDH 3' UTR, (T7)CCTCAACGACCACTTT GTCA and GGTTGAGCACAGGGTACTTTAT.

Biotin pull-down assays were carried out by incubating 40 µg of whole-cell lysates with equimolar (~4 µg) biotinylated transcripts for 1 h at room temperature. Complexes were isolated with paramagnetic streptavidin-conjugated Dynabeads (Dyna), and bound proteins in the pull-down material were analyzed by Western blotting using antibodies recognizing AUF1, HuR, KSRP, NF90, TIA-1, and TIAR. After secondary-antibody incubations, the signals were visualized by chemiluminescence (Amersham Biosciences).

Analysis of nascent protein. De novo TIAR and GAPDH protein synthesis was measured by incubating HeLa cells with 1 mCi L-[³⁵S]methionine and L-[³⁵S]cysteine (Easy Tag EXPRESS; NEN/PerkinElmer) for 15 min, followed by lysis with RIPA buffer and IP with 10 µg anti-TIAR or anti-GAPDH antibodies (Santa Cruz Biotechnology); IgG was used in control IP reactions. Beads were washed in RIPA buffer, and the IP material was resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and visualized and quantified using a phosphorimager.

RESULTS

Analysis of RNP complexes containing endogenous TTR-RBPs and endogenous TTR-RBP mRNAs. In order to investigate systematically the possible existence of RNP complexes comprising TTR-RBPs and the mRNAs that encode them, we first performed IP assays to examine these associations in human cervical carcinoma HeLa cells. Six major TTR-RBPs were chosen for analysis: AUF1, HuR, KSRP, NF90, TIA-1, and TIAR. The specificity and quality of the IP reactions were tested by Western blot analysis of each TTR-RBP (Fig. 1); despite repeated attempts, the KSRP antibody (a gift from C. Y. Chen, University of Alabama) did not yield adequate IP signals in HeLa cells, and therefore, KSRP IP materials were omitted from this part of the analysis. In each IP sample, the presence of TTR-RBP mRNAs was tested for by extraction and RT of the RNA present in the pellets, followed by real-time qPCR amplification of gene-specific products. As shown, several mRNAs tested were found to associate distinctly with TTR-RBPs, since RT-qPCR analysis revealed their presence to be significantly enriched (twofold or higher) in TTR-RBP IP samples compared with control IgG IP samples (Fig. 1). Interestingly, each TTR-RBP showed binding to its own mRNA; in fact, besides the known target mRNAs included as positive controls in each IP set, the cognate mRNA was found in several cases to be the most highly enriched target mRNA (as seen for HuR and TIA-1 [Fig. 1B and D]). Several other prominent complexes were also identified in this analysis; for example, HuR and AUF1 bound all of the TTR-RBP mRNAs tested (Fig. 1A and B), and TIA-1 and TIAR bound reciprocally the respective mRNAs (Fig. 1D and E). The levels of the housekeeping GAPDH and UBC mRNAs, which are routinely found as low-level contaminating mRNAs in the RNP IP samples, were comparable between IgG IPs and TTR-RBP IPs, providing evidence that equivalent amounts of sample were used in each IP (Fig. 1A to E).

Endogenous TTR-RBPs bind biotinylated TTR-RBP transcripts. To further investigate the complexes detected (Fig. 1), we tested the binding of TTR-RBPs to biotinylated tran-

FIG. 3. Compiled results from TTR-RBP binding to endogenous and biotinylated TTR-RBP transcripts. Shown are schematic representations of the results from the RNP IP analysis in Fig. 1 (A) and from the biotin pull-down analysis in Fig. 2 (B).

scripts spanning different regions of the 3' UTR or the CR of TTR-RBPs (Fig. 2A). Equimolar amounts of the corresponding biotinylated RNAs (synthesized as described in Materials and Methods) were incubated with HeLa whole-cell lysates, whereupon the formation of complexes of TTR-RBPs and biotinylated RNAs was tested by Western blot analysis. Again, KSRP could not be studied by this approach due to undetectable Western blotting signals (not shown). Compared with the binding to a negative control RNA (biotinylated 3' UTR of the GAPDH housekeeping gene), several positive interactions were seen in the biotin pull-down assays (twofold or greater intensity by densitometry compared to GAPDH signals, when measurable, or simply detectable when biotinylated GAPDH signals were below the detection level). For example, HuR and AUF1 associated with all of the biotinylated transcripts tested and generally displayed stronger binding to the 3' UTR than CR fragments. Similarly, TIAR bound all of the transcripts shown in Fig. 1E, but not biotinylated AUF1 RNA (Fig. 2B), possibly because the region of TIAR association with the AUF1 mRNA was not present in the biotinylated transcript or because a putative additional factor(s) was necessary for binding but was unavailable in the in vitro binding reaction. Other unanticipated binding events included TIA-1 binding

FIG. 4. Effects of TTR-RBP silencing on the expression of TTR-RBPs. (A) Forty-eight hours after transfection of HeLa cells with siRNAs specifically targeting each of the TTR-RBPs indicated (or control [Ctrl.] siRNAs), the levels of the corresponding TTR-RBPs were tested by Western blot analysis; the membranes were reprobed to test the levels of the housekeeping proteins GAPDH and α -tubulin in order to monitor loading differences. In cultures with silenced TTR-RBPs (processed as described for panel A), the levels of AUF1 (B), HuR (C), KSRP (D), NF90 (E), TIA-1 (F), and TIAR (G) were assessed by Western blot analysis, quantified by densitometric scanning, normalized to loading controls (GAPDH or α -tubulin levels), and represented as “relative TTR-RBP levels” compared with the levels seen in control siRNA-transfected cultures. Black bars, TTR-RBPs showing twofold or higher levels, as well as TTR-RBPs showing 50% or lower levels, in TTR-RBP-silenced cultures compared with control silenced cells; gray bars, all other TTR-RBPs. The data represent the means and standard errors of the mean of four independent experiments.

to biotinylated NF90 3' UTR, TIAR binding to biotinylated HuR 3' UTR, and the general binding of NF90 to most biotinylated transcripts tested, including CR transcripts. Some of these complexes were likely absent in the RNP IP

analyses (Fig. 1) because the endogenous mRNAs might have been unavailable to the TTR-RBPs. For some 3' UTRs, additional segments were tested; a more proximal fragment of the HuR 3' UTR revealed similar binding pat-

FIG. 5. Analysis of the levels of TTR-RBPs. (A) Western blot analysis of the abundance of proteins corresponding to the TTR-RBPs shown in Fig. 4 (10 μ g of whole-cell HeLa lysates per lane). Ctrl., control. (B) Using uterine sarcoma MES-SA/Dx5 cells that were transfected as described for HeLa cells in the legend to Fig. 4A, whole-cell lysates were tested for expression of the TTR-RBPs shown. The levels of the control proteins β -actin and GAPDH were also tested. The data in panels A and B are representative of three independent experiments.

terns (i.e., it was bound by all TTR-RBPs except NF90) (not shown).

In summary, the binding of cytoplasmic TTR-RBPs to biotinylated transcripts spanning various regions of TTR-RBP mRNAs largely recapitulated the binding patterns observed by RNP-IP analysis in HeLa cells, as summarized in Fig. 3. Furthermore, most RNP complexes were found to implicate the 3' UTRs of target mRNAs (Fig. 3B).

Influence of TTR-RBP silencing on the expression of TTR-RBPs encoded by target mRNAs. In order to examine the functional consequences of the RNP associations under investigation, each TTR-RBP was individually silenced by transfecting HeLa cells with siRNAs. As shown by Western blot analysis (Fig. 4A), the specific siRNAs strongly reduced the levels of endogenous TTR-RBPs (from undetectable to ~25% of the levels seen in control cultures) by 48 h after transfection. The influence of each TTR-RBP silencing intervention upon the expression levels of all other TTR-RBPs was tested by Western blot analysis, followed by densitometric quantification. The results, summarized in Fig. 4A, revealed several prominent changes in the TTR-RBPs. In addition to seeing reductions in the levels of the particular TTR-RBP targeted in a transfection group (Fig. 4B to G), TIAR silencing increased TIA-1 protein levels (Fig. 4F), TIA-1 silencing increased TIAR levels (Fig. 4G), and HuR silencing reduced TIA-1 and KSRP levels (Fig. 4D and F). Unexpectedly, HuR silencing elevated AUF1 levels

in HeLa cells (Fig. 4B), despite the general positive influence of HuR on the expression of many target mRNAs in several systems (9) (see Discussion). All other RNP associations did not appear to have a measurable influence on the expression of the encoded TTR-RBP in untreated HeLa cells, as determined using siRNA-based approaches.

Representative changes in protein expression in HeLa cells are shown in Fig. 5A. To investigate whether these results were specific to HeLa cells or were observed more broadly, the silencing experiments were extended to Dx5 cells (derived from the human uterine sarcoma cell line MES-SA). By 48 h after siRNA transfections to silence HuR, TIA-1, or TIAR, the expression levels of HuR, AUF1, TIA-1, KSRP, TIAR, and loading controls were tested by Western blot analysis (Fig. 5C). The increase in AUF1 levels after HuR silencing that was documented in HeLa cells (Fig. 4B and 5A) was not seen in Dx5 cells, where AUF1 abundance remained unchanged (Fig. 5B). However, all other modulations were readily observed in Dx5 cells, supporting the view that the effects of TTR-RBPs upon the expression of target TTR-RBPs were a general occurrence: HuR silencing lowered KSRP and TIA-1 expression, TIAR silencing increased TIA-1 abundance, and TIA-1 silencing increased TIAR levels (Fig. 5B).

To further study the changes in TTR-RBP expression levels that occurred after TTR-RBP silencing in HeLa cells, we tested if there were differences in the levels of mRNAs

FIG. 6. Analysis of the effects of TTR-RBP silencing on specific TTR-RBP target mRNAs and proteins. (A) Total RNA was extracted from HeLa cell cultures that were processed as described in the legend to Fig. 4A, and the levels of AUF1, KSRP, and TIA-1 mRNAs in HuR-silenced cells (left), TIAR mRNA in TIA-1-silenced cells (center), and TIA-1 mRNA in TIAR-silenced cells (right) were tested by RT-qPCR analysis. The data represent the means and standard errors of the mean from three independent experiments. (B) The stability of the TIA-1 mRNA was measured in HeLa cells expressing either normal or reduced levels of HuR (48 h after transfection with either control (Ctrl.) siRNA or HuR siRNA, respectively) by incubating cells with actinomycin D to block de novo transcription by RNA polymerase II. At the times shown following the addition of actinomycin D, total RNA was extracted and the levels of TIA-1 mRNA and housekeeping control GAPDH mRNA were measured by RT-qPCR. After normalization of each mRNA to the level of 18S rRNA in each sample, mRNA clearance was visualized by comparing the levels of remaining mRNA to the levels of mRNA before the addition of actinomycin D (time zero). The mRNA half-life was calculated as the time required to reach one-half of its initial mRNA abundance.

encoding these TTR-RBPs (Fig. 6A). No measurable changes in TIAR or TIA-1 mRNA levels were seen in cultures with silenced TIA-1 or TIAR, respectively (Fig. 6A). By contrast, silencing of HuR caused a reduction in the

levels of KSRP and TIA-1 mRNAs (as measured by RT-qPCR analysis of total RNA) by 48 h after siRNA transfection, indicating that the changes in mRNA steady-state levels contributed to reducing KSRP and/or TIA-1 protein abundance (Fig. 4D and F). These decreases likely involved reductions in mRNA stability, as HuR has repeatedly been shown to stabilize target mRNAs (reviewed in reference 9). This possibility was tested experimentally for the TIA-1 mRNA. The half-life of the TIA-1 mRNA was tested by addition of actinomycin D to block de novo transcription, followed by measurement (by RT-qPCR) of the time required for the transcript to reach 50% of its initial abundance. HuR silencing was found to decrease the stability of TIA-1 mRNA from >10 h to ~3.5 h by this assay (Fig. 6B), revealing that HuR likely stabilizes the TIA-1 mRNA.

Posttranscriptional regulation of TIA-1 expression by HuR. In order to gain a more mechanistic insight into the regulation of one of these TTR-RBPs, we focused on TIA-1, whose expression levels were reduced in HuR-silenced cells and were elevated in TIAR-silenced cells. First, we increased HuR levels in order to test the levels of TIA-1 mRNA and protein. Forty-eight hours after the transfection of HeLa cells with a vector that expressed HuR-TAP (a chimeric HuR protein linked to a tandem-affinity purification [TAP] tag), TIA-1 protein levels increased by ~2-fold (Fig. 7A), mirroring the changes in steady-state TIA-1 mRNA levels, which were also elevated by ~2-fold as measured by RT-qPCR analysis from total RNA (Fig. 7B). We next performed a “rescue” experiment in which HuR was silenced using siRNAs that targeted the HuR 3' UTR (HuR3); HuR expression was then restored by ectopic expression of HuR-TAP, encoded by an mRNA which lacked the HuR 3' UTR and was thus refractory to this particular siRNA. While the HuR3 siRNA did not suppress HuR expression as robustly as the CR-targeting siRNA used earlier (Fig. 2A), it still lowered TIA-1 protein levels; a concomitant overexpression of HuR-TAP in cultures transfected in parallel caused TIA-1 protein levels to increase above those seen in cells transfected with pTAP and control siRNA and to remain elevated (Fig. 7C). The differences in TIA-1 protein seen in each transfection group reflected the differences in steady-state TIA-1 mRNA abundance measured after HuR levels were modulated (Fig. 7D).

For additional analysis of the posttranscriptional regulation of TIA-1 expression by HuR, the reporter plasmid pEGFP-TIA-1(3') was constructed to express a chimeric mRNA containing the TIA-1 3' UTR [EGFP-TIA-1(3') mRNA] in transfected cells; control populations were transfected with pEGFP, which expressed only the EGFP mRNA lacking any TIA-1 3' UTR regulatory sequences (Fig. 8A). As shown in Fig. 8B, the levels of EGFP protein expressed from the EGFP-TIA-1(3') mRNA were significantly reduced in the HuR-silenced cultures and routinely exhibited ~50% of the protein levels seen in the control siRNA transfection group. This regulation was linked to the presence of the TIA-1 3' UTR, since EGFP expression in cell populations transfected with the control reporter pEGFP was unchanged regardless of HuR expression levels. Finally, the reduction in expression of pEGFP-TIA-1(3') in HuR-silenced cells was associated with a decrease in the levels of reporter EGFP-TIA-1(3') mRNA in these cells. As shown in Fig. 8C, while EGFP mRNA levels were virtually

FIG. 7. Effects of HuR on TIA-1 mRNA and protein expression. (A) HeLa cells were transfected with a plasmid expressing a TAP-tagged HuR (pHuR-TAP) or the control pTAP vector. Forty-eight hours after transfection, the levels of endogenous HuR, HuR-TAP, and TIA-1 (as well as loading control β -actin) were tested by Western blot analysis. (B) Total RNA was prepared from HeLa cells that had been transfected as described in the legend to Fig. 6A; the levels of TIA-1 mRNA were measured by RT-qPCR, normalized to the levels of housekeeping control GAPDH mRNA, and represented as the relative TIA-1 mRNA levels in pHuR-TAP-transfected cells compared with pTAP-transfected cells. (C) HeLa cells were simultaneously transfected with siRNAs (an siRNA targeting the HuR 3' UTR [HuR3] or control [Ctrl.] siRNA) and with plasmid DNA (pHuR-TAP or pTAP). Forty-eight hours later, the levels of endogenous HuR, HuR-TAP, TIA-1, and loading control β -actin were assessed by Western blot analysis. (D) TIA-1 mRNA levels in cells that were transfected as described in the legend to Fig. 6C were measured by RT-qPCR, normalized to the levels of housekeeping control GAPDH mRNA, and represented as the relative TIA-1 mRNA levels in pHuR-TAP-transfected cells compared with pTAP- and control siRNA-transfected cells. Panels A and C display representative results from three independent experiments; panels B and D show the mean values and standard errors of the mean from three independent experiments.

unchanged in HuR-silenced cells, the expression of EGFP-TIA-1(3') mRNA was reduced to ~55% of the values seen in the control siRNA population. Together, these results indicate that TTR-RBP HuR influences the expression of another TTR-RBP, TIA-1. Together with earlier data on HuR binding to TIA-1 transcripts (Fig. 1B and 2B), our results indicate that HuR contributes to maintaining elevated TIA-1 mRNA levels, and hence TIA-1 expression, via effects on the TIA-1 3' UTR.

Posttranscriptional regulation of TIA-1 expression by TIAR.

To strengthen the notion of a regulatory circuitry among TTR-RBPs, we next investigated another regulatory interaction: the influence of TIAR upon TIA-1 expression. First, we tested the hypothesis that TIAR might suppress the translation of TIA-1. As shown in Fig. 9A, de novo TIA-1 translation was comparatively higher in populations with silenced TIAR, as monitored by nascent translation analysis using a brief incubation with [35 S]methionine and [35 S]cysteine, immediately followed by IP

reactions using anti-TIA-1 or control IgG antibodies. The radiolabeled signals revealed >1.7-fold-higher nascent TIA-1 translation in the TIAR siRNA transfection group than in the control populations, while the nascent translation of a control housekeeping protein (GAPDH) was unaffected. For this set of "rescue" experiments, TIAR was silenced using siRNAs that targeted the TIAR 3' UTR (TIAR3); TIAR expression was then restored by ectopic expression by transfecting cells with plasmid pMT2-TIAR, which lacked TIAR 3' UTR sequences and was therefore refractory to TIAR3 siRNA. Like HuR3 siRNA, TIAR3 siRNA did not suppress TIAR expression as robustly as the CR-targeting siRNA (compare Fig. 9A and B), but it did elevate TIA-1 expression by >2-fold; the concomitant overexpression of TIAR caused TIA-1 protein levels to remain reduced (Fig. 9B); overexpression of TIAR alone did not suppress TIA-1 expression levels (data not shown). The differences in TIA-1 protein levels in each transfection group

FIG. 8. TIA-1 3' UTR reporter analysis after HuR silencing. (A) Schematic of plasmids pEGFP and pEGFP-TIA-1(3'UTR), which were constructed in order to investigate the influence of the TIA-1 3' UTR on the expression of the heterologous reporter EGFP. (B) HeLa cells were cotransfected with 2 μ g of plasmid DNA [either pEGFP or pEGFP-TIA-1(3')] and with 100 nM of siRNA (either control [Ctrl.] siRNA or HuR siRNA); 48 h later, protein lysates were prepared from each transfection group and the levels of EGFP, HuR, and loading control β -actin were assessed by Western blot analysis. (C) The levels of EGFP and EGFP-TIA-1(3' UTR) mRNAs in cells that were transfected as described in the legend to Fig. 7B were measured by RT-qPCR analysis, using 18S rRNA for normalization. The data represent the means and standard errors of the mean from three independent experiments.

were not due to changes in steady-state TIA-1 mRNA abundance (Fig. 9C), suggesting instead that TIAR was acting as a negative regulator of TIA-1 translation.

Additional analysis of TIAR's influence on TIA-1 expression was performed by using the reporter plasmid pEGFP-TIA-1(3') (Fig. 8A). The levels of EGFP protein expressed from the EGFP-TIA-1(3') mRNA were significantly elevated (2.4-fold) in the TIAR-silenced cultures compared with the control siRNA transfection group (Fig. 10A). These differences in reporter expression did not arise from changes in EGFP-TIA-1(3') mRNA levels, as these remained unchanged (as did the levels of control EGFP mRNA) (Fig. 10B). Instead, these data indicate that the silencing of TIAR selectively elevates TIA-1 translation, and they further support the view that TIAR functions as a translational suppressor of TIA-1. Collectively, our

results provide mechanistic details of two regulatory influences of TTR-RBPs upon the expression of other TTR-RBPs: HuR binding to TIA-1 transcripts (Fig. 1B and 2B) contributes to maintaining elevated TIA-1 mRNA and protein levels (Fig. 7 and 8), and TIAR binding to TIA-1 transcripts (Fig. 1E and 2B) contributes to maintaining reduced TIA-1 expression by repressing its translation (Fig. 9 and 10).

DISCUSSION

Here, we have undertaken a systematic analysis of RNP complexes that comprise TTR-RBPs and their cognate mRNAs. Six TTR-RBPs (AUF1, HuR, KSRP, NF90, TIA-1, and TIAR) were surveyed for their individual abilities to bind each of the six mRNAs that encode these TTR-RBPs (Fig. 1 and 2). A complex matrix of interactions (Fig. 3) emerged from these studies, with each TTR-RBP showing affinity for its cognate mRNA and for multiple other TTR-RBP mRNAs (endogenous and recombinant in each case). This interesting circuitry of RNP associations suggests that the expression of these TTR-RBPs likely has an important posttranscriptional component and that their expression levels could be tightly interdependent.

The significance of RNPs comprising a given TTR-RBP and its cognate mRNA is unclear, but it likely represents instances of autoregulatory loops. Negative self-regulation would be expected for RNPs comprising AUF1-AUF1 mRNA and KSRP-KSRP mRNA, which are anticipated to limit the availability of the respective mRNAs, while TIAR-TIAR mRNA and TIA-1-TIA-1 mRNA RNPs would be predicted to suppress the translation of the respective proteins. On the other hand, a positive autoregulatory loop would be expected in cases such as HuR-HuR mRNA and NF90-NF90 mRNA RNPs, resulting in rapid enhancement of HuR mRNA stability and/or translation. However, it must be acknowledged that HuR and NF90 have also been shown to suppress the translation of several target mRNAs (31, 35, 44, 60), so negative-feedback loops may also be envisioned. A technical consideration to recognize here is the potential that the sensitive RNP IP analysis may have detected nascent TTR-RBP polypeptides whose template mRNAs were still associated with the respective proteins because translation was not terminated and the polysome was still assembled. We cannot exclude such interactions, although the biotin pull-down analyses indicated that these complexes also formed in the absence of ongoing translation.

The finding that HuR silencing caused an upregulation of AUF1 levels (Fig. 4B) was unexpected. The increased AUF1 expression did not appear to involve the stabilization of AUF1 mRNA in HuR-silenced cells, since the AUF1 mRNA was not significantly elevated in this population (Fig. 4A). Instead, it may arise from an enhancement of AUF1 translation after HuR silencing, consistent with a role for HuR as a suppressor of AUF1 translation. Should this possibility be confirmed experimentally, it would constitute an example, akin to that reported in the Wnt5a mRNA (35), of HuR repressing the translation of a target mRNA to which it binds on the 3' UTR (although HuR was also shown to have affinity for CR sequences [Fig. 2B]). In the cases of IFG-IR and p27, such repression was reportedly elicited via complexes that formed on internal ribosomal entry site-bearing 5' UTRs (31, 44). By

FIG. 9. Effects of TIAR on TIA-1 expression. (A) Forty-eight hours after transfection of HeLa cells with either an siRNA targeting the TIAR CR (TIAR siRNA) or a control (Ctrl.) siRNA, the levels of endogenous TIAR and loading control α -tubulin were assessed by Western blot analysis (left). (Right) In the same cultures, the levels of nascent TIA-1 and control GAPDH were measured following incubation of cells with L-[35 S]methionine and L-[35 S]cysteine for 15 min; following IP using either anti-TIA-1 (left) or anti-GAPDH (right) antibodies, along with control IgG (center), the incorporation of radiolabeled amino acids into newly synthesized TIA-1 and GAPDH polypeptides (arrowheads) was visualized after SDS-PAGE and transfer and was quantified using a phosphorimager; the change in de novo-translated TIA-1 is shown. (B) HeLa cells were simultaneously transfected with siRNAs (an siRNA targeting the TIAR 3' UTR [TIAR3] or control siRNA) and with plasmid DNA (pMT2-TIAR or pMT2). Forty-eight hours later, the levels of endogenous TIAR, TIA-1, and loading control β -actin were assessed by Western blot analysis. The differences in TIA-1 expression levels are indicated. (C) TIA-1 mRNA levels in cells that were transfected as described in the legend to panel B were measured by RT-qPCR, normalized to the levels of housekeeping control GAPDH mRNA, and represented as the relative TIA-1 mRNA levels in pMT2-TIAR-transfected cells compared with pMT2- and control siRNA-transfected cells. Panels A and B display representative results from three independent experiments; panel C shows the mean values and standard errors of the mean from three independent experiments.

contrast, HuR might enhance the translation of KSRP, since interventions to silence HuR moderately reduced KSRP mRNA levels (Fig. 6A) but strongly lowered KSRP protein abundance (Fig. 4D and 5A). Such a role for HuR as a putative enhancer of KSRP translation is in keeping with the translation-promoting function of HuR upon target prothymosin α , p53, CAT-1, cytochrome *c* (7, 27, 32, 42), and the hypoxia-inducible protein 1 α (S. Galban and M. Gorospe, submitted for publication). The discrepancy between the positive influence of HuR upon the translation of certain target mRNAs and its negative effects upon other target mRNAs remains unresolved.

Curiously, despite many other binding events detected (Fig. 1 and 3), most TTR-RBPs were not found to influence the expression of the corresponding target TTR-RBP mRNAs in unstimulated cultures. It is possible that the overexpression (which did not influence the expression of other TTR-RBPs [data not shown]) or the silencing interventions were insufficient in magnitude or that the kinetics of the effects were not studied in adequate detail. However, it is more plausible that the lack of appreciable consequences from these interactions arises from the fact that the functional roles of these TTR-

RBPs have often become apparent only after stimulation of the cell with agents of various types (stressful, proliferative, immune, etc.). The studies reported here did not include any such treatments. Ongoing efforts are aimed at testing whether exposure to the aforementioned stimuli will unmask the regulation of TTR-RBPs by other TTR-RBPs, as anticipated from the RNP analyses.

Among the exceptions was TIA-1, whose expression was found to be influenced positively by HuR and negatively by TIAR in cell populations that were otherwise unstimulated (Fig. 7 to 10). HuR was found to bind the endogenous TIA-1 mRNA, as well as biotinylated TIA-1 transcripts (Fig. 1B and 2B). HuR silencing caused a reduction in TIA-1 mRNA and protein levels (Fig. 4F and 5A), and conversely, HuR overexpression elevated TIA-1 mRNA and protein levels (Fig. 7A). Importantly, ectopic reexpression of HuR after the endogenous HuR was silenced restored TIA-1 mRNA and protein levels (Fig. 7B), indicating that HuR could rescue the loss of TIA-1 and demonstrating HuR's direct influence on TIA-1 expression. HuR elicited these effects via its interaction with the TIA-1 3' UTR, since a heterologous reporter comprising the EGFP CR and the TIA-1 3' UTR displayed a similar

FIG. 10. TIA-1 3' UTR reporter analysis after TIAR silencing. (A) Two micrograms of plasmids pEGFP and pEGFP-TIA-1(3'UTR), depicted in Fig. 8A, were cotransfected with 100 nM of siRNA (either control [Ctrl.] siRNA or TIAR siRNA); 48 h later, protein lysates were prepared from each transfection group and the levels of EGFP, TIA-1, and loading control β -actin were assessed by Western blot analysis. (B) The levels of EGFP and EGFP-TIA-1(3') mRNAs in cells that were transfected as described in the legend to panel A were measured by RT-qPCR analysis, using 18S rRNA for normalization. The data represent the means and standard errors of the mean from three independent experiments.

regulatory pattern in response to modulation of HuR levels (Fig. 8). These results are in keeping with earlier observations that HuR silencing was associated with reductions in TIA-1 protein abundance (27). Studies are under way to investigate the influence of HuR upon the expression of AUF1 and KSRP, two regulatory interactions that also occurred in the absence of additional stimuli (Fig. 4 to 6). TIAR was also found to bind the endogenous TIA-1 mRNA, as well as biotinylated TIA-1 transcripts (Fig. 1E and 2B), and silencing TIAR enhanced TIA-1 expression levels (Fig. 4F and 5A). Further analysis of this regulatory RNP complex revealed that TIAR suppressed the translation and expression levels of the endogenous TIA-1 (Fig. 9) and reduced the levels of the aforementioned heterologous reporter protein (EGFP) that was expressed from a chimeric mRNA including the TIA-1 3' UTR (Fig. 10). Taken together, these two regulatory processes (HuR and TIAR influencing TIA-1 expression levels) support the notion that TTR-RBPs are subject to control by a posttranscriptional regulatory circuitry involving TTR-RBPs.

It is worth noting that in addition to RBPs, there is another

major group of *trans*-acting factors that interact with mRNAs and negatively regulate their expression, microRNAs (miRNAs). miRNAs (typically ~21 nucleotides long) form complexes with various degrees of complementarity to the mRNA and can lower the stability and translation of target mRNAs (reviewed in reference 53). The overlapping functions of miRNAs and TTR-RBPs, both influencing mRNA half-life and translation, may not be coincidental, given mounting evidence that physical and functional interactions exist between miRNAs and TTR-RBPs (7, 19, 24). In this regard, it is likely that miRNAs also contribute to the posttranscriptional regulation of TTR-RBP expression; we further anticipate that miRNA biogenesis is, in turn, influenced by TTR-RBPs.

In closing, the studies reported here highlight the complexity of a particular area of posttranscriptional gene control: regulatory RBPs modulating the expression of regulatory RBPs. With a growing interest in understanding the molecular underpinnings of posttranscriptional gene regulation, our results raise awareness of the need to consider additional regulatory interactions involving TTR-RBPs. The notion of "regulating the regulator" is well established in the transcription field, with numerous documented examples of transcription factors controlling the transcription of genes that encode transcription factors. Through the systematic analysis of a subset of RBPs that regulate mRNA turnover and translation, our findings reported here provide experimental support to the concept that analogous regulatory processes are in effect at the posttranscriptional level.

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