**cAMP-Dependent Posttranscriptional Regulation of Steroidogenic Acute Regulatory (STAR) Protein by the Zinc Finger Protein ZFP36L1/TIS11b**

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**Star** is expressed in steroidogenic cells as 3.5- and 1.6-kb transcripts that differ only in their 3′-untranslated regions (3′-UTR). In mouse MA10 testis and Y-1 adrenal lines, Br-cAMP preferentially stimulates 3.5-kb mRNA. ACTH is similarly selective in primary bovine adrenocortical cells. The 3.5-kb form harbors AU-rich elements (AURE) in the extended 3′-UTR, which enhance turnover. After peak stimulation of 3.5-kb mRNA, degradation is seen. Star mRNA turnover is enhanced by the zinc finger protein ZFP36L1/TIS11b, which binds to UAUUUAUU repeats in the extended 3′-UTR. TIS11b is rapidly stimulated in each cell type in parallel with Star mRNA. Co-transfection of TIS11b selectively decreases cytomegalovirus-promoted Star mRNA and luciferase-Star 3′-UTR reporters harboring the extended 3′-UTR. Direct complex formation was demonstrated between TIS11b and the extended 3′-UTR of the 3.5-kb Star. AURE mutations revealed that TIS11b-mediated destabilization required the first two UAUUUAUU motifs. HuR, which also binds AURE, did not affect Star expression. Targeted small interfering RNA knockdown of TIS11b specifically enhanced stimulation of 3.5-kb Star mRNA in bovine adrenocortical cells, MA-10, and Y-1 cells but did not affect the reversals seen after peak stimulation. Direct transfection of Star mRNA demonstrated that Br-cAMP stimulated a selective turnover of 3.5-kb mRNA independent of AURE, which may correspond to these reversal processes. Steroidogenic acute regulatory (STAR) protein induction was halved by TIS11b knockdown, concomitant with decreased cholesterol metabolism. TIS11b suppression of 3.5-kb mRNA is therefore surprisingly coupled to enhanced Star translation leading to increased cholesterol metabolism. (*Molecular Endocrinology* 23: 497–509, 2009)
2.8 kb, respectively). An approximately 300-base region containing AU-rich elements (AURE) is only found at the 3′-end of the 3.5-kb transcript (20). Mouse, rat, bovine, and human Star retains similar polyadenylation sites in exon 7 that direct equivalent alternative transcripts with AURE in the extended 3′-UTR. These extended transcripts are seen in bovine primary adrenal cells (21, 22) and human H295R adrenal cells (23).

The stability of many labile transcripts is regulated by signal transduction pathways, most commonly through the interaction between AURE and AURE-binding proteins (AURE-BP) (24–26). Stabilization and destabilization of AURE-harboring mRNA by AURE-BP provides the means to very rapidly change the expression of key transcripts. In an earlier publication, we showed that the 3.5-kb Star message is preferentially synthesized relative to the 1.6-kb transcript after Br-cAMP stimulation and then preferentially degraded after removal of the stimulus. Because the two transcripts share the same promoter, this suggests that mRNA stability mechanisms are involved in regulating the long transcript (27). We further described the use of deletion/mutation of luciferase and Star constructs to study 3′-UTR sequences that affect steady-state expression of Star mRNA in the absence of any stimulation (28). We identified two regions in the extended 3′-UTR (a basal instability region and the AURE) that selectively enhance basal transcript destabilization. However, the question of how cAMP/PKA activation elicits changes in Star mRNA stability has not been addressed.

AURE are regulatory sequences usually found at the 3′-UTR of labile transcripts such as cytokines, growth factors and proto-oncogenes. They consist of pentamers of AUUUA, nonamers of UUAUUUA(U/A)(U/A), or U-rich elements (26). Over 900 genes in the human genome database have been found to contain AURE within their 3′-UTR, underlying the importance of this sequence element (29). Many of these genes express early response transcription factors (fos, jun, and myc), cytokines [TNFα and granulocyte-macrophage colony-stimulating factor (GM-CSF)], inflammatory regulators [cyclooxygenase 2 (COX2) and endothelial nitric oxide synthase (eNOS)] that play an important role in acute cellular responses to a changing environment. The stability of PTH mRNA has also been shown to be regulated through AURE (30).

A number of proteins have been characterized to interact with AURE sequences. The TTP family of tandem zinc finger proteins includes TTP/ZFP36, TIS11b/ZFP36L1, and TIS11d/ZFP36L2, all of which have been shown to directly bind AURE and promote degradation of the host transcript (31, 32). Their central RNA-binding domain interacts with AURE, whereas the N- and C-terminal domains recruit enzymes involved in the mRNA degradation pathway. Crystal structures show that the TIS11d tandem zinc finger domains bind as a homodimer on the 8-base sequence UAUUUAUU (33). Mouse, rat, bovine, and human Star sequences each have two or three repeats of this octamer in the extended 3′-UTR (27).

AURE are also regulated by other proteins including AUFI/ heterogeneous nuclear ribonuclear protein D (34–36), HuR (37), and other more specific AURE-BP (38). The four isoforms of AUFI can either stabilize or destabilize host transcripts. HuR typically causes mRNA stabilization, in part by competing with destabilizing AURE-BP such as TIS11b (36, 37, 39).

Destabilizing AURE-BP recruit proteins and enhance the deadenylation and de-capping processes preceding ribonuclease degradation at the 5′- or 3′-ends of the transcript. Stabilizing AURE-BP protect the message from access to this degradation machinery (40, 41). Inhibition of translation commonly slows mRNA degradation, including Star mRNA (27). Stress particles that include TIS11b have been characterized that retain translationally arrested mRNA through AURE interactions (41). TIS11b, HuR, and AUFI all undergo constant CRM1-dependent nuclear-cytoplasmic shuttling directed by localization signals (42–44). The nuclear packaging of mRNA before reaching the cytoplasm may involve these proteins and can determine posttranscriptional regulation (45, 46).

Signal transduction pathways regulate the stability of AURE-harboring transcripts by altering the cellular level of AURE-BP, by changing their subcellular localization, or by affecting their binding affinities for AURE. Protein kinase B (PKB/Akt) phosphorylation of TIS11b induces complex formation with the scaffolding protein 14-3-3 and sequesters the protein from binding to AURE (47). ACTH rapidly induces TIS11b in bovine adrenocortical cells (BAC) (48) which destabilizes the vascular endothelial growth factor (VEGF) transcripts through interaction with two AURE (49) in competition with a stabilizing effect from HuR (50). Besides regulating the turnover of labile transcripts through AURE, some AURE-BP have also been shown to affect the translation of mRNAs (51).

In a previous paper, we showed that basal stability in the 3.5-kb mRNA depends on sequences in a 700-base basal instability region located in the extended sequence upstream of the AURE (28). Here we show that TIS11b is highly induced by Br-cAMP in mouse Y-1 cells and MA10 cells and by ACTH in BAC, each in parallel with stimulation of Star. Paradoxically, this stimulation of TIS11b selectively restricts the increase in the 3.5-kb mRNA but also enhances StAR protein translation. We establish that Star mRNA degradation can be mediated by a complex between AURE and TIS11b and that unlike VEGF mRNA, Star mRNA turnover is independent of HuR. We also provide evidence that this stimulation of TIS11b can enhance STAR-mediated cholesterol metabolism.

Results

Br-cAMP induces expression of the two Star transcripts with different kinetics

The extended 3′-UTR sequences of mouse and bovine Star mRNA each contain repeats of the sequence UAUUUAUU (Fig. 1A). There are three repeats in the mouse Star 3′-UTR separated by about 20 bases that are conserved in the rat Star sequence (27). Polyadenylation sequences compatible with, respectively, short (1.6 kb) and long (3–3.5 kb) mRNA forms are present in exon 7 of the mouse, rat, bovine, and human genes (27). The bovine Star sequence retains two repeats of the AU-rich octamer immediately upstream of the polyadenylation site for the 3-kb mRNA but with a different separation (~40 bases) and a com-
Br-cAMP induces TIS11b, which exhibits multiple phosphorylated forms

The extended Star transcripts in mouse and bovine cells provide the additional possibility of regulation through the AURE shown in Fig. 1A. Because TIS11b/ZFP36L1 is highly elevated by ACTH in BAC (48), we tested whether stimulation of TIS11b expression by Br-cAMP in MA-10 and Y-1 cells was comparable (Fig. 2, A and B). The substantial stimulations were rapid and similar in each cell type to the stimulation of BAC by ACTH (4- to 10-fold) and closely paralleled 3.5-kb Star mRNA increases.

TIS11b protein exhibited at least five bands between 37 and 50 kDa (Fig. 2, C and D), which collapsed to a single lower mobility band after treatment with A-protein phosphatase (Fig. 2D). MA-10 cells transfected with a human TIS11b vector showed similar multiple bands (Fig. 2C). Previous work showed that S92 of TIS11b is phosphorylated by Akt and that TIS11b phosphorylation both promotes binding to 14-3-3 and inhibition of activity (47). Multiple sites are evidently phosphorylated based on the number of phosphatase-sensitive bands.

TIS11b selectively down-regulates luciferase chimeric constructs harboring extended Star 3'-UTR through interaction with the AURE

To test whether TIS11b destabilizes Star mRNA through its 3’-UTR, we cloned luciferase upstream of different Star 3’-UTR. MA-10 cells were co-transfected with luciferase-Star chimera (Fig. 3A) and a TIS11b expression vector. The TIS11b protein levels corresponded to 1 h stimulation by Br-cAMP (Fig. 2C). UTRS, UTRL, and UTRdARE luciferase reporters exhibited steady-state expression levels in the ratio of 8:1:1.4, respectively (28). Increasing amounts of the TIS11b vector decreased the steady-state expression of the UTRL by 50% compared with only 10% for UTRS (P < 0.05). Deletion of the 350-base AURE removed most of the destabilizing effect of the UTRL (P < 0.05) (Fig. 3B). TIS11b therefore primarily targets the 2-kb extended 3’-UTR sequence and destabilizes the luciferase-3’-UTR transcript through the AURE sequence. This destabilization of Star 3’-UTR by TIS11b was similar to that previously reported for the equivalent Vegf 3’-UTR construct (49).

We also tested whether TIS11b could destabilize luciferase reporters that were linked only to the 350-base AURE sequence (Fig. 3A, ARE). Figure 3C shows that TIS11b suppresses the AURE luciferase reporter to a much greater extent than the parent luciferase construct pGL3P. Mutation of the two upstream octamers that bind TIS11b largely reversed this effect (P < 0.05). Either one of these AUUUAAUU motifs are therefore necessary for this TIS11b activity.

We carried out similar titrations of TIS11b in BAC with this set of three Star 3’-UTR luciferase reporters. The responses in these primary cells to TIS11b were appreciably greater than in the mouse cell lines. The UTRS luciferase response to TIS11b was doubled when TIS11b targeted the UTRL vector (Fig. 3D). Again, the extra response was almost completely lost when the AURE was deleted. ACTH stimulation for 3 h, which elevated TIS11b (Fig. 2B), specifically lowered UTRL twice as much as UTRS, and again this difference was removed when the AURE

Completely different surrounding sequence. The human Star 3’-UTR has three octamer repeats in the extended 3’-UTR sequence, but otherwise the 3’-UTR sequence has no homology to either rodent or bovine sequences (27).

Br-cAMP stimulation of either MA-10 or Y-1 cells predominantly produces the 3.5-kb transcript. In addition, the stimulation of the 1.6-kb transcript follows different time courses from the 3.5-kb transcript (Fig. 1B). In MA-10 cells, the 3.5-kb mRNA peaks in 3 h but then declines rapidly whereas the 1.6-kb mRNA, reaches a steady-state level at 6 h, which is maintained (Fig. 1B). This result is typical of time courses for Star expression in MA-10 cells obtained in other laboratories (27, 52). In Y-1 cells, the predominant 3.5-kb mRNA also peaks several hours before the 1.6-kb mRNA, whereas after removal of PKA stimulation, the 3.5-kb form declines more rapidly (27). These different time courses for 3.5- and 1.6-kb Star transcripts probably arise from differences in posttranscriptional regulation. In BAC, the long transcript (3.0 kb) is again more extensively induced by ACTH (53) (Fig. 1C), but the short (1.7-kb) form follows a similar time course. Each stimulation was transient with a full reversal between 6 and 24 h.

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cotransfection selectively and similarly suppressed STAR protein and mRNA derived from the Star3.5k by 3-fold (Fig. 4, B and C) (P < 0.02) but did not affect expression from either Star3'UTR or Star1.6k. Therefore, TIS11b-mediated suppression is targeted selectively to the AURE of the Star 3'-UTR.

The parallel effects of TIS11b on mRNA and protein indicate that translational efficiency is not additionally affected by TIS11b. The similar effects on luciferase chimeric reporters compared with the equivalent Star constructs show that the Star 5'-UTR and translated sequence contribute minimally to these TIS11b-induced effects.

Prolonged Br-cAMP treatment selectively destabilizes the 3.5-kb Star transcript but without involvement of AURE

We have found that actinomycin D and other transcription inhibitors slow Star degradation by at least 5-fold (27). This excludes the use of transcription inhibitors to measure Star mRNA half-life. Instead, we directly transfected MA-10 cells with presynthesized rat Star mRNA in which we varied the 3'-UTR (Fig. 5A). These were generated with T7-reverse transcriptase from rat Star cDNA that contained a 90-base polyA sequence downstream of the natural polyadenylation element. The levels of transfected rat Star mRNA were quantified by RT-PCR, using primers that discriminated this transcript from endogenous mouse Star mRNA. Twelve hours after transfection, Star mRNA reached a steady state between uptake and degradation, which was appreciably higher than endogenous Star mRNA (data not shown). After removal of extracellular mRNA, the transfected Star mRNA in the cells declined with first-order kinetics (Fig. 5, B and C). Both the short and the long 3'-UTR increased the basal degradation rates (P < 0.05). A 12-h pretreatment with Br-cAMP specifically halved the half-life of for the 3.5-kb mRNA (P < 0.05, Fig. 5C). When we introduced the same two mutations into the Star 3.5-kb messages which prevented the TIS11b effects on luciferase chimeric reporters (Fig. 3C), the increased degradation rate and the stimulation of turnover by Br-cAMP were unaffected (P < 0.05, Fig. 5C). These destabilization mechanisms apparently target the extended 3'-UTR without participation of these octamers or intervention of TIS11b.

These direct transfections establish the presence of an AURE-independent mechanism that targets the 3.5-kb Star mRNA. We previously observed that sequences immediately upstream of the AURE enhanced basal degradation of 3.5-kb transcripts (28). These direct transfections may, however, diminish the TIS11b mechanism by avoiding nuclear packaging and export of mRNA (45, 46). It is notable that TIS11b undergoes nuclear-cytoplasmic shuttling (41). The high levels of directly transfected rat Star mRNA may also exceed the availability of endogenous TIS11b or other key binding proteins.

Suppression of TIS11b decreases STAR protein translation and steroidogenesis

We next used small interfering RNA (siRNA) to suppress TIS11b in MA-10 and Y-1 cells by about 75% for both mRNA
of additional interest because it decreases the potency of cAMP induction of STAR in these cells (57, 58). A similar 75% suppression of TIS11b by siRNA in Y-1 cells similarly halved STAR protein induction by Br-cAMP (Fig. 6B).

STAR mediates import of cholesterol into mitochondria and subsequent metabolism through a mechanism that depends on newly synthesized STAR protein (14). Stimulation of cholesterol metabolism by Br-cAMP was halved by TIS11b depletion, paralleling the decreased rate of translation of new STAR protein (Fig. 6C). There was no equivalent decrease in activity in Y-1 cells (data not shown) where we have previously shown that optimal cholesterol metabolism requires only phosphorylation of basal STAR (14).

Previous work has shown that the AURE-BP HuR stabilizes Vegf mRNA in BAC through interactions with AURE (50). HuR was constitutively expressed at high basal levels in both MA-10 and Y-1 cells (data not shown), and total expression was unaffected by Br-cAMP (Fig. 6D). Previous work has shown that ACTH stimulates the translocation of HuR from nucleus to cytoplasm in BAC, which then stabilizes Vegf mRNA (50). However, effective siRNA suppression of HuR had no effect on Star expression (Fig. 6D).

**Suppression of TIS11b selectively enhances Br-cAMP stimulation of the 3.5-kb transcript**

In BAC, Star produces 3.0-kb mRNA and 1.6-kb mRNA species (Fig. 1). Suppression of TIS11b in BAC by siRNA led to a 50% increase in total Star mRNA at all time points but did not affect the decline in Star mRNA between 3 and 6 h (Fig. 7A). Because short and long Star transcripts (Fig. 1C) and TIS11b mRNA (Fig. 2B) decrease similarly, this may reflect a general down-regulation of the ACTH receptor.

To establish the selectivity of TIS11b, we tested whether the 3.5-kb mRNA was specifically targeted. To discriminate between 3.5- and 1.6-kb Star transcripts, we designed a primer pair that targeted only the extended 3′-UTR (Fig. 7B, primer pair 1). A second primer pair was targeted to the beginning of the 3′-UTR. Although shared by both transcripts, after the use of a poly-dT primer for reverse transcription, the RT-PCR response corresponded to that expected for 1.6-kb transcript. Thus, TIS11b suppression increased Br-cAMP induction of 3.5-kb Star mRNA (primer pair 1), which doubled at all time points, whereas the primer pair 2

![Diagram](https://example.com/diagram.png)

**FIG. 3.** Effects of transfected TIS11b on luciferase vectors modified by Star 3′-UTR. A. Diagram of luciferase-Star 3′-UTR chimeric constructs used in the cotransfection experiments. Deletion of the AURE (UTRdARE) represents removal of the terminal 250 bases that include three AURE octameric motifs. AURE chimeric constructs include only this 250-bp sequence. AU/CG mutations were made in the upstream pairs of sequences (54–56) was induced by Br-cAMP as previously described (57, 58) but unaffected by the TIS11b siRNA. Cox-2 is

(data not shown) and protein (Fig. 6A). Surprisingly, the induction of STAR protein by Br-cAMP was approximately halved at all time points by this removal of TIS11b despite the expected increase in 3.5-kb mRNA (Fig. 6A). Cox-2 whose transcript harbors multiple AURE in the 3′-UTR including UAUAUUAU sequences (54–56) was induced by Br-cAMP as previously described (57, 58) but unaffected by the TIS11b siRNA. Cox-2 is

3′-UTR (Fig. 7B, primer pair 1). A second primer pair was targeted to the beginning of the 3′-UTR. Although shared by both transcripts, after the use of a poly-dT primer for reverse transcription, the RT-PCR response corresponded to that expected for 1.6-kb transcript. Thus, TIS11b suppression increased Br-cAMP induction of 3.5-kb Star mRNA (primer pair 1), which doubled at all time points, whereas the primer pair 2

![Diagram](https://example.com/diagram.png)
response was apparently much more efficient in providing a cDNA from the 1.6-kb transcript than for the 3.5-kb transcript at each time point (primer pair 1) (Fig. 7D). The reverse transcriptase efficiency of 1.6-kb mRNA (27) and was again independent of TIS11b suppression to mRNA translation.

CIIS11b binds selectively to Star AURE in vitro and in vivo

To determine whether CIIS11b interacts with the 3'-UTR from Star 1.6- and 3.5-kb transcripts, radiolabeled UTRS and UTRL RNA probes were incubated with bacterial extracts containing glutathione-S-transferase (GST)-CIIS11b fusion protein. Analysis by SDS-PAGE after UV cross-linking shows that a covalent ribonucleoprotein (RNP) complex forms only with the extended UTRL sequence from the 3.5-kb mRNA. The apparent molecular size of 38 kDa is consistent with CIIS11b (Fig. 9A). An excess of unlabeled UTRL completely inhibits the interaction, confirming that UTRL harbors the CIIS11b binding domain.

Our next goal was to evaluate whether this interaction could also occur in live cells. BAC primary cultures were subjected to in vivo cross-linking with formaldehyde (49). CIIS11b-containing RNP complexes were then immunoprecipitated from cell lysates using a specific antibody, and the immunoprecipitates were subjected to RT-PCR amplification of Star mRNA. As shown in Fig. 9B, Star mRNA was specifically detected in anti-CIIS11b immuno precipitates. These results demonstrate that CIIS11b-Star 3'-UTR interaction occurs not only in reconstituted in vitro systems but also in living cells.

Discussion

Star is a central regulator of steroidogenesis, which can fluctuate rapidly in response to stress-induced hormonal changes. An increase in mRNA stability provides a mean to acutely stimulate

The 1.6-kb mRNA was unaffected as indicated by the primer pair 2 responses. CIIS11b suppression specifically increased the 3.5-kb Star mRNA by 50% at all times from 3–24 h. There was no effect of CIIS11b suppression on the late decline in the 3.5-kb mRNA. The 1.6-kb transcript remained constant from 3–24 h as measured by either Northern blots or primer pair 2 (see also Fig. 1B). The lack of involvement of CIIS11b in this decline in 3.5-kb Star mRNA may correspond to the Br-cAMP-stimulated degradation of directly transfected rat 3.5-kb Star mRNA. This was also independent of the CIIS11b binding elements (Fig. 5C). Because removal of CIIS11b stimulates 3.5-kb Star mRNA while halving STAR protein synthesis (Fig. 6), the Br-cAMP-induced increase in CIIS11b should produce the opposite effect: an increased turnover of 3.5-kb mRNA coupled to greatly increased efficiency of Star mRNA translation.

FIG. 4. Effects of CIIS11b in MA-10 cells on the expression of Star vectors that differ in the length of the 3'-UTR. A, Diagram of rat Star expression vectors used in the cotransfections. B, Effect of cotransfected CIIS11b on STAR protein expression after 24 h. Upper panel shows STAR immunoblots, whereas lower panel shows quantification of STAR protein levels at these exposures. C, Effect of cotransfected CIIS11b on the expression of total Star mRNA measured by RT-PCR using primers specific for rat Star mRNA. Data represent mean ± sd for three separate cultures. *, P < 0.05 compared with empty vector cotransfection.
many fast-responding cytokines, growth factors, and protooncogenes (24–26). The stability of transcripts is commonly regulated through interactions between AURE and specific binding proteins (AURE-BP). Transcription of mouse Star generates 1.6-kb and 3.5-kb mRNA through alternative polyadenylation sites in exon 7 that extends the 3′-UTR in the longer form (20). The 3.5-kb Star mRNA is intrinsically much less stable when expressed from vectors but is similarly translated (28). This selective polyadenylation, which generates the extended 3′-UTR, is a likely target of PKA regulation that is coordinated with effects on transcription. In mouse MA-10 and Y-1 cells, Br-cAMP stimulates that sequences immediately upstream of the AURE primarily mediate basal instability (28).

TIS11b selectively changes steady-state levels of the 3.5-kb transcript without affecting the 1.6-kb mRNA. Suppression of TIS11b by siRNA elevated the stimulation of 3.5-kb Star mRNA by Br-cAMP similarly from 3–24 h in both MA-10 and Y-1 cells despite appreciable differences in the TIS11b levels at these times. Total TIS11b may not be a good indicator of activity. TIS11b may participate early in the generation of Star RNP complexes (41), before becoming inactivated by phosphorylation (47) (Figs. 2D and 6). The turnover of transfected Star mRNA also provided evidence for appreciable influences of the 3′-UTR before export of mRNA from the nucleus to the cytoplasm (28).

ACTH stimulation of TIS11b in BAC destabilizes Vegf transcripts through complex formation with two AURE located in a 75-base 3′-UTR domain (48, 49). This suppression is critical in regulating angiogenesis. Removal of this mechanism in TIS11b−/− embryos probably accounts for their loss at gestational d 8 when the vascular connection to the placenta is first developed (59). The magnitude of the effects of TIS11b on Star is similar to
expression is a determining factor but not in Y-1 cells where STAR phosphorylation is limiting (14). Extensive previous work has shown that de novo synthesis of STAR and cotranslational phosphorylation are essential for intramitochondrial cholesterol transport (1, 5, 12, 15). This disparity between mRNA turnover and translation was not seen after coexpression of TIS11b and CMV promoted 3.5-kb Star vectors (Fig. 4), possibly due to additional contributions from Br-cAMP. Specificity was demonstrated by the absence of effects on COX2, a cAMP-responsive protein whose expression can be affected through TTP/AURE interactions (61).

TIS11b protein has previously been implicated in mRNA/protein assemblages as an inhibitor of translation (41). Alternative TIS11b interactions within AURE complexes may explain this novel coupling of mRNA degradation and translation. First, translational arrest commonly decreases AURE-mediated mRNA degradation through complexes involving the stall ribosome (62, 63). Notably, cycloheximide inhibition of translation decreases the degradation of Star mRNA (27). Ribosomes stabilize myc mRNA through a protein complex involving a specific coding region (CRD) sequence that prevents endonuclease degradation (64). For c-fos, a different CRD stabilization complex includes interactions of poly-A binding protein with the poly-A tail and AUF1 with the AURE. Displacement of this complex by passage of the ribosome opens up the poly-A tail to degradation (65, 66). Star appears to contain not only a CRD-like sequence (27) but also an adjacent cluster of rare codons (arginine/CGA and threonine/ACA) that will additionally slow ribosome transit (64). We hypothesize that TIS11b enhances STAR translation by displacing AUFI from the AURE that forms the core of the poly-A binding protein/CRD complex that otherwise stabilizes Star 3.5-kb mRNA while inhibiting translation (65, 66).

A second mechanism is suggested by similarities in the regulation of Star and Tnfα through their respective AURE. The primary AU-rich region in the Tnfα 3′-UTR (1291–1329) (Tnf-AURE) mediates rapid TTP-induced degradation (67, 68) via three adjacent UAUUUAUU octamers. This Tnf-AURE binds a complex consisting of Fragile X mental retardation-related protein (FXR1), Argopyrifos, and a possible one other protein (64).

FIG. 6. Effects of Tis11b and HuR suppression on STAR protein induction and steroidogenesis. A, Expression of TIS11b, STAR, COX-2, and β-actin proteins. At 48 h after transfection of MA-10 cells with TIS11b siRNA or scrambled siRNA, cells were then stimulated with Br-cAMP for the indicated times. β-Actin is used for standardization. B, Y-1 cells were similarly treated with TIS11b siRNA and Br-cAMP. C, Effects of TIS11b siRNA on the stimulation of cholesterol metabolism by Br-cAMP in MA-10 cells. Shown are the rates of pregnenolone synthesis in 5-min periods after addition of trilostane to inhibit the rapid conversion of pregnenolone to progesterone. Trilostane was added at the indicated times after Br-cAMP stimulation. Pregnenolone was determined by RIA and standardized to total cellular protein levels. Data represent mean of duplicate samples. Experiments were repeated two times with similar results. D, Effective knockdown of HuR by siRNA does not change the expression of STAR in MA-10 cells.

those seen in cotransfection studies with Vegf in 3T3 cells and with various AURE constructs in other cell types (49, 60).

Although TIS11b suppresses 3.5-kb Star mRNA, we show that STAR protein levels increase, presumably because translation of this mRNA increases. Stimulation of cholesterol metabolism occurs in MA-10 cells (Fig. 6) where increased STAR
were transfected with siRNA or scrambled siRNA. After 48 h, cells were either lysed to evaluate Star mRNA expression levels. HPRT mRNA levels were used for quantification of 3.5- and 1.6-kb transcripts. D, Equivalent analyses were greatly increased by the transcription inhibitor actinomycin D with concomitant stabilization of the transcript (74). The great stabilization afforded Star mRNA by actinomycin D (27) suggests that HuR may stabilize the Star-AURE when shifted to the cytoplasm by actinomycin D.

The extensive phosphorylation of TIS11b in MA-10 and Y-1 cells (Fig. 2, C and D) suggests that the effectiveness of most TIS11b is compromised by binding to 14-3-3 (47). The possible phosphorylation of TIS11b after activation of the AMP signal transduction pathway remains to be investigated. PKA phosphorylates TIS11b in vitro and in vivo, and ACTH rapidly induces TIS11b phosphorylation in BAC (Cherradi, N., unpublished). Other work indicates that Akt and p38 MAPK phosphorylate TIS11b (47). We have previously shown that activation of p38 by arsenite or actinomycin selectively elevates 3.5-kb Star mRNA by an amount similar to the effect of TIS11b suppression shown here (75). This paper establishes that the extended Star mRNA found in mouse steroidogenic cell lines and in primary bovine adrenocortical cells is specifically regulated by TIS11b in parallel with hormonal stimulation of both proteins. Although TIS11b halves the expression of these extended transcripts through enhanced turnover, this is also coupled to enhanced Star translation, which in turn increases cholesterol metabolism (14). Participation of TIS11b in this mechanism and in enhanced turnover of Star mRNA facilitates a rapid shutoff of steroidogenesis after removal of the hormonal stimulus. These interactions may explain the preferred formation of a much extended Star transcript and the coregulation of TIS11b with Star despite an adverse affect on mRNA stability.
Materials and Methods

Materials

Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) at the highest grade unless otherwise stated. Cell culture media and horse serum were bought from Invitrogen/GIBCO Co. (Carlsbad, CA). Fetal bovine serum was purchased through Atlanta Biologicals, Inc. (Lawrenceville, GA). The Transf-LT1 reagent for DNA vector transfection and Trans-it-mRNA kit for mRNA transfection were from Mirus Bio Corp. (Madison, WI). Pfu Ultra enzyme for PCR cloning was purchased from Stratagene (La Jolla, CA). Restriction enzymes were purchased from Promega (Madison, WI). Plasmid and RNA preparation kits were purchased from Qiagen (Valencia, CA). Cell culture flasks, dishes, and plates were purchased from Corning Inc. (Corning, NY). The rabbit polyclonal antibody against STAR was a generous gift from Dr. Dale Buck Hales (University of Illinois at Chicago). The murine polyclonal antibody against human TIS11b was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The rabbit polyclonal antibody against COX-2 was purchased from New England Biolabs, Inc. (Beverly, MA). The horseradish peroxidase-conjugated secondary antibodies against rabbit or mouse IgG were purchased from Promega.

Plasmid constructs

The human TIS11b expression vector pTarget-hTIS11b, luciferase-Star 3'-UTR chimeric vectors as well as Star expression constructs were described previously (28, 49). The Luciferase-Star 3'-UTR chimeric vectors, Star expression constructs, and the in vitro transcription constructs have been previously described (28).

Cell culture and DNA vector transfection

MA-10 mouse Leydig tumor cells were a generous gift from Dr. Mario Ascoli (University of Iowa College of Medicine). They were maintained in DMEM/F-12 medium (GIBCO) supplemented with 5% horse serum, 2.5% fetal bovine serum, 26.66 mM NaHCO₃, and 50 μg/ml gentamicin. MA-10 cells were cultured on 0.1% gelatin-coated plates. Cells were incubated at 37°C in a humidified atmosphere with 5% CO₂. Y-1 mouse adrenocortical tumor cells were expanded from a subclone obtained from Dr. Bernard Schimmer (University of Toronto) that has a lower passage number than those available from ATCC. These cells were cultured in F12K medium (Sigma) supplemented with 15% horse serum, 2.5% fetal bovine serum, 17.86 mM NaHCO₃, 50 IU penicillin, and 50 μg/ml streptomycin. Bovine adrenocortical fasciculata-retticularis cells were prepared by enzymatic dispersion with trypsin, and primary cultures were established as described elsewhere (76). BAC were kept at 37°C in Ham’s F12 medium supplemented with 10% horse serum, 2.5% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 μg/ml gentamicin, under a 5% CO₂/95% air atmosphere. On d 4, cells cultured in 10-cm petri dishes (3 × 10⁶ cells per dish) were stimulated with 10 nM ACTH for the indicated periods of time.

Star and luciferase expression vectors were co-transfected with TIS11b vector using Trans-mLT1 (Mirus Bio) according to the manufacturer’s protocol. Briefly, cells were seeded in 24-or six-well plates at 25% density 24 h before transfection. Triplicate cultures were transfected with the same amounts of luciferase or Star vectors and increasing amounts of TIS11b vector together with 40 ng/ml pRLTK control vector (Promega). An empty vector corresponding to the TIS11b vector backbone was used to supplement each condition to make the total DNA transfected 500 ng/well. This was scaled up accordingly for six-well plates. Ratio of total DNA:Trans-mLT1 reagent:OPTI-MEM medium is 1 μg:2.5 μl:50 μl. OPTI-MEM media (GIBCO) were mixed with Trans-mLT1 and incubated for 15 min at room temperature. DNA vectors were then added, mixed thoroughly, and incubated for another 15 min. This transfection medium mixture was directly aliquoted to cells cultured in complete medium and incubated for 24 h. For luciferase activity measurements, cells were harvested in 1× passive lysis buffer and assayed using Promega’s Dual-Luciferase kit on a Pharmigen Monolight 3010 luminescence reader. Data from luciferase transfections are expressed as mean ± sd calculated from the triplicate cultures.

In vitro transcription and mRNA transfection

In vitro transcription vectors for rat StarDUTR and 1.6- and 3.5-kb mouse mRNA vector were made by site-directed mutagenesis (Stratagene, La Jolla, CA) using the following primers: first motif, 5'-CTTGGAAAGTTGCGGCTGTCGTTAGAACGACAAACGTT-3' and 5'-ACGTGGTTCATGGTCAATACGAAAGCCGAGTCTGTCGAGG-3', and second motif, 5'-CAAGGTCGAAACCCGAGCTGTTGACATTTGAAATTCGGAAGAC-3' and 5'-AGTCCTTCACAGCTCAATACGAAACCCGAGCTGTTGACATTTGAAATTCGGAAGAC-3'. Vectors were linearized with HindIII enzyme and transcribed using Ambion’s Message Machine T7 kit per manufacturer’s protocol. Transcribed messengers are complete with a functional 5' cap as well as a 90-base poly-A tail from the vector.

mRNAs were transfected using Mirus Bio’s TransIt-mRNA transfection kit. Cells were plated in 12-well plates at 25% density 24 h before transfection, and 1 μg mRNA was transfected in each well in the presence of Transit-mRNA reagent, 1 μl mRNA boost reagent, and 100 μl serum-free medium. mRNA was first mixed with serum-free medium, after which mRNA boost reagent was added and mixed, followed immediately by Transit-mRNA reagent. The mixture was incubated at room temperature for 3 min and then aliquoted directly to cells cultured in complete medium. Twelve hours after transfection, cells were washed once and changed to complete medium without transfection mixture to stop uptake of transcripts and start the 0-h time point. The degradation of mRNA within the cells was determined by harvesting at appropriate time points using RNeasy Mini Kit (Qiagen) and isolating total RNA according to the manufacturer’s protocol. Rat Star mRNA levels were determined by reverse transcription and quantitative real-time PCR. The half-life...
turer’s protocol. Cells were plated 1 d before transfection in six-well plates into MA-10 cells using the DharmaFECT 1 reagent according to manufacturer’s protocols. cDNA products were diluted to 100 μl, from which 3 μl was used for each well in a 96-well plate for real-time PCR. Two primer pairs targeting different regions within mouse Star mRNA were used (Fig. 5B). Their sequences are as follows: primer pair 1, 5'-TTTCTACCCGCAGTGGCAATT-3' and 5'-ACAGCATGAAAGAATGG-3', and primer pair 2, 5'-AAAAGACCCAGCACTGACAG-3' and 5'-GGTAAGAACACACTCCCGATCCT-3'. The primers used for detecting TIS11b were 5'-CCACATTTTCTCTGCATGG-3' and 5'-TGAGCATCTTGTATAACCTGCG-3'. Specificity of these primers was indicated by a single sharp peak within the dissociation curves. Real-time PCR was performed on a Bio-Rad (Hercules, CA) MyIQ single-channel real-time PCR machine, using reagents purchased from Bio-Rad. Data were collected by computer and analyzed using Bio-Rad MyIQ software. All samples were done in triplicate.

Real-time RT-PCR

Star and TIS11b mRNA levels were determined by real-time RT-PCR. Total cellular RNA was isolated using the RNeasy Mini Kit (QIAGEN) according to the manufacturer’s protocol. When mRNAs produced from transfected DNA vectors were measured, an additional step of extensive deoxyribonuclease (DNase) I digestion was employed to remove transfected DNA vector contamination in real-time PCR. The DNase I treatment was performed using QIAGEN’s ribonuclease-free DNase kit at 37 C for 4 h. Effective removal of DNA vectors was confirmed by the extremely low signal of no reverse transcriptase controls in real-time PCR (<0.1% of samples with reverse transcriptase). RNA concentrations were quantitated in triplicates. Total RNA (1.5 μg) was used for cDNA synthesis by Superscript III reverse transcriptase (Invitrogen) per manufacturer’s protocols. cDNA products were diluted to 100 μl, from which 3 μl was used for each well in a 96-well plate for real-time PCR. Two primer pairs targeting different regions within mouse Star mRNA were used (Fig. 5B). Their sequences are as follows: primer pair 1, 5'-TTTCTACCCGCAGTGGCAATT-3' and 5'-ACAGCATGAAAGAATGG-3', and primer pair 2, 5'-AAAAGACCCAGCACTGACAG-3' and 5'-GGTAAGAACACACTCCCGATCCT-3'. The primers used for detecting TIS11b were 5'-CCACATTTTCTCTGCATGG-3' and 5'-TGAGCATCTTGTATAACCTGCG-3'. Specificity of these primers was indicated by a single sharp peak within the dissociation curves. Real-time PCR was performed on a Bio-Rad (Hercules, CA) MyIQ single-channel real-time PCR machine, using reagents purchased from Bio-Rad. Data were collected by computer and analyzed using Bio-Rad MyIQ software. All samples were done in triplicate.

Northern blot

Total RNA was isolated using QIAGEN RNeasy mini kit per manufacturer’s instructions. About 10 μg total RNA was resolved by electrophoresis in a 1% (wt/vol) agarose-formaldehyde-formamide denaturing gel and transferred to Hybond-N+ membrane (Amersham Biosciences) for approximately 16 h by the capillary method. RNA on the membrane was immobilized by UV Stratalinker 1800 on auto mode (1900 J × 100 for 30 sec). The membrane was then prehybridized at 65 C for 1 h in QuickHyb hybridization solution (Stratagene). Hybridization was performed at 65 C for 2 h in QuickHyb with the probes of 0.9-kb mouse Star cDNA probe date, 1 mM phenylmethylsulphonyl fluoride, and 1% protease inhibitors cocktail from Sigma. Lysate was passed through a 25-gauge needle six times, centrifuged at 12,000 × g for 10 min at 4 C and the supernatant collected. The lysates were assayed for protein concentrations using BCA protein assay kit (Pierce Biotechnology Inc., Rockford, IL), and 20–60 μg total cellular proteins were loaded on each lane, resolved on 10% SDS-PAGE gel, and electrophoto-thermically transferred to nitrocellulose membranes. After transfer, the membrane was incubated in blocking buffer (Tris-buffered saline containing 0.1% Tween with 5% nonfat milk, TBST) for 1 h, washed with TBST, and incubated with the appropriate primary antibodies overnight. The membrane was then washed three times with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies containing 1% milk, followed by three washes. Protein bands were visualized using ECL reagent (Amersham Biosciences, Arlington Heights, IL) and Hyperfilm from Amersham Biosciences.

siRNA transfection

The ON-TARGETplus SMARTpool siRNA sequences against mouse TIS11b and HsR along with nontarget siRNA sequences were purchased from Dharmacon, Inc. (Lafayette, CO). They were transiently transfected into MA-10 cells using the DharmaFECT 1 reagent according to manufacturer’s protocol. Cells were plated 1 d before transfection in six-well plates at a split ratio of 1:8. For each well, 10 μl 20 μM siRNA was added to 190 μl serum-free medium and incubated at room temperature for 5 min; 4 μl DharmaFECT 1 reagent was added to 196 μl serum-free medium and also incubated for 5 min. siRNA and DharmaFECT 1 were then mixed and incubated at room temperature for 20 min, after which the mixture was added directly to the cells cultured in 1.6 ml fresh complete medium. Approximately 2–3 d after transfection, cells were treated with Br-cAMP and harvested at various time points for protein or RT-PCR analysis.

Western blot analysis

To harvest total cellular proteins, cells were washed once in PBS and harvested with RIPA buffer [50 mM Tris- HCI (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 1% Nonider P-40, 0.25% deoxycholic acid, 0.05% SDS, 40 mM NaF, 10 mM sodium molyb-
that was radiolabeled with [α-32P]dCTP (PerkinElmer, Norwalk, CT; 3000 Ci/mmol) using a Ready to Go DNA labeling kit (Amersham Biosciences). The membrane was then washed twice in 2× standard saline citrate 0.1% SDS buffer at room temperature followed by a stringency wash in 0.1× standard saline citrate/0.1% SDS at 65°C for 0.5 h. The membrane was exposed overnight and scanned using phosphorimager (Molecular Dynamics, Sunnyvale, CA). Quantification was performed with the ImageQuant 5.2 software. The level of 28S rRNA was measured as internal standard for the RNA loading.

**RNA-protein UV cross-linking assay**

Star UTRS and UTRL sequences were digested with KpnI and BamHI from pGL3-LucUTRS or pGL3-LucUTRL plasmids and inserted into the same cutting sites in pGEM-4z plasmid. Star-UTRS and UTRL were transcribed in vitro using the Riboprobe in vitro transcription system (Promega). Radioactive RNA probes were mixed with bacterial cell extracts containing either GST alone or the fusion protein GST-Tis11b (2 μg) in the presence or in the absence of cold RNA competitor. The reaction mixtures were treated with UV radiation and analyzed by 12% SDS

RIP complex immunoprecipitation and analysis by RT-PCR

RNP complexes were immunoprecipitated after reversible cross-linking between RNA and proteins as previously described (49). Briefly, BAC suspensions were incubated in 1% formaldehyde for 10 min at room temperature. The reaction was stopped by 0.25 M glycine, and cells were lysed in RIPA buffer containing protease inhibitors. Protein A-agarose preadsorbed cell lysates were immunoprecipitated using protein A-agarose beads preincubated with 2 μg anti-Tis11b antibody (anti-N-terminal or anti-C-terminal antibody), 2 μg nonimmune IgG (nonimmune serum), or no IgG. After cross-linking reversion at 70°C for 45 min, RNA was isolated from immunoprecipitates, treated with DNase I (Invitrogen), and reverse transcribed with 2 μL of 5× first-strand buffer at room temperature followed by a stringency wash in 0.1× standard saline citrate/0.1% SDS at 65°C for 0.5 h. The membrane was exposed over night and scanned using phosphoimager (Molecular Dynamics, Sunnyvale, CA). Involves TIS11b Mol Endocrinol, April 2009, 23(4):497–509

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