The RNA binding protein PTB exerts translational control on 3'-untranslated region of the mRNA for the ATP synthase beta subunit

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Abstract

We have recently reported that RNA binding proteins TIA-1 (T-cell Intracellular Antigen-1), TIAR (TIA-1 related protein) and HuR (Hu antigen R) modulate the expression of the ATP synthase beta subunit mRNA (β-F1-ATPase mRNA) (J.M. Izquierdo, *Biochem. Biophys. Res. Commun.* 348 (2006) 703-711). Here we found that PTB (Polypyrimidine Tract Binding protein) is a novel member of the ribonucleoprotein complex that interacts with the β-F1-ATPase mRNA through an adenosine/uridine (AU)-rich element located to the β-F1-ATPase 3'-untranslated region (β-3'-UTR). Co-expression of GFP from a reporter mRNA quimera containing human β-3'-UTR and recombinant PTB in HeLa cells increased the amount of GFP protein. Interestingly, this effect is not due to increased steady-state levels of GFP-β-3'-UTR mRNA. Taken together, these results suggest that PTB regulates post-transcriptional expression of the β-F1-ATPase mRNA at the translational level.

Keywords: PTB, TIA-1, TIAR, β-F1-ATPase mRNA, gene expression control.

Introduction

RNA binding proteins and/or microRNAs (small non-coding RNAs) are the molecular executors of the post-transcriptional control of gene expression and, therefore, the major determinants of the fate of mRNAs in eukaryotic cells [1-4]. This level of regulation introduces the spatial and temporal as well as a more dynamic and versatile adaptation to the rapid switch in the responses of mammalian cells to changes that occur in several environmental conditions [1-4]. RNA binding proteins together with microRNAs define macromolecular and multifunctional associations known as ribonucleoprotein (RNP) complexes [2,3,5,6]. These dynamic structures contain each RNA during its life since its synthesis until its destruction, governing the complex metabolism of these ancient biopolymers [5,6]. Multiple interactions into RNP micro-scenario are the molecular basis to the fine-tuning of gene expression in response to epigenetic changes of many cellular processes, including immune response, apoptosis, neuronal differentiation, intracellular transport, inflammation, cell cycle, and oncogenesis [1-8].

The mitochondrial H^+-ATP synthase plays an essential role in cellular function [9,10] and in apoptosis [11]. The β-subunit of the mitochondrial H^+-ATP synthase (β-F1-ATPase) catalyzes the rate-limiting step of ATP formation in eukaryotic cells [9]. The expression of the nuclear gene encoding the β-F1-ATPase subunit is regulated at the post-transcriptional level during development [12-15], in several tissues [16,17] and in carcinogenesis [18,19]. Post-transcriptional regulation of β-F1-ATPase mRNA involves the control of its stability [14] and of its translational efficiency [12,13,15,18,20-23]. From a cellular viewpoint, this mRNA is localized into granules associated with the mitochondrial outer membrane [24,25]. The translation and cellular localization of this

mRNA, both in lower [26,27] and higher [24,25] eukaryotes, appears to depend on its 3'-untranslated region (3'-UTR) [15,18,20,21,23-27]. Recently, we have reported that RNA binding proteins TIA-1 (T-cell intracellular antigen-1), TIAR (TIA-1 related protein), and HuR (Hu antigen R) modulate the post-transcriptional expression of this mRNA through its 3'-UTR [23].

The aim of this work was to identify novel factors involved in the post-transcriptional control of human β-F1-ATPase mRNA. We report here that the RNA binding protein PTB (Polypyrimidine Tract-Binding protein), also known as hnRNP I, directly binds to the AU-rich sequence present in the 3'-UTR of this mRNA. We also show that the expression level of a chimeric mRNA containing gfp fused to the β-3'-UTR sequence is modulated by recombinant PTB protein, in the absence of significant changes at the steady-state mRNA level. These results suggest a specific role for PTB in the translational control of the human β-F1-ATPase mRNA in living cells.

Materials and methods

Reagents

HeLa adherents cells were grown as described [28]. The constructs used were obtained as described previously [23]. Antibodies: anti-TIA-1 (C-20, Santa Cruz Biotechnology), anti-TIAR (C-18, Santa Cruz Biotechnology), anti-PTB (BB7, kindly provided by Dr. C.W. Smith, University of Cambridge, UK), anti-HA-epitope (16B12, Covance), anti-GFP (JL-8, BD Biosciences Clontech), anti-α-Tubulin (B-5-1-2, Sigma), and anti-U2AF65 (MC3, kindly provided by Dr. J. Valcárcel, CRG, Spain). Recombinant His-TIA-1, His-PTB and GST-TIAR proteins were expressed in *E. coli* and purified by affinity chromatography.

Protein analysis and RNA isolation

Whole-cell HeLa extracts were prepared as described previously [23]. Cytoplasmic RNAs were prepared using the RNeasy kit (Qiagen) as described [28]. Cytoplasmic RNA was quantified by optical density at 260 nm and treated with RNase-free DNase (Promega). RNA was reverse-transcribed (RT) with High-Capacity cDNA Archive kit (Applied Biosystems). Real time PCR (qPCR) was performed using three different RT reactions. Oligonucleotides used for qPCR amplification were: 5'-CCTGCTGGAGTTCGTGACCGC-3' and 5'-CTGAGGGGTGTACATTTTATTG-3' for chimeric gfp-β-3' mRNA, and 5'-AAAGACCTGTACGCCAACAC-3' and 5'-GTCATACTCCTGCTTGCTGA-3' for β-actin mRNA [23]. For qPCR, an aliquot (5 ng) of the cDNAs and 0.5 μM of each primer together with Power Sybr Green PCR Master mix (Applied Biosystems) were used following the protocol: 10 min at 95ºC followed by 20-40 cycles of denaturation (15 sec at 95ºC) and annealing-elongation (1 min at 60ºC)

with fluorescence acquisition at 60ºC. A melting curve (15 sec at 95ºC, 15 second at 60ºC and 15 sec at 95ºC) with fluorescence acquisition at 60ºC to 95ºC was included in each qPCR. The amplification efficiency of each primer was empirically determined and applied to the relative quantification of the data using qbase software (http://medgen.ugent.be/qbase/).

Preparation of *in vitro* synthesized RNA probes. UV-crosslinking-immunoprecipitation (UV-CXL-IP) and immunoprecipitation-RT-PCR (IP-RT-PCR) assays

In vitro transcription reactions were carried out with T7 RNA polymerase as described [23]. As specific binding controls, an RNA probe identified as β-3'-UTR (ΔAU), lacking the last 51 nt of the human β-3'-UTR end sequence (where resides the AU-rich sequence) and an unrelated RNA (identifies as C) [23] were used. UV cross-linking assays were carried out as described [15,23,28]. UV-CXL-IP and IP-RT-PCR analysis were performed as previously described [23,29].

Results and Discussion

The human β-3'-UTR ribonucleoprotein (mRNP) complex comprises of at least four protein sets of which we previously identified the species with apparent molecular masses of 40- and 42-kDa as the RNA binding proteins TIA-1 (T-cell intracellular antigen-1), TIAR (TIA-1 related protein) and HuR (Hu antigen R) (Figure 1A, lane 2, and see Figure 1 in [23]). The identities of the remaining proteins (p57 and p80) remained unknown. Our previous results also indicated that the formation of human β-3'-RNP complexes depended of poly(U) sequences present at the 3'-UTR (Figure 1A, lane 3, and see Figure 1 in [23]). Several RNA-binding proteins are able to bind U-rich sequences and have been shown to play a defined role in RNA metabolism [30-33]. Among these, the Polypyrimidine Tract- Binding protein (PTB), also known as heterogeneous nuclear ribonucleoprotein I (hnRNP I). PTB is a well-characterized multifunctional RNA binding protein that plays a relevant role in the control of RNA post-transcriptional metabolism [34-44]. One of the β-3'-RNP crosslinked complexes (Figure 1A, lane 2) had an apparent molecular mass (57 kDa) close to that of PTB. We therefore test whether PTB is a component of the β-3'-RNP. Radiolabeled β-3'-UTR riboprobe was incubated with HeLa extracts, UV crosslinked and immunoprecipitated with the anti-TIA-1, anti-TIAR, anti-PTB, and anti-U2AF65 antibodies (Figure 1B, lanes 3-6, respectively). PTB was immunoprecipitated together with β-3'-UTR (Figure 1B, lane 5), indicating that PTB is part of the RNP complex that forms on β-3'-UTR RNA. TIA-1 and TIAR immunoprecipitations were used as positive controls of the experiment. No RNA-protein immunoprecipitates were detected with empty beads or anti-U2AF65 antibody (Figure 1B, lanes 2 and 6). As an additional evidence toward identifying whether PTB was present in

the β-3'-UTR RNP, band shift assays and antibody supershifts were carried out (Figure 1C). This type of assay reveal either the formation of a higher molecular weight RNA-protein complex (supershift) or disruption of one or more of the RNA-protein complexes containing the epitope recognized by the antibody. Our results show that the presence of anti-TIA-1, anti-TIAR, or anti-PTB antibodies in the reaction mixture produced a supershift (Figure 2C, lanes 3-5), with a concomitant reduction in the RNA-protein complex for TIA-1 and TIAR (Figure 2C, lanes 3 and 4). The intensity of the supershift increased notably when the anti-PTB antibody was used (Figure 2C, lane 5), suggesting that a more stable complex between PTB protein and β-3'-UTR riboprobe is formed. These results support the notion that PTB can form *in vitro* ribonucleoprotein complexes with β-3'-UTR RNA.

We next investigated the *in vivo* interaction between PTB and the 3'-UTR of β-F1-ATPase mRNA. Whole-cell extracts were prepared and the β-F1-ATPase mRNA associated with PTB was immunoprecipitated with the corresponding antibody and analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) (Figure 1D). The β-3'-UTR RT-PCR signal was detected in the samples immunoprecipitated with TIA-1, TIAR and PTB antibodies (Figure 1D, lanes 2-4, respectively). This signal was absent when the cell extract were incubated either with RNase A (Figure 1D, lane 1) or in the presence of anti-U2AF65 antibody (Figure 1D, lane 5). Taken together, the results of panels B to D indicate that PTB associates with 3'-UTR sequence of human β-F1-ATPase mRNA *in vitro* and *in vivo*.

To illustrate a direct interaction of PTB to the β-3'-UTR of β-F1-ATPase mRNA, β-3'-UTR riboprobe was incubated with affinity-purified GST, His-TIA-1, GST-TIAR or

His-PTB proteins, crosslinked, and separated on SDS-10% PAGE gel (Figure 1E). Recombinant PTB, as well as TIA-1 and TIAR, were found to bind the β-3'-UTR (Figure 1D, lanes 2, 5, and 8). To further demonstrate the specificity of this binding, we used a deletion mutant lacking the AU-rich sequence (β-3', ΔAU) (Figure 1E, lanes 3, 6, and 9) as well as an unrelated RNA (c) (Figure 1E, lanes 4, 7, and 10). No complex was detected in the control sample containing GST (Figure 1E, lane 1).

Altogether the above results support the interaction of PTB with the 3'-UTR of β-F1-ATPase mRNA, suggesting among others, a potential role of PTB in the post-transcriptional regulation of β-F1-ATPase expression. To test this possibility HeLa cells were cotransfected with a reporter GFP-β-3'-UTR chimera, *Drosophila* Hsp 83 mRNA and a recombinant plasmid expressing either TIA-1, TIAR, PTB or HuR proteins (Figure 2). The synthesis of GFP reporter protein and of recombinant proteins was analyzed after 24 h by Western blot using antibodies against GFP, Xpress-epitope, HA-epitope, and α-Tubulin (used as loading control) (Figure 2, panels A and B). The results indicated that PTB over-expression, as well as TIA-1, TIAR, or HuR, resulted in a significant increase of GFP synthesis (Figure 2, panels A-C). The effects appeared to be specific for the GFP-β-3'-UTR chimera because *Drosophila* HA-Hsp 83 expression remained unchanged (Figure 2, panels A and B). The increased expression of GFP protein in transfected HeLa cells with the plasmids indicated is not due to higher GFP-β-3'-UTR mRNA levels. Similar steady-state levels of GFP-β-3'-UTR and β-actin mRNAs (used as a loading control) were found by reverse transcription and quantitative PCR in the cytoplasmic RNAs analyzed (Figure 2D). These results suggest that not only TIA-1, TIAR, HuR, but also PTB can

modulate β-F1-ATPase gene expression. Taken together, these findings suggest that PTB regulates human β-F1-ATPase expression at the translational level.

During the course of our study, Gama-Carvalho and colleagues [45], from Maria Carmo-Fonseca´s lab, reported a collection of PTB target mRNAs implicated in intracellular transport, vesicle trafficking and apoptosis. Our results show for the first time a direct interaction and regulation of β-F1-ATPase mRNA by PTB.

PTB represents one of the first well-characterized paradigms of a multifunctional RNA binding protein. PTB regulates RNA metabolism events occurring in the nucleus and the cytoplasm. In the nucleus, PTB functions as pre-mRNA splicing regulator probably as a general repressor of exon inclusion, as a consequence of its capacity to bind to pyrimidine-rich tracts located in intronic and exonic sequence elements on the pre-mRNA [28,34-38]. Additionally, PTB has been implicated in the regulation of 3' end processing [39]. In the cytoplasm, PTB is involved in the regulation of cap-independent translation of viral and cellular mRNAs driven by the internal ribosome entry sites (IRES) [40,41]. PTB has also been implicated in the control of mRNA localization [42,43] and stability [44]. We report here a role for PTB in the post-transcriptional control of β-F1-ATPase mRNA in living cells mediated by an AU-rich element (ARE) present in the 3'-UTR of this mRNA. Interestingly, the 3'-UTR of the β-F1-ATPase mRNA regulates the translation of this messenger in a cap-independent manner, showing functional features similar to the IRES [21]. PTB contains four RNA recognition motifs (RRMs) and recognizes short motifs, such as UCUU and UCUCU, located within a pyrimidine-rich contex [34-38]. In most systems regulated by PTB, regulation is achieved through the interaction of PTB with multiple PTB binding sites surrounding the target sequence [36-38]. The last 51-nt of

the 3'-UTR sequence end of β-F1-ATPase mRNA, where we have demonstrated PTB is bound, contains two motifs (UUCUCUCU) within a pyrimidine-rich context [23]. So, we suggest that (i) this sequence on β-3′-UTR contains the *cis*-acting element recognized by PTB and (ii) the features of the 3'-UTR of the β-F1-ATPase mRNA as an IRES can be bound to the capacity of this sequence to recruit PTB.

It is nowadays recognized that the subcellular localization and translation of certain mRNA species influences the spatial and temporal distribution of protein and affects the function of eukaryotic cells [42,43]. It has been suggested that localized mRNAs do not become translationally active until placed or anchored in their appropriate subcellular site. For the localized β-F1-ATPase mRNA this would imply that cytoplasmic granules containing β-F1-ATPase mRNA can not be translationally active until they reach the vicinity of mitochondria or are anchored to the outer mitochondrial membrane. Given that a same RNA-binding protein can repress or activate mRNA translation under different environmental conditions, it is tempting to speculate that a multifunctional effector as PTB is compatible to be involved in the coupling between different events affecting to the post-transcriptional control, including cellular localization and translation. Thus, PTB is an additional candidate to regulate these processes in a concerted way with other specific-β-F1-ATPase mRNA binding proteins, such as TIA-1, TIAR and HuR [23]. This set of proteins comprise the β-F1-ATPase post-transcriptional operon [1], which might determine the timing of the biosynthesis of β-F1-ATPase protein during several stages of active/inactive mitochondrial biogenesis, including the development, cell cycle or oncogenesis [14,15,18-21,46].

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Figure legends

Figure 1 PTB is a component of human β-F1-ATPase mRNA RNP particle

(A) Specific binding of the HeLa extract proteins to the AU-rich sequence. Protein (25 μg) derived from HeLa cells (lanes 2 and 3) was incubated with ^{32}P-labeled β-3'UTR (lane 2) or AU-rich region deleted β-3'UTR (β-3' ΔAU; lacking the last 51 nt of the 3'-UTR sequence end) (lane 3) riboprobes. Then, the samples were UV crosslinked, digested with RNase T1 and loaded on a 10% SDS-PAGE. Lane 1 shows the T1 digested radiolabeled β-3'UTR riboprobe in the absence of the added protein. The arrowheads indicate the nature of the RNA-protein complexes. Molecular mass markers (kDa) are shown on the left. (**B**) UV cross-linking and immunoprecipitation (UV-CXL-IP) assays. HeLa cell extracts were incubated with radiolabeled β-3'UTR riboprobes, UV cross-linked and precipitated with empty beads (lane 2) or immunoprecipitated with either anti-TIA-1 (lane 3), anti-TIAR (lane 4), anti-PTB (lane 5) or anti-U2AF65 (lane 6) antibodies. The RNA-antigen-antibody complexes were resolved on a 10% SDS-PAGE gel. Lane 1 shows the overexposed profile of proteins bound to ^{32}P-labeled β-3'-UTR in HeLa cells. The arrowheads indicate the apparent migration of the immunoprecipitated RNA-protein complexes. Molecular mass markers (kDa) are shown on the left. (**C**) Electrophoretic mobility shift assay (EMSA) and antibody supershifts. Radiolabeled β-3'-UTR riboprobes were incubated with HeLa extracts (30 μg) in the absence (lane 2) or presence of anti-TIA-1 (lane 3), anti-TIAR (lane 4), anti-PTB (lane 5) antibodies. Lane 1 shows the T1 digested riboprobe in the absence of extract. The native RNA-protein complexes were resolved on 5% PAGE gel. The identities of RNA-protein complexes are indicated by arrowheads (C1, C2, and supershift). (**D**) Immunoprecipitation and reverse transcription plus polymerase

chain reaction (IP-RT-PCR) analysis. HeLa cell extracts were immunoprecipitated with anti-TIA-1 in the presence of RNase A (lane 1), anti-TIA-1 (lane 2), anti-TIAR (lane 3), anti-PTB (lane 4) or anti-U2AF65 (lane 5) antibodies and the β-F1-ATPase mRNA associated to the antigen-antibody complexes was analyzed by RT-PCR on a 2% agarose gel. The arrowhead shows the migration of the resulting PCR products on the right. Molecular mass markers (MW) are indicated on the left. (**E**) UV cross-linking analysis with purified recombinant proteins. Equal amounts (100 ng) of purified GST (lane 1), His-TIA-1 (lanes 2-4), GST-TIAR (lanes 5-7) or MBP-PTB (lanes 8-10) fusion proteins were incubated with ^{32}P-labeled full-length β-3'UTR (lanes 1, 2, 5 and 8), ΔAU β-3'UTR (a deletion mutant version lacking the last 51 nt containing AU-rich sequence) (lanes 3, 6 and 9) or unrelated RNA (c) (lanes 4, 7 and 10) riboprobes, UV cross-linked, and fractionated on 10% SDS-PAGE as before. The electrophoretic mobilities of the fusion proteins are indicated by arrowheads. Molecular mass markers (kDa) are indicated on the left.

Figure 2 Over-expression of recombinant PTB increases the GFP expression from a GFP-β-3'-UTR mRNA in HeLa cells

(**A and B)** Effects of TIA-1, TIAR, HuR and PTB over-expression on the translation of a GFP-β3' mRNA quimera. HeLa cells were cotransfected with chimeric GFP-β-3' reporter and HA-Hsp 83 plasmids as well as with a plasmid encoding recombinant versions of either TIA-1, TIAR, PTB or HuR proteins. Protein extracts (20 μg) were analyzed by Western blot with anti-GFP, anti-Xpress epitope, anti-HA-epitope, and anti-α-tubulin antibodies. Representative Western blots are shown to illustrate the ectopic expression

levels of GFP* (truncated version of GFP [23]), GFP-TIA-1, GFP-TIAR, Xpress-PTB, HA-Hsp 83, and endogenous α-Tubulin (panels A and B). Molecular weight markers are indicated on the left. The positions of the expressed recombinant proteins are indicated on the right by arrowheads. (**C**) The values of GFP* expression normalized by α-tubulin levels were expressed relative to empty pCMV56 plasmid expression, whose value was fixed arbitrarily to 1. The values are means ± SEM for at least 2-3 independent experiments. (**D**) Cytoplasmic RNAs from HeLa cells transfected with recombinant proteins indicated before were analyzed by reverse-transcription and quantitative PCR. The values of GFP-β3' mRNA normalized by β-actin mRNA levels were expressed relative to empty pCMV56 plasmid expression, whose value was fixed arbitrarily to 1. The values are means ± SEM for at least 2-3 independent experiments.

FIGURE 1

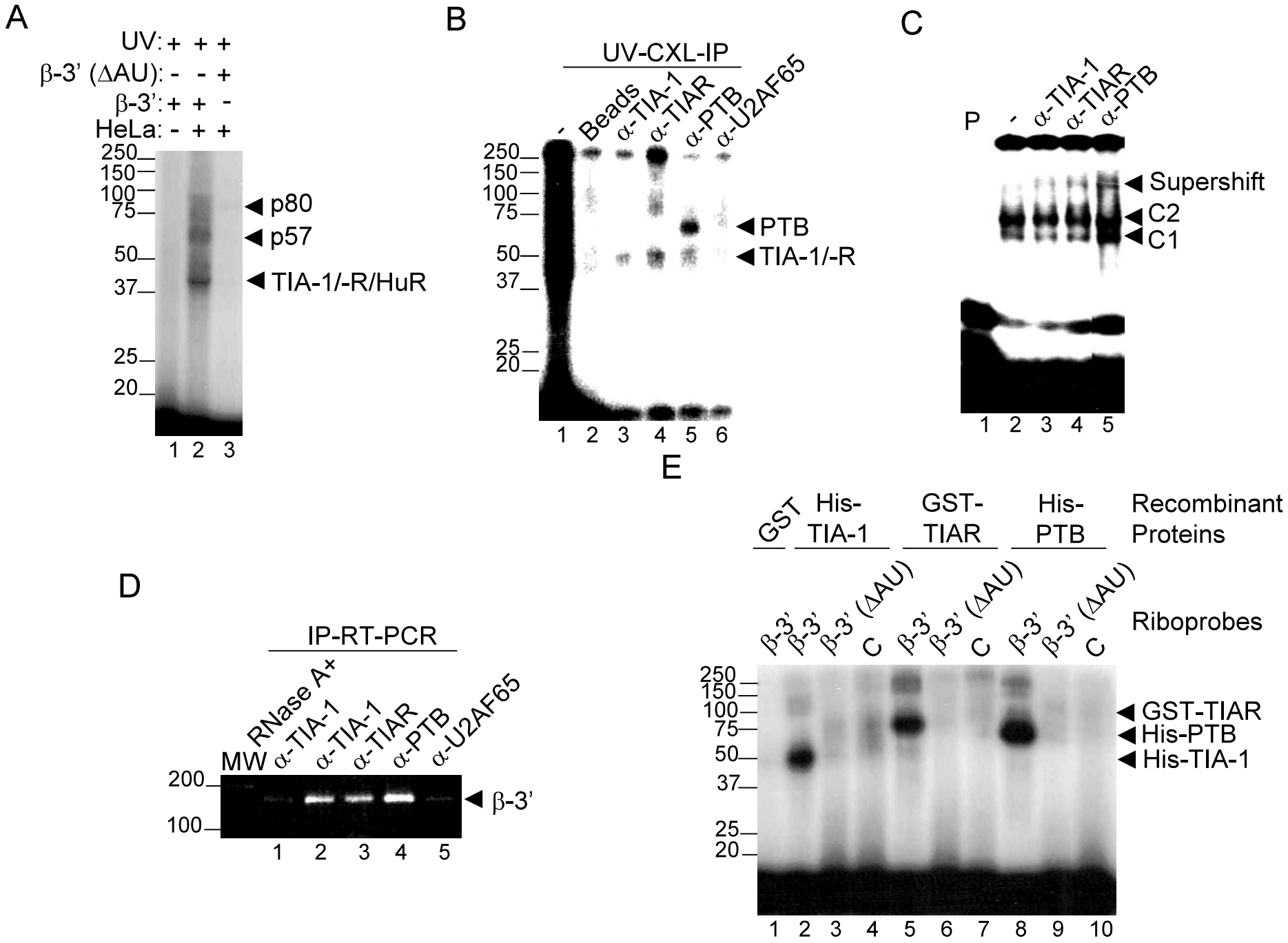
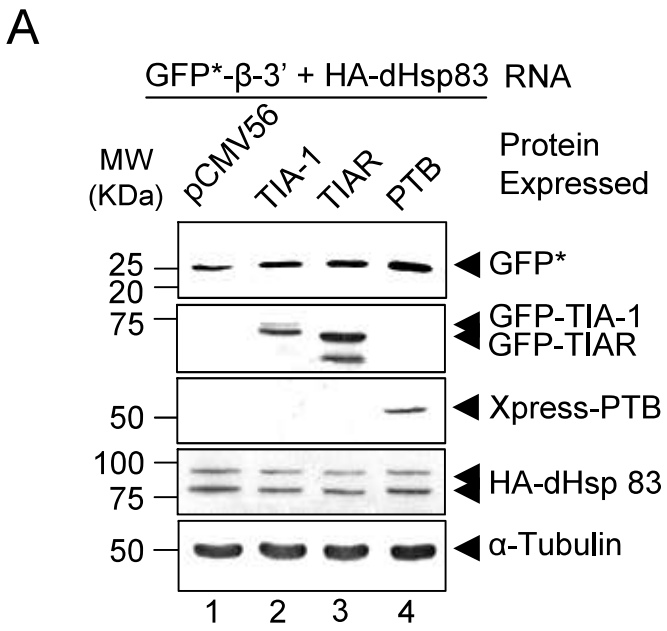
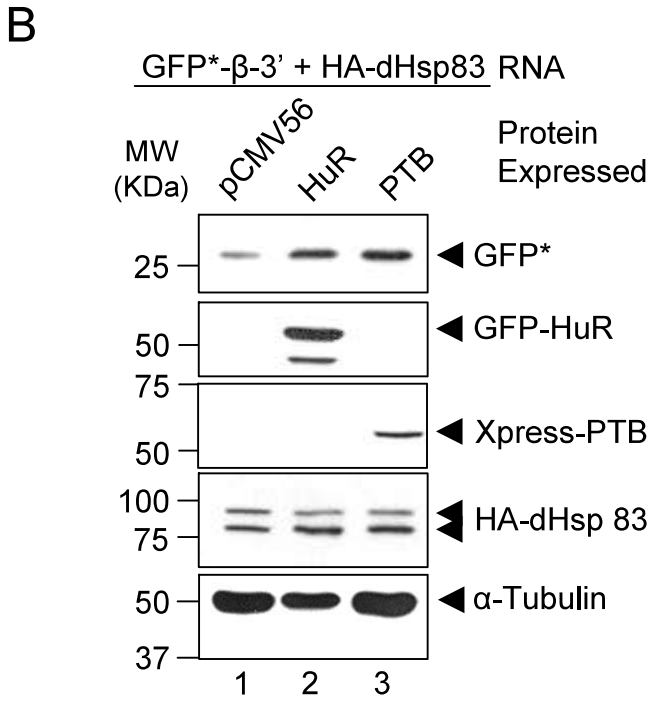
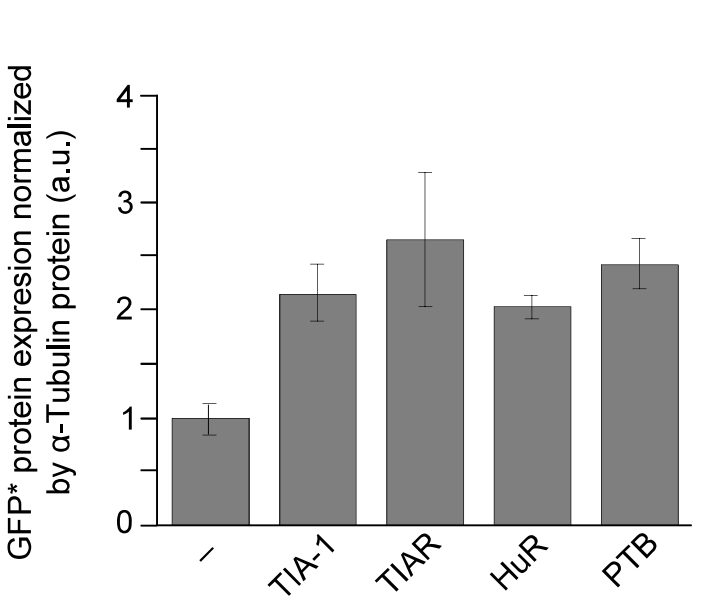
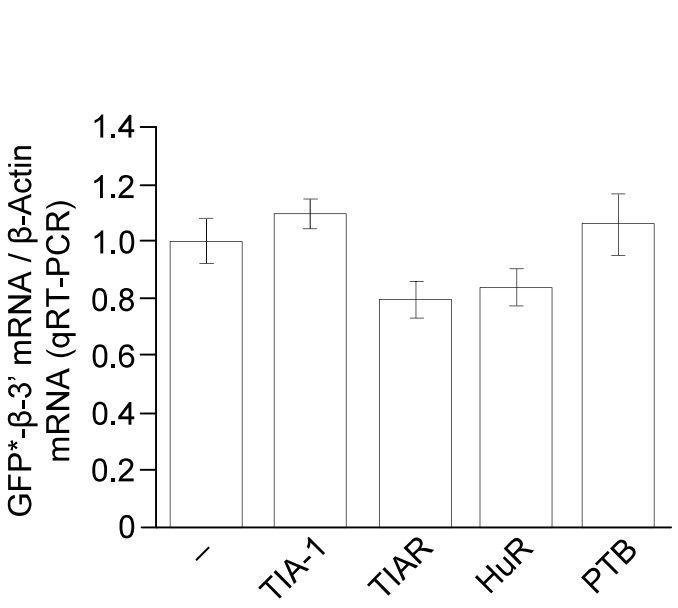

A
UV: + + +
β-3' (ΔAU): - - +
β-3': + + -
HeLa: - + +
250
150
100
75
50
37
25
20
p80
p57
TIA-1/-R/HuR
1 2 3
B
UV-CXL-IP
- Beads α-TIA-1 α-TIAR α-PTB α-U2AF65
250
150
100
75
50
37
25
20
PTB
TIA-1/-R
1 2 3 4 5 6
C
P - α-TIA-1 α-TIAR α-PTB
Supershift
C2
C1
1 2 3 4 5
D
IP-RT-PCR
MW RNase A+ α-TIA-1 α-TIA-1 α-TIAR α-PTB α-U2AF65
200
100
β-3'
1 2 3 4 5
E
GST
His-TIA-1
GST-TIAR
His-PTB
Recombinant Proteins
β-3' β-3' β-3' (ΔAU) C β-3' β-3' (ΔAU) C β-3' β-3' (ΔAU) C
Riboprobes
250
150
100
75
50
37
25
20
GST-TIAR
His-PTB
His-TIA-1
1 2 3 4 5 6 7 8 9 10

FIGURE 2


A
GFP*-β-3' + HA-dHsp83 RNA
MW (KDa)
pCMV56
TIA-1
TIAR
PTB
Protein Expressed
25
20
75
50
100
75
50
GFP*
GFP-TIA-1
GFP-TIAR
Xpress-PTB
HA-dHsp 83
α-Tubulin
1 2 3 4


B
GFP*-β-3' + HA-dHsp83 RNA
MW (KDa)
pCMV56
HuR
PTB
Protein Expressed
25
50
75
50
100
75
50
37
GFP*
GFP-HuR
Xpress-PTB
HA-dHsp 83
α-Tubulin
1 2 3


C
GFP* protein expresion normalized by α-Tubulin protein (a.u.)
0
1
2
3
4
–
TIA-1
TIAR
HuR
PTB


D
GFP*-β-3' mRNA / β-Actin mRNA (qRT-PCR)
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
–
TIA-1
TIAR
HuR
PTB