Post-transcriptional regulation of androgen receptor mRNA by an ErbB3 binding protein 1 in prostate cancer

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ABSTRACT

Androgen receptor (AR)-mediated pathways play a critical role in the development and progression of prostate cancer. However, little is known about the regulation of AR mRNA stability and translation, two central processes that control AR expression. The ErbB3 binding protein 1 (EBP1), an AR corepressor, negatively regulates crosstalk between ErbB3 ligand heregulin (HRG)-triggered signaling and the AR axis, affecting biological properties of prostate cancer cells. EBP1 protein expression is also decreased in clinical prostate cancer. We previously demonstrated that *EBP1* overexpression results in decreased AR protein levels by affecting AR promoter activity. However, EBP1 has recently been demonstrated to be an RNA binding protein. We therefore examined the ability of EBP1 to regulate AR post-transcriptionally. Here we show that EBP1 promoted AR mRNA decay through physical interaction with a conserved UC-rich motif within the 3′-UTR of AR. The ability of EBP1 to accelerate AR mRNA decay was further enhanced by HRG treatment. EBP1 also bound to a CAG-formed stem-loop in the 5′ coding region of AR mRNA and was able to inhibit AR translation. Thus, decreases of EBP1 in prostate cancer could be important for the post-transcriptional up-regulation of AR contributing to aberrant AR expression and disease progression.

INTRODUCTION

Prostate cancer remains a commonly diagnosed invasive cancer in men in the USA and other industrial countries. Androgen receptor (AR), a member of the nuclear receptor superfamily of transcription factors, is a critical molecule in the etiology of both early and advanced stages of the disease (1–3). A correlation between AR levels and disease progression has been reported in both humans and animal models (4). Strategies targeting AR *in vitro* and in animal models (5–8) have been shown to ameliorate hormone refractory disease. However, insights into AR mRNA stability and translation, central to the regulation of AR expression, are still largely lacking.

EBP1, an ErbB3 binding protein, was identified as an AR corepressor (9). A role for EBP1 in the progression of prostate cancer was suggested by studies indicating that EBP1 expression is significantly reduced in preclinical models of hormone-refractory prostate cancer (7) and in advanced stages of clinical prostate cancer (8). Ectopic expression of *EBP1* inhibited prostate cancer cell growth both *in vitro* and in xenograft mouse models of prostate cancer (9), decreased expression of androgen-regulated genes such as PSA, and altered the cellular response to HRG and androgens (7). Conversely, ablation of EBP1 expression resulted in increased cell growth in the absence of androgen, increased PSA production and activated AKT signaling (8).

These changes in biological responsiveness were postulated to be due in part to changes in levels of AR. For example, forced expression of exogenous EBP1 cDNA in hormone responsive LNCaP cells led to decreases of several AR-target genes including AR itself

at the mRNA and protein levels (7,9). EBP1-knock out mice expressed higher levels of AR protein in prostate tissue as compared to age-matched controls (10). EBP1 was demonstrated to bind to androgen response elements in AR-regulated promoters (11,12), leading to decreases in AR mRNA transcription. The ability of EBP1 to decrease AR transcription was mediated in part by its interactions with Sin3A and HDAC2. Although AR mRNA transcription was decreased, AR protein levels were down-regulated to a greater extent than mRNA levels (7). This is an important observation, as EBP1, a highly conserved and ubiquitously expressed protein, also interacts with other proteins (13,14), and RNA (15–18), suggesting a versatile role in the transcription and post-transcriptional regulation of an array of genes. These observations are supported by the crystal structure of EBP1 (19,20), which demonstrates the ability of EBP1 to bind DNA, RNA and protein. We thus hypothesized that EBP1 regulation of mRNA stability and translation is an important determinant of AR gene expression (21). Our current work indentified EBP1 as a novel AR mRNA-binding protein and suggests that targeting EBP1 has potential mechanistic and functional significance in the therapeutic management of prostate cancer.

MATERIALS AND METHODS

Cell culture and reagents

The generation of LNCaP cells stably transfected with *EBP1* cDNA, mutant (^{354}LXXLL^{358} to ^{354}LKAAA^{358}) *EBP1* cDNA or a control vector and LNCaP EBP1-silenced C13 and control A16 cells, was previously described (8,9). 22Rv1 cells (a gift from Dr Yun Qiu, University of Maryland) stably transfected with CMV10-EBP1 cDNA or a control vector, were established as previously described (22). HRG and EGF were purchased from R&D Systems (Mpls, MN, USA) and Cell Signaling (Danvers, MA, USA), respectively.

Analysis of mRNA stability by Actinomycin D chase assays

Cells were serum-starved overnight, then incubated with or without heregulin (20 ng/ml) for 12 h followed by addition of Actinomycin D (Act D) (5 µg/ml). Cells were harvested at subsequent time intervals (0, 2, 4, 6 and 8 h). Total RNA was extracted with Trizol and DNAse-treated for quantitative real-time PCR (RT-qPCR) analysis. To monitor reporter pGL3-ARUC and control pGL3-Luc (PGL3-con) mRNA decay, A16 and C13 cells were transfected with pGL3-ARUC and control pGL3-luc vector. Forty-eight hours later, cells were treated with Act D (5 µg/ml) and RNA was extracted at 0, 2, 4, 6 and 8 h, followed by RT-qPCR for luc mRNA. Data from Act D assays were processed using the Prism 3.03 software to calculate the time required for each mRNA to reach one-half of its initial abundance and *P*-value. Error bars represent the SD of three independent experiments.

Ribonucleoprotein immunoprecipitation assays

For immunoprecipitation (IP) of endogenous AR mRNA-EBP1 protein complexes [ribonucleoprotein (RNP)], cell lysates (1.5 mg) were incubated for 2 h at 4°C with protein A-sepharose beads (Calbiochem) that had been precoated with 3 µg of either rabbit IgG (BD Biosciences, San Jose, CA, USA), mouse IgG (Sigma, St Louis, MO, USA) or antibodies recognizing EBP1 (Upstate, Lake Placid, NY, USA) or HuR, a ubiquitous RNA-binding protein (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Beads were washed with NT2 buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$ and 0.05% Nonidet P-40], incubated with 20 U of RNase-free DNase I (15 min, 30°C), followed by incubation with 100 µl NT2 buffer containing 0.1% SDS and 0.5 mg/ml proteinase K for another 30 min at 55°C. The RNA isolated from the IP was converted to cDNA using gene-specific primer pairs and amplified by RT-qPCR as described (11).

Linear sucrose gradient fractionation

Linear sucrose gradient fractionation was performed as described previously (23) with minor modifications. A total of 50-million cells were incubated with cycloheximide (100 µg/ml) for 15 min to arrest polyribosome migration. Then 0.5 ml of each cell lysate was loaded onto sucrose gradients [10–50 w/v, 100 mM KCl, 20 mM Tris–HCl (pH 7.5) and 5 mM $MgCl_2$]. After centrifugation (Beckman SW41 rotor at 35 000 r.p.m. for 3 h at 4°C), each gradient was fractionated into 1-ml aliquots using a gradient fractionator (Brandel) and monitored by optical density measurement (A_{254}). For RT-qPCR, each fraction was diluted with an equal volume of water and RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA). For western blotting, 15 µl of each fraction was denatured in 15 µl of 1× Laemmli buffer.

RNA isolation and PCR analysis

RNA was extracted from whole-cell lysates, IP materials or sucrose gradient fractions, DNAse-treated and converted into cDNA using the AMV reverse-transcription system (Promega, Madison, WI, USA) in the presence of random hexamers (Invitrogen). The cDNA was used for conventional PCR or RT-qPCR with gene-specific primers as shown in Supplementary Data. An MYIQ RT-PCR detection system and SYBR green PCR mix (Bio-Rad, Richmond, CA, USA) were used to carry out the RT-PCR. The relative quantization of targeted genes was determined by the comparative $\Delta\Delta C_t$ (threshold) method using actin as an internal control (7). All data were analyzed from three independent experiments and statistical significance was validated by Student's *t*-test.

Affinity purification of GST- or His-EBP1 fusion proteins

In vitro expression and purification of recombinant His-EBP1 (a gift from Dr E. Spicer, Medical University of South Carolina) or GST-EBP1 fusion proteins were prepared as described (24).

In vitro transcription and biotin pull-down assays

For *in vitro* synthesis of biotinylated AR and GAPDH 3′UTR transcripts, cDNA from LNCaP cells was used as a template for PCRs. The T7 RNA polymerase promoter sequence (CCAAGCTTCTAATACGACTCA CTATAGGGAGA) was added to the 5′-end of all fragments. Primers used for the amplification of sequences of partial GAPDH 3′UTR, AR 3′UTR-A, B, C or D are shown in Supplementary Data. Biotinylated transcripts of AR CAG9, 20, 44 were transcribed using a MaxiScript T7 kit (Ambion) according to the manufacturer's instructions with linearized pcDNA-AR-Q9, Q20, Q44 (25) as templates. Bcl2 transcripts were transcribed using the pCR4-ARE constructs containing the 137-nt ARE^{bcl-2} motif (921–1057-nt of *bcl-2* cDNA) (18). Biotin pull-down assays were carried out as described previously (12). Briefly, whole-cell lysates (40 μg for each sample) or 150 ng of His- or GST-EBP1 fusion proteins were incubated with 1 μg of biotinylated transcripts for 1 h at room temperature. Complexes were isolated with paramagnetic streptavidin particles (Promega) and bound proteins were assayed by western blotting using antibodies recognizing EBP1 or GST (Santa Cruz).

Transient transfection and luciferase assays

The pGL3-ARUC plasmid was constructed as previously described (26) by fusing the UC-rich region of the 3′UTR of AR mRNA in frame to the 3′-end of the Firefly-luciferase (Luc) coding sequence. A16 and C13 cells were grown to 70% confluence prior to transfection in triplicate wells with 1 μg of either pGL3-ARUC or pGL3-Luc control vector (PGL3-con) using FuGene 6 (Roche). Cells were cotransfected with 5 ng of TK-*Renilla* reporter as a transfection control. Luciferase activity was determined using the Promega Dual luciferase activity kit according to the manufacturer's instructions. Data from three independent experiments were processed as previously described (9,27).

Western blotting analysis

Western blot analysis was done as described previously (8). The EBP1 antibody was from Upstate, the monoclonal anti-β-actin and anti-GADPH antibodies were from Sigma, and the polyclonal antibodies against eIF2α and phospho-eIF2α (Ser51) were from Cell Signaling. The monoclonal antibody against Rrp46 was from Novus Biologicals (Littleton, CO, USA). The monoclonal antibody against AR was from Santa Cruz.

Statistical analysis

qRT-PCR and luciferase assays were performed in triplicate and repeated at least three times and western blotting assays were repeated three times. All data presented represent one individual experiment. Where appropriate, means comparison were made using a two-tailed Student's *t*-test with α = 0.05.

RESULTS

EBP1 promotes AR mRNA decay

Previous work from our laboratory showed that ectopic expression of EBP1 decreases the levels of AR mRNA and protein (7). However, AR protein stability was not changed by EBP1 overexpression (Supplementary Figure S1). To test if EBP1 is involved in post-transcriptional regulation of AR, we first measured AR mRNA half-life in LNCaP cells overexpressing *EBP1*. As shown in Figure 1A(i), AR mRNA stability was significantly ($P < 0.05$) reduced in LNCaP cells stably transfected with EBP1 cDNA ($t_{1/2} = 2.98$ h) compared with vector control ($t_{1/2} = 5.19$ h). The results for AR mRNA half life in LNCaP cells are consistent with previously published data (28). Since EBP1 negatively regulates the HRG-triggered signaling and the AR axis (7–9,27), we examined the effect of HRG on EBP1-induced acceleration of AR mRNA decay. As shown in Figure 1A(i), HRG treatment led to a small but significant ($P < 0.05$) decrease in the stability of AR mRNA in LNCaP vector controls ($t_{1/2} = 4.1$ h with HRG treatment versus 5.19 h without treatment) in keeping with recently published data (29). However, HRG treatment had an even greater effect on AR mRNA decay rates in LNCaP cells stably transfected with *EBP1* ($t_{1/2} = 1.29$ h with HRG treatment versus $t_{1/2} = 2.98$ h no treatment, $P < 0.05$). The half-life of a control GAPDH mRNA was not significantly affected ($P > 0.05$) by either EBP1 overexpression or HRG treatment [Figure 1A(ii)]. Moreover, ectopic expression of EBP1 led to a significant decrease in the steady-state levels of AR mRNA and protein [Figure 1B(i and ii)] in the LNCaP unrelated 22Rv1 line, indicating that EBP1 inhibition of AR is not cell type-specific.

We studied the role of EBP1 in regulating AR mRNA stability using LNCaP EBP1-silenced C13 cells and the control A16 cells that were previously described (8). Briefly, to derive the A16 and C13 cell lines, LNCaP cells were transduced with lentiviral particles corresponding to different shRNA constructs targeted to the *PA2G4* *(EBP1)* gene. Five individual shRNA lentiviral particle constructs were tested. Only one construct (13C), corresponding to NT 302–322 of *PA2G4* (Genbank NM_006191.1), inhibited EBP1 expression. Another construct, 16A, did not inhibit EBP1 expression and served as a control.

A16 cells express a comparable level of EBP1 as LNCaP cells stably transfected with pcDNA vector (data not shown). Abrogation of EBP1 expression significantly elevated the basal steady-state level of AR mRNA [Figure 1C(i)]. HRG treatment resulted in a further significant ($P < 0.05$) decrease in AR mRNA in A16, but not in C13 cells. Under regular culture conditions, EBP1 silencing (C13) led to an increase in basal steady-state levels of endogenous AR protein [Figure 1C(ii)], consistent with the observation in EBP1-knock-out animals (10). HRG treatment also reduced the AR protein level in C13 cells. However, this level was still significantly higher than that in A16 cells [Figure 1C(ii)]. To determine if the increased expression of AR mRNA in EBP1 ablated cells was due to changes in mRNA stability, we measured
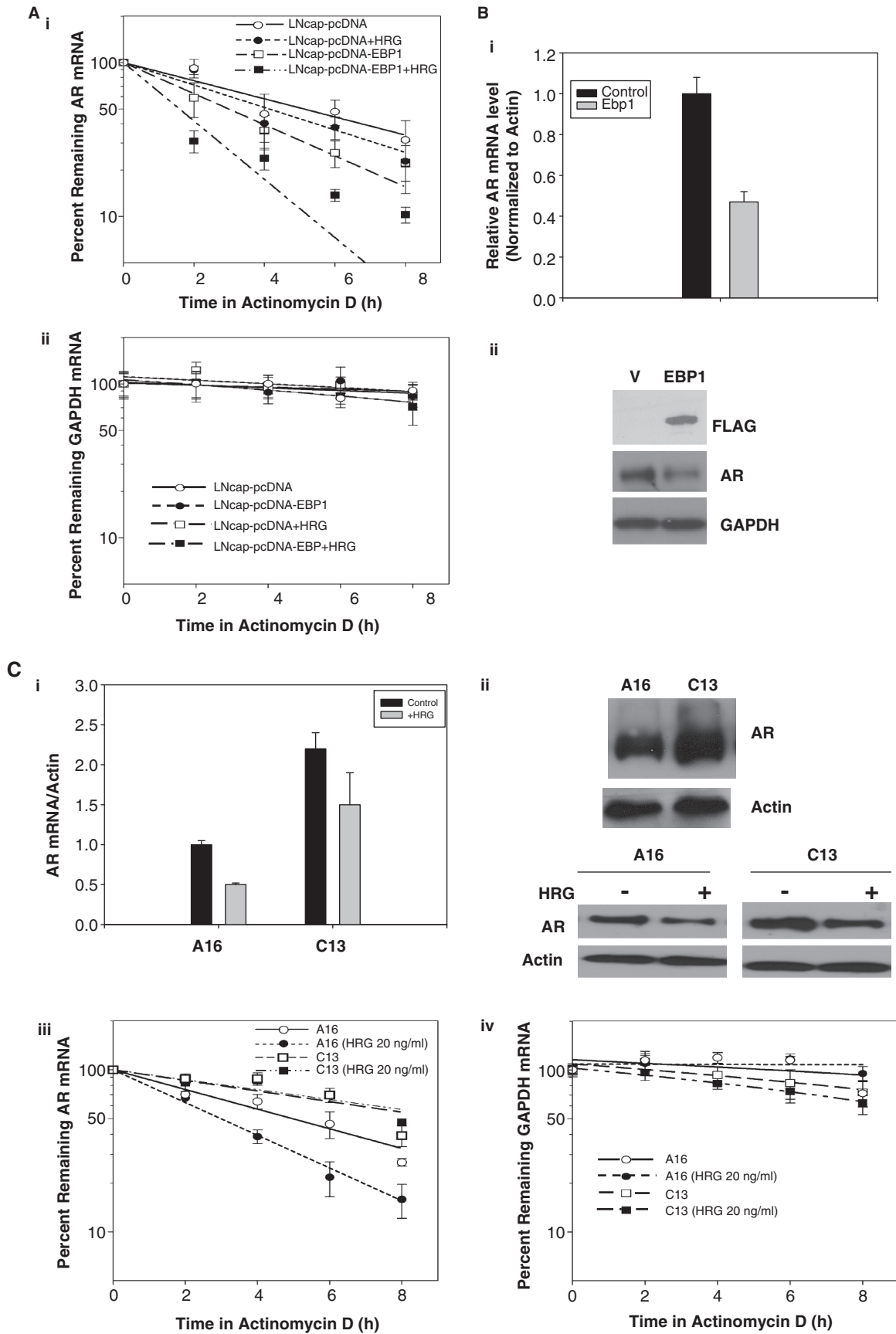
Figure 1. EBP1 promotes AR mRNA decay. (**A**) The stability of AR and GAPDH mRNA was analyzed in LNCaP cells transfected with vector control (pcDNA) or with *EBP1*. Total cellular RNA was isolated at the indicated times after treatment with Act D. The remaining levels of AR and

AR half-lives. The half-life of AR mRNA was 4.96 h in the control A16 cells, but 9.8 h in C13 cells [($P < 0.0001$), Figure 1C(iii)], suggesting that ablation of EBP1 stabilized AR mRNA. HRG treatment decreased the AR mRNA half-life from 4.96 h to 2.98 h ($P < 0.0001$) (in A16 cells, similar to what was observed in parental LNCaP cells [Figure 1C(iv)]. In contrast, HRG did not significantly change AR mRNA half-life in C13 cells ($t_{1/2} = 9.8$ h without treatment versus $t_{1/2} = 9.2$ h with HRG) ($P > 0.05$). The half-life of a control house-keeping transcript (GAPDH mRNA) was not markedly different in these cell lines in the presence or absence of HRG ($P > 0.05$) [Figure 1C(iii)].

EBP1 interacts with AR mRNA

EBP1 is an RNA-binding protein with broadly similar affinity for single- and double-stranded RNA (20). To address if EBP1 functions as an AR mRNA binding protein, we determined if AR mRNA was present in EBP1 immunoprecipitates. We found AR mRNA in EBP1 immunoprecipitates from the A16 cell line (Figure 2A), but not from the EBP1 knockout C13 cell line (Figure 2A). A known EBP1-interacting transcript, Bcl-2 mRNA (18) was also detected in EBP1 immunopre-cipitates from A16 but not C13 cells (Figure 2A). Neither AR nor Bcl-2 mRNA was precipitated by control IgG (Figure 2A). Actin mRNA is a highly abundant mRNA that is present as a low-level contaminant in the IP mate-rials, and thus served to monitor the equal input of lysate (30) (Figure 2A). The assay was also validated by the positive association between HuR and its target AR mRNA (26) in A16 but not C13 cells (Figure 2B). To determine if the EBP1-AR mRNA interaction was direct, we measured the ability of recombinant EBP1 to bind AR mRNA. GST-tagged recombinant EBP1 was incubated with A16 and C13 lysates, both sources of AR mRNA. *In vitro* GST-pull-down assays demonstrated that AR mRNA from both A16 and C13 lysates bound to recombinant EBP1 (Figure 2C).
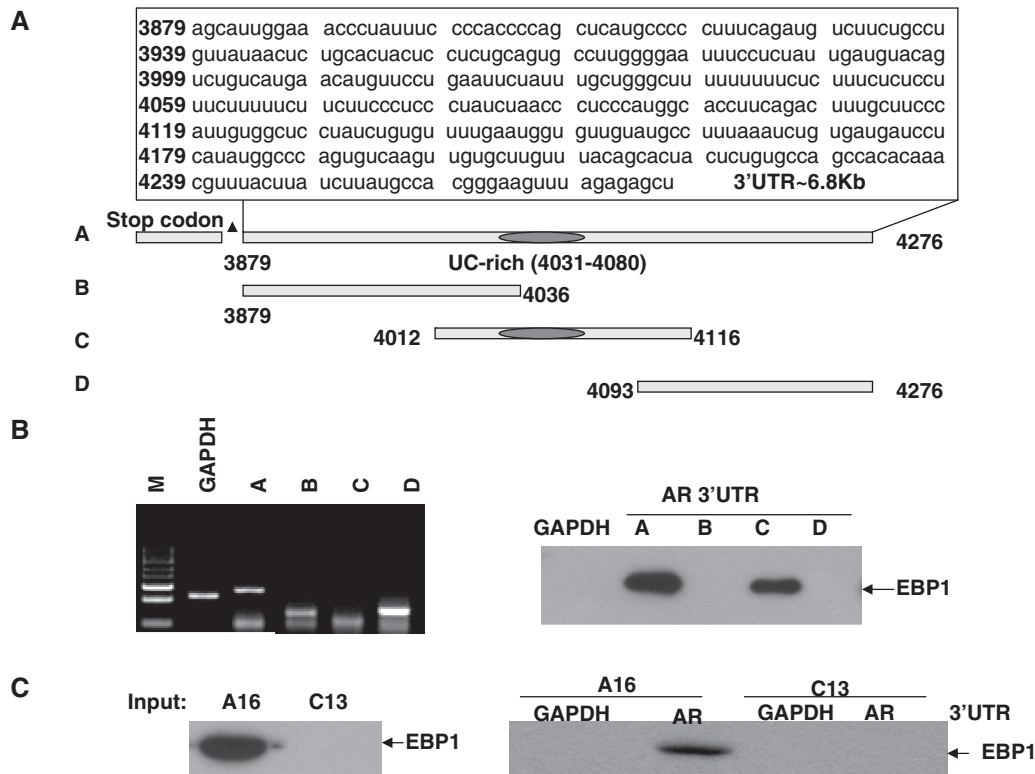
A UC-rich sequence has been identified in the proximal AR 3′ UTR that is implicated in AR mRNA stability (26). As EBP1 affected AR mRNA decay, we examined the ability of EBP1 to interact with this region. Several biotinylated transcripts spanning this region were synthesized (Figure 3A) and the interaction between the biotinylated transcripts and recombinant His-EBP1 fusion proteins was assessed by biotin pull-down assays followed

by western blot analysis. As shown in Figure 3B, equal amounts of purified full-length EBP1 protein interacted specifically with a 397-nt region of the 3′UTR containing the AR UC-rich element (fragment A). A shorter 104-nt fragment (fragment C) containing the UC-rich fragment also bound EBP1. The fact that binding was decreased between EBP1 and the UC-rich region alone (fragment C) compared to the full-length region suggests that the sequences spanning both sides of the UC-rich region
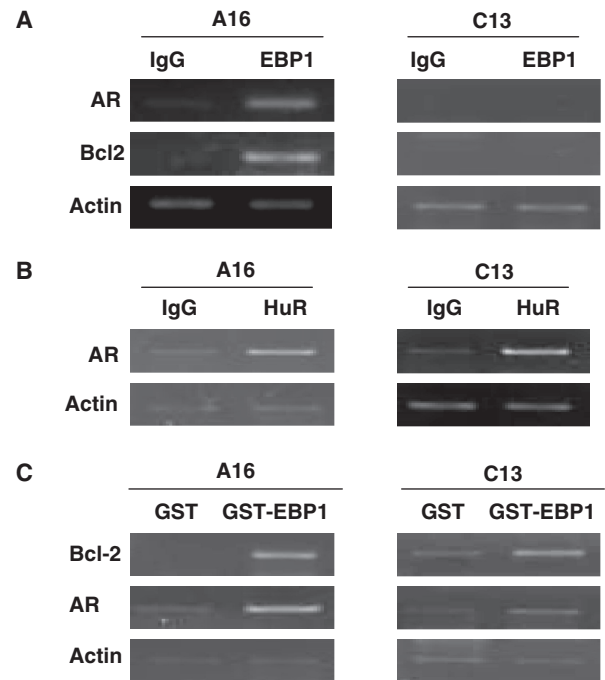
Figure 2. Endogenous EBP1 interacts with AR mRNA. (**A** and **B**) To assess the association of endogenous EBP1 and HuR with endogenous AR mRNA, lysates of A16 control lysates or C13 EBP1-silenced cells were immunoprecipitated with antibodies recognizing EBP1 or with control IgG (A), or with an antibody recognizing HuR and control IgG (B). RNA was extracted and used as a template for cDNA synthesis followed by PCR amplification using primers specific for AR, Bcl-2 or actin. PCR products were visualized after electrophoresis in ethidium bromide-stained agarose gels. Actin mRNA is a highly abundant mRNA that is present as a low-level contaminant in the IP materials, and thus serves to monitor the equal input of lysate. (**C**) A16 and C13 lysates were incubated with GST or GST-EBP1 as described in the 'Materials and Methods' section. GST complexes were isolated with glutathione–agarose beads and RNA present in the pull-down was detected by RT-PCR as described in (A and B).

GAPDH mRNAs were measured by RT-qPCR analysis. Data from Act D assays were processed by Prism 3.03 software to calculate the time required for each mRNA to reach one-half of its initial abundance and *P*-value. Values are means ± SD of triplicates. Results are representative among three independent experiments. (**B**) AR expression in 22Rv1 cells: (i) Total RNA was extracted from vector control and EBP1 transfected cells to detect the steady-state levels of AR and house-keeping actin mRNAs by RT-qPCR. Graph (mean ± SEM) is shown from three independent experiments. (ii) The steady-state level of AR protein was measured by immunoblotting with antibodies to AR and actin. Flag antibody verified the stable expression of Flag-tagged EBP1. (**C**) (i) A16 and C13 cells were either left untreated or treated with HRG (20 ng/ml) for 24 h. Total RNA (triplicate wells) was then extracted to detect the steady-state levels of AR and β-actin mRNAs by RT-qPCR. (ii) *Upper panel*: The steady-state levels of AR and actin in control A16 and C13 EBP1-silenced cells were measured by western blot analysis as described in the 'Materials and Methods' section. *Lower panel*: A16 and C13 cells were serum starved and then treated with HRG (20 ng/ml) for 12 h. (iii and iv) The stability of AR and GAPDH mRNA in A16 control and C13 EBP1-silenced cells was analyzed. Total cellular RNA was isolated at indicated times after treatment with Act D. The remaining levels of AR and GAPDH mRNAs were measured by RT-qPCR analysis. Data from Act D assays were processed by Prism 3.03 software to calculate the time required for each mRNA to reach one-half of its initial abundance and *P*-value. Values are means ± SD of triplicates. Results represent one of three independent experiments.

A

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3879 agcauuggaa  acccuauuuc  cccaccccag  cucaugcccc  cuuucagaug  ucuucugccu
3939 guuauaacuc  ugcacuacuc  cucugcagug  ccuuggggaa  uuuccucuau  ugauguacag
3999 ucugucauga  acauguuccu  gaauucuauu  ugcugggcuu  uuuuuuucuc  uuucucuccu
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4179 cauauggccc  agugucaagu  ugugcuuguu  uacagcacua  cucugugcca  gccacacaaa
4239 cguuuacuua  ucuuauugcca cgggaaguuu  agagagcu          3'UTR~6.8Kb
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B

C

Figure 3. EBP1 binds a UC-rich region in the 3′ UTR of AR mRNA. (**A**) Schematic of partial 3′UTR fragments in the AR mRNA and the biotinylated transcripts used for *in vitro* binding reactions. (**B**) Biotin pull-down assays were performed by incubating the transcripts indicated in left panel with 150 ng of purified recombinant His-tagged EBP1 per reaction. Bound proteins were detected by immunoblotting with anti-EBP1 antibody (right panel). (**C**) Lysates of A16 and C13 were incubated with the 3′UTR of AR (transcript A) or the GAPDH 3′ UTR. Associated proteins were determined by biotin pull-down followed by western blotting.

may be important for the EBP1 interaction. Fragments B and D that did not contain the UC-rich region were unable to bind EBP1 (Figure 3B). Recombinant EBP1 did not interact with the GAPDH 3′UTR (Figure 3B). We further examined the ability of endogenous EBP1 to bind the UC-rich sequence. A16 and C13 lysates were incubated with fragment C containing the UC-rich region. EBP1 was detected by immunoblotting. Lysates of A16 but not C13 cells bound the UC-rich sequence (Figure 3C).

RNA-binding proteins have also been postulated to influence AR gene expression by interacting with the CAG polyglutamine repeat, which is predicted to form a stable stem-loop structure (21) at the 5′-end of the coding sequence in the AR mRNA. Considerable interest has been focused on this CAG trinucleotide repeat sequence as repeats of differing length are associated with different risks for prostate cancer (31,32). Short CAG repeats (<21) are associated with higher risk of prostate cancer, while longer repeats are associated with lower transcriptional activity. An average repeat length of 20 is considered to be associated with a normal phenotype (21). We therefore examined the ability of EBP1 to interact with the CAG repeat. CAG repeat probes were first transcribed *in vitro* with T7 RNA polymerase in the presence of linearized pcDNA-AR-Q9, Q20 or Q44 constructs (25) and biotin-11-CTP. The results of *in vitro* biotin pull-down assays indicated that CAG9, 20 and 44 repeats associated

with recombinant His-tagged EBP1 (Figure 4A) (18). To detect binding *in vivo*, CAG20 transcripts were incubated with A16 and C13 cell lysates and associated proteins detected by biotin pull-down assays. As the CAG20 repeat is most commonly found in normal prostate epithelial tissue, it was used for further studies. The biotinylated CAG20 transcript associated with endogenous EBP1 from A16 cell lysates (Figure 4B). As expected, EBP1 was not detected in biotin pull-down assays using EBP1-silenced C13 cells. EBP1 derived from A16 lysates was unable to bind the GAPDH 3′UTR (Figure 4C), suggesting the interaction of EBP1 with the AR 3′UTR was specific.

We next mapped the domain of EBP1 responsible for the binding to CAG and UC-rich sequences. A schematic of the constructs and the expression of the GST proteins are presented in Figure 5A and B. We found that the C-terminus of EBP1 (aa 322–394) bound both the CAG and 3′UTR of AR mRNA. Mutation of [354]LKALL[358] to LKAAA abolished the interaction of EBP1 with the UC-rich sequence, but had no effect on binding to the CAG repeat (Figure 5C). It is of interest that stable overexpression of the LKAAA mutant form of EBP1 in LNCaP cells does not decrease expression of AR protein (Figure 5D) and thus would be unlikely to affect AR mRNA stability. Previous data have indicated that mutant and wild-type EBP1 are expressed at the same level (9).

### EBP1 controls AR mRNA turnover via a conserved UC-rich motif within the 3′-UTR

Previous work has demonstrated that the UC-rich element in the AR 3′UTR to which EBP1 binds accelerates AR
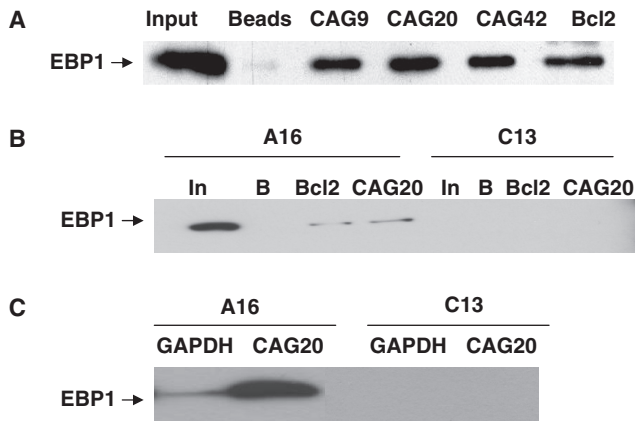
Figure 4. EBP1 binds the CAG repeat sequence in AR codon 1. (**A**) His- EBP1 (150 ng total) was incubated with equimolar amounts of biotinylated CAG repeats of the indicated sizes. The complexes were trapped on magnetic streptavidin beads and associated proteins detected by western blotting. The Bcl-2 UTR was used as a positive control. (**B**) A16 or C13 (EBP1-silenced) cell lysates were incubated with the biotinylated CAG20 repeat or the biotinylated Bcl-2 3′ UTR. Associated proteins were determined by biotin pull-down followed by western blotting. (**C**) A16 or C13 lysates were incubated with the biotinylated GAPDH 3′UTR (GAPDH) or the AR CAG20 repeat. Associated proteins were determined by biotin pull-down followed by western blotting.

mRNA decay (26). To determine if EBP1 affects the ability of the UC-rich region to accelerate AR mRNA decay, we performed luciferase reporter assays in EBP1-expressing and EBP1-silenced cells. The UC-rich region in the 3′UTR of AR mRNA was fused in-frame to the 3′-end of the firefly-luciferase coding sequence (pGL3-ARUC) (26). A reporter plasmid lacking the UC-rich region (pGL3-con) served as a control (Figure 6A). As shown in Figure 6B, the presence of the AR UC-rich sequence significantly ($P = 0.0087$) reduced reporter activity in A16 cells, as previously demonstrated in the parental LNCaP cell line (26). In contrast, the activity of the chimeric pGL3-ARUC plasmid significantly ($P = 0.0023$) increased after silencing EBP1 in C13 cells. Deletion of EBP1 thus appeared to abolish the inhibitory effects of the UC 3′ UTR.

Considering the fact that luciferase activity measures the effect of EBP1 on both translation and mRNA stability, we performed a parallel set of transfections to determine the Luc mRNA decay rate. Degradation of the pGL3-ARUC transcript ($t_{1/2} = 3.45$ h) was significantly ($P < 0.0001$) accelerated compared with the control pGl3-con ($t_{1/2} = 5.18$ h) in A16 cells, indicating that inserting the UC-rich sequence led to a significant destabilization of the reporter transcript. Moreover, the half-life of pGL3-ARUC was significantly ($P < 0.05$) prolonged from 3.45 h in A16 to 4.21 h in the EBP1-silenced C13 cell line [Figure 6C(i)]. Thus, the reduced reporter activity resulted from the accelerated decay of the reporter mRNA. The half life of GAPDH mRNA was
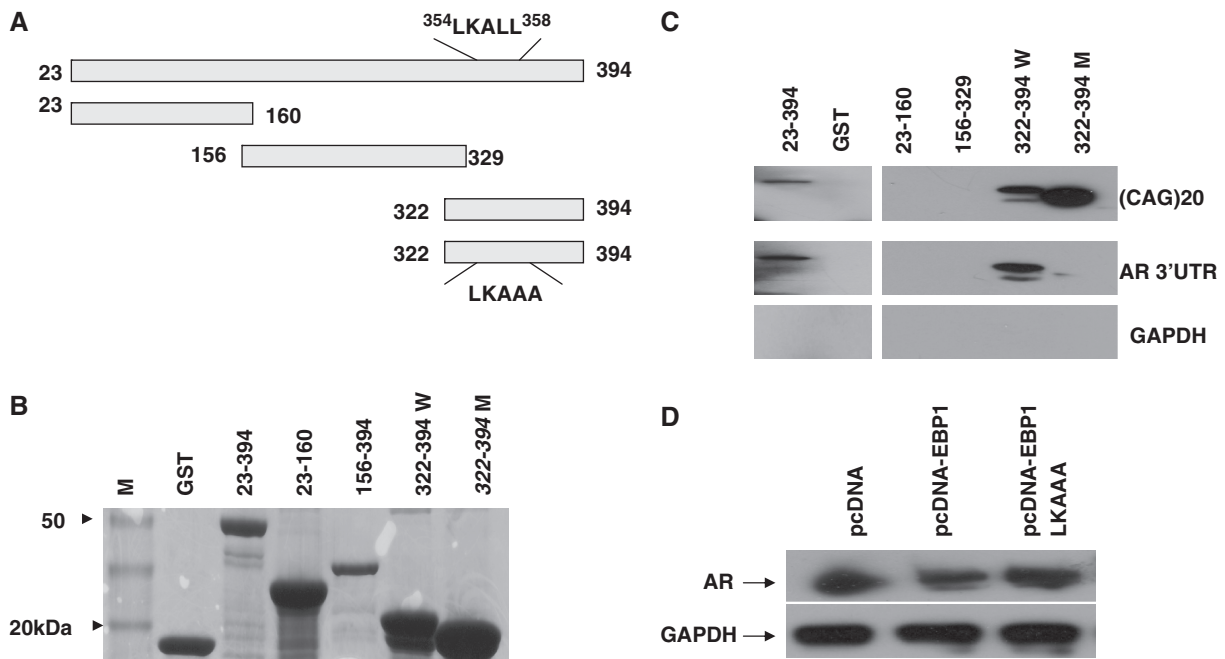
Figure 5. The C terminal region of EBP1 binds AR mRNA. (**A**) Schematic of EBP1 and fragments used for GST-pull down assay. (**B**) The expression of the GST proteins was analyzed by Coomassie blue staining. 322–394 W = C-terminal end, wild-type sequence; 322–394 M = C-terminal sequence, LKAAA mutation. (**C**) Equal amounts of GST or GST-EBP1 fusion proteins were incubated with biotin-labeled CAG20, the AR 3′UTR fragment A (Figure 3) and the GAPDH 3′UTR. GST complexes were isolated with glutathione–agarose beads and proteins present in the pull-downs were detected by western blot analysis. (**D**) The steady-state level of AR protein in LNCaP cells stably transfected with *EBP1* cDNA, mutant ([354]LXXLL[358] to [354]LKAAA[358]) *EBP1* cDNA or a control vector was measured by immunoblotting with antibodies to AR and GAPDH.
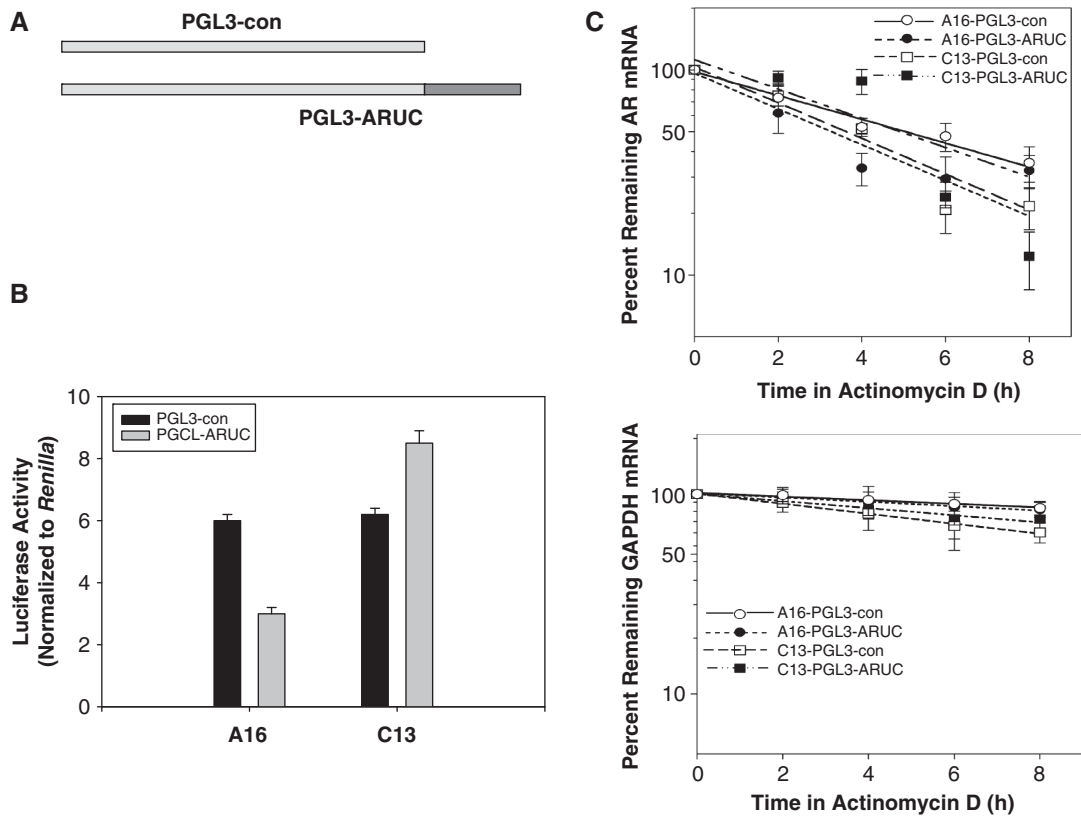
Figure 6. The activity of the AR mRNA UC-rich region is influenced by EBP1. (**A**) Schematic of the control and UC-containing reporter plasmids. (**B**) A16 and C13 cells were transfected with control pGL3-Luc (PGL3-con) or pGL3-Luc+ARUC containing the AR UC-rich region (PGL3-ARUC) plus a Renilla control plasmid. Luciferase activity was determined as described in 'Materials and Methods' section. The graph is representative of three experiments, each performed with six replicates per condition. (**C**) The stability of a PGL3-con or PGL3-ARUC transcript or GAPDH mRNA was analyzed in A16 or C13 cells transfected with PGL3-con or PGL3-ARUC as indicated. Total cellular RNA was isolated at the indicated times after treatment with Act D. The remaining levels of the indicated transcripts were measured by RT-qPCR analysis. Values are means ± SD of triplicates. Results are representative among three independent experiments.

the same in A16 and C13 cells as previously shown whether the luciferase control of the Luc-UC construct was transfected [Figure 6C(ii)].

EBP1 affects AR mRNA association with polysomes

We have previously demonstrated that ectopic expression of *EBP1* results in a 5-fold decrease in AR protein levels. However, AR mRNA levels were decreased only 2-fold (7). In addition, AR protein stability was not changed by EBP1 overexpression (Supplementary Figure S1). As EBP1 binds to CAG regions in AR mRNA postulated to play a role in translational control (21,26), we hypothesized that EBP1 may modulate not only AR stability, but also its translation. To test this possibility, we investigated the distribution of AR mRNA associated with polysomes in EBP1 depleted LNCaP cells.

Equal amounts of cytoplasmic extracts of logarithmically growing A16 and C13 cells were fractionated by sucrose gradient centrifugation in the absence of EDTA to preserve polysome integrity. Reading of absorbance at A254 indicates the distribution of monosomes and polysomes. The pattern is comparable in A16 (Figure 7A, left top panel) and C13 cells (Figure 7A, right top panel), indicating that ablation of EBP1

expression did not trigger significant changes in general translation. Western blot analysis of the different fractions of the gradient identified the association of EBP1 with 40s, 60s and 80s ribosomes. EBP1 co-purified with eIF2α, a component of the translation initiation complex, as has been previously reported in HeLa cells (17,24,33,34). eIF2α was not phosphorylated in either A16 or C13 cells, consistent with the active translation of AR that was observed. We also observed that EBP1 and Rrp46 cosedimented. Rrp46p is one of six RNase-PH domain subunits in the exosome, an evolutionarily highly conserved 3′–5′ exoribonuclease complex existing in both the nucleus and the cytoplasm to degrade target mRNA (35). Rrp46 has been previously shown to interact with EBP1 in a yeast-two hybrid assay (36).

The relative abundance of AR mRNA in each polysome fraction was next used to measure the degree of engagement of the AR mRNA with the translational apparatus. The percentage of AR mRNA shifted towards a more translationally active polysome pool in EBP1 knockout C13 as cells compared with control A16 cells (Figure 7A, bottom left panel). The association of a control house-keeping transcript (Actin mRNA) with polysomes was not different in the A16 versus C13 groups (Figure 7A, bottom right panel).
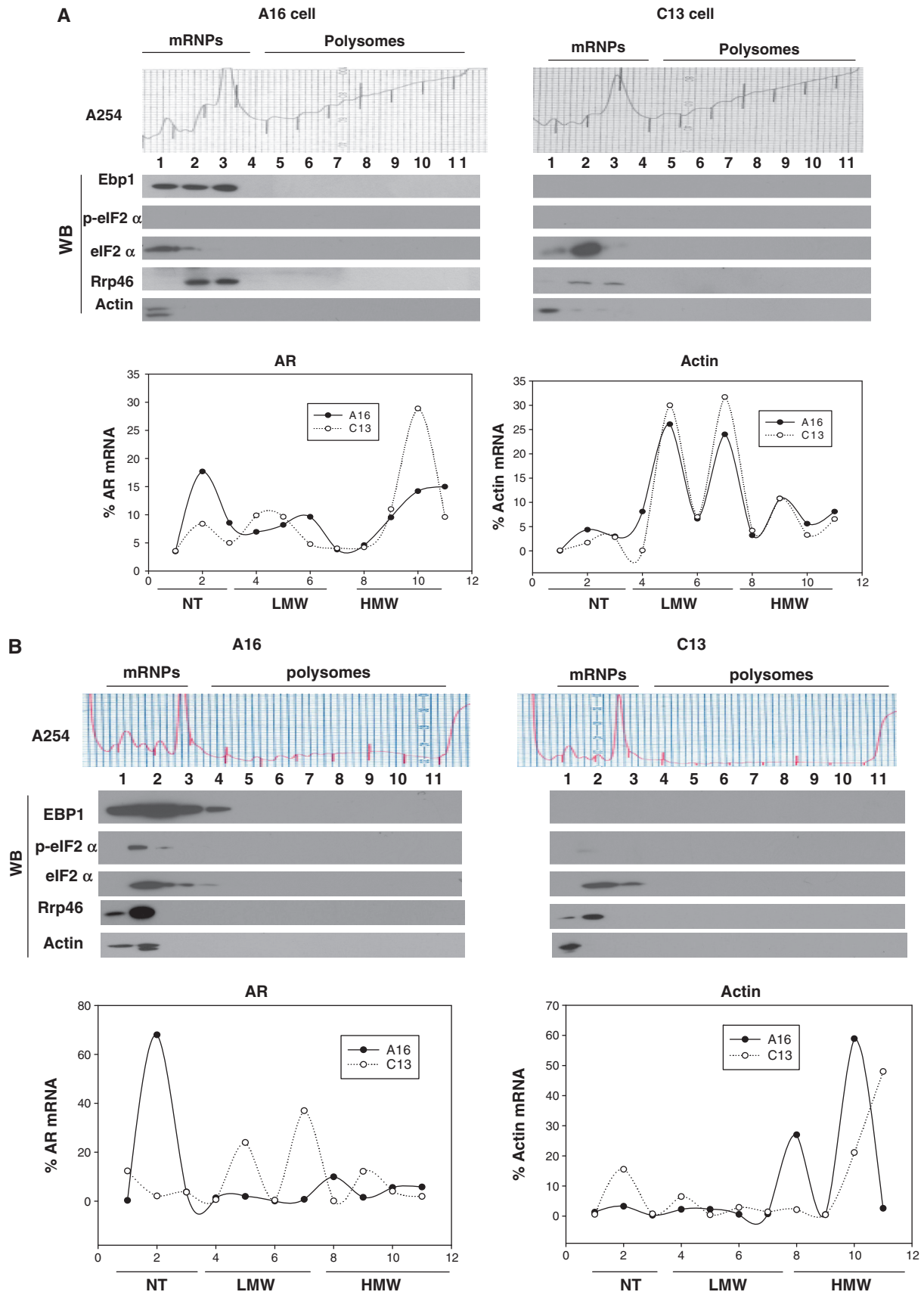
Figure 7. Loss of EBP1 enhances AR mRNA translation. (**A**) *Upper panel*: Cytoplasmic extracts of A16 (left top panel) and C13 cells (right top panel) growing in logarithmic culture conditions were fractioned by centrifugation on a 10–50 w/v sucrose gradients. Eleven fractions were collected

This suggests that loss of EBP1 accelerates AR protein translation.

To investigate if HRG is involved in regulating the association of EBP1 with polysomes and/or the AR translational profile, A16 and C13 cell lines were treated with HRG and processed as described above. HRG treatment led to a reduction in general translation in both A16 and C13 cells (Figure 7B, upper panel). HRG treatment did not change the distribution of actin, Rrp46 or eIF2α protein in either cell line. However, HRG treatment induced the phosphorylation of eIF2α in A16, but not C13 EBP1 knock out cells (Figure 7A, middle panel). This finding was of interest, as phosphorylated eIF2α is inactive, leading to inhibition of translation initiation.

HRG elicited a dramatic shift in the distribution of AR mRNA in A16 cells towards untranslated fractions. In contrast, EBP1 silencing in C13 cells abrogated such a shift, as the majority of AR mRNA was still being moderately translated after HRG treatment (Figure 7B, bottom left panel). Actin continued to be actively translated in both A16 and C13 cells after HRG treatment (Figure 7B, bottom right).

DISCUSSION

Our current study has identified EBP1 as a novel AR mRNA-binding protein that promotes AR mRNA decay. The EBP1-mediated decay depended on the presence of a UC-rich region in the 3′-UTR of AR mRNA that was previously identified as a destabilizing element for interaction with RNA-binding proteins (21). The fact that EBP1 expression is decreased in prostate cancer suggests that its control of AR mRNA decay may play a role in prostate cancer progression. Similarly, HuR destabilizes AR mRNA and its subcellular localization is de-regulated in a subset of prostate carcinomas (37). However, the functional consequence of HuR binding to AR mRNA remains to be defined.

The fact that EBP1, HuR (a member of the ELAV/Hu group) and PCBPs [poly(C) RNA-binding proteins] all bind to this region of AR mRNA suggests that this short UC-rich region is capable of docking with several RNA-binding proteins at once. It will thus be interesting to dissect the kinetics of these interactions and the functional impact of these proteins on AR expression. Further, the fact that HuR and PCBPs jointly interact with the 3′-UTRs of AR, p21[WAF1] and renin mRNAs suggests a common model of multiple RNA-binding protein interaction with a single RNA sequence (21). However, whether EBP1 can bind to the 3′UTRs of these other genes has not

yet been determined. In addition, the association of exosome protein Rrp46 with EBP1 in polysome gradients suggests another mechanism for EBP1-mediated decay of AR mRNA. The ability of EBP1 to bind to RNA in concert with other proteins is in line with the crystal structure of EBP1 that demonstrates that the C-terminal helix 10 with the LXXLL motif close to the putative RNA-binding platform may provide an adjustable interface for the interaction of EBP1 with different partners (19).

EBP1 was able to bind both the CAG and 3′UTR sequences of AR mRNA. Similarly, heterogeneous nuclear ribonucleoprtotein K (hnRNP K) was demonstrated to bind to several regions within the AR mRNA (38). A simultaneous interaction of EBP1 with two structured RNAs seems feasible. Of note, EBP1 that was mutated in its LXXLL domain was able to bind the CAG repeat, but not the AR 3′UTR. The LKALL sequence adopts the helical structure common to LXXLL motifs and was hypothesized to be important in EBP1's interactions with binding partners (20). However, the basis for the differential affinity for the CAG and 3′UTR motifs is not known. Nevertheless, we have demonstrated that the LXAAA mutant unable to bind the 3′UTR mRNA lost its ability to inhibit AR expression.

In keeping with a recent report (28), we found that treatment of LNCaP cells with HRG decreased expression of endogenous AR mRNA due to increased AR mRNA degradation. However, specific RNA binding proteins that might have contributed to these HRG-induced changes in AR mRNA stability were not identified in that study. Many other ErbB3 binding proteins with a possible role in prostate cancer have been identified and may play a role in HRG-induced changes in AR mRNA stability (13). We found that the HRG-induced decrease in AR mRNA stability was greatly enhanced when EBP1 was ectopically expressed. In addition, the HRG-induced accelerated decay of AR mRNA was abrogated in the absence of EBP1. These findings suggest that EBP1 may be an important mediator of the HRG-induced decrease in AR mRNA. However, the fact that HRG still slightly diminished AR levels in the absence of EBP1 suggests that other proteins are involved in the HRG-induced AR regulation. EBP1 was initially isolated as an ErbB3-binding protein and its phosphorylation and activity are significantly affected by treatment with the ErbB3 ligand HRG (26,39). EBP1 is phosphorylated on Thr261 after HRG treatment by PAK1(40). Thus, it is possible that PAK1 may be important in mediating HRG's effect on AR mRNA decay. In keeping with this, AR down-regulation in response to HRG was not affected by

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and 254-nm absorbance was recorded. *Middle panel*: Western blot analysis (WB) was used to measure the levels of EBP1, p-eIF2α, eIF2α, Rrp46 and Actin in each fraction from A16 (left panel) and C13 (right panel) cells. *Lower panel*: The levels of AR and house-keeping Actin mRNAs in each gradient fraction were measured by RT-qPCR and plotted as a percentage of the total AR (bottom left panel) or Actin mRNA levels (bottom right panel) in that sample. The translational activity associated with each fraction is indicated as untranslated (NT, not translated), moderately translated (LMW, low-molecular-weight polysomes) and actively translated (HMW, high-molecular-weight polysomes) as previously described (29). Data represent one of three independent experiments showing similar results. (**B**) A16 and C13 cells were treated with HRG (20 ng/ml) for 16 h. Cell lysates were processed as described in (A). *Upper panel*: Two-hundred and fifty-four nanomolar absorbance profiles. *Middle panel*: Western blot analysis (WB) of polysome fractions. *Lower panel*: The levels of AR and house-keeping Actin mRNAs in each gradient fraction were measured by RT-qPCR and plotted as a percentage of the total AR (bottom left panel) or Actin mRNA levels (bottom right panel) in that sample. Data represent one of three independent experiments showing similar results.

ERK or PI3-kinase inhibition (28). Thus, EBP1 is the first endogenous protein demonstrated to promote AR mRNA decay in an HRG-inducible manner, providing novel insights into cross-talk between HRG-activated pathways and AR signaling (1,41,42).

AR protein levels have also been shown to be regulated by changes in AR mRNA translation. For example, hnRNP K has been shown to regulate AR translation (38). In our study, EBP1 also repressed AR translation. A shift towards more translationally active ribosomes was observed in polysome fractions in the absence of EBP1. In contrast, loss of EBP1 had no effect on the distribution of Actin mRNA. Further, HRG treatment shifted AR mRNA to translationally inactive ribosomes in the presence, but not the absence, of EBP1. These findings, taken with work of others indicating an involvement of EBP1 in translation (17,20), suggest that EBP1 might inhibit translation initiation of AR mRNA. Indeed, our analysis of sucrose density gradients showed that EBP1 cosediments with 40S, 60S and 80S ribosomes, consistent with previous reports that EBP1 is part of RNP complexes (16,17,19). Moreover, EBP1 has been identified as an internal ribosomal entry site (IRES)-*trans*-acting factor (43).

At present, we do not know which region of the AR message is responsible for the effects of EBP1 on translation. Mukhopadhyay *et al*. (38) demonstrated that hnRNP-K uses regions within the AR coding region to regulate translation. We suggest that the CAG trinucleotide repeat at the 5′-end of AR exon 1, which is predicted to form a stable stem-loop structure, might function similar to an IRES element, which usually consists of a highly structured RNA regions located at the 5′UTR of viral or cellular mRNAs (44). A possible link between EBP1 and both mature ribosomal subunits and CAG-formed stem-loop of AR mRNA might constitute a basis for cell-specific translational control of AR mRNA by affecting their assembly into a stable RNP complex and/or efficient elongation. Such repression is well characterized in ferritin mRNA containing a stem-loop structure (iron-responsive element) in a cap-proximal 5′UTR. Binding of the iron regulatory protein to the iron-responsive element prevents recruitment by eIF4F of the 43S ribosomal complex to the cap-proximal region of the 5′UTR before scanning to the initiation cordon (45).

Our previous work indicated that EBP1 diminishes transcription of AR and several of its target genes (7,8). These findings, in combination with the current work, indicate that EBP1 may affect AR at both the transcriptional and post-transcriptional levels. The integrated action of EBP1 at through these mechanisms provides an effective program to attenuate AR signaling. Such biology has been reported for several proteins (46). PCBPs, members of the hnRNP K homology domain family of RNA-binding proteins, modulate RNA stability and also facilitate or inhibit translation. HnRNP K can similarly function to both regulate AR translation (28) and transcription (47). EBP1 is a member of the *Proliferation-associated 2G4* gene family which is comprised of 30 identified homologs that are highly conserved in organisms ranging from *Danio rerio* to *Pan troglodytes* (48). These proteins are involved in the regulation of cell growth, differentiation and apoptosis (13,14) by a variety of mechanisms. As the human member of the family, EBP1 has a profound impact on physiologic and pathologic process (13,14), further evidenced by our current study.

Our findings also provide a new rationale for clinical treatment of prostate cancer. Reduction of EBP1 in prostate cancer, as recently documented (7,8), might first impair the co-repressor machinery for transcription; second, EBP1 reduction could result in changes in post-transcriptional regulation of AR contributing to aberrant expression of AR and a subsequent impact on castration-resistant metastasis. An inverse relationship between EBP1 and AR mRNA levels in metastatic and organ-confined proste tumors has recently been reported (49,50) (Supplementary Figure S2). The development of EBP1-based modifiers of AR mRNA in target tissues is thus envisaged as a potent therapeutic approach for the treatment of prostate cancer.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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