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)

A critical step in trophic hormone-activated steroidogenesis is the delivery of cholesterol from the outer to the inner mitochondrial membrane, where the conversion to pregnenolone by P450sc (CYP11A1) takes place (1–3). The steroidogenic acute regulatory (STAR) protein mediates this intramitochondrial cholesterol transport in most steroidogenic tissues (4–7). The physiological function of STAR is highlighted by the human genetic disease lipoid congenital adrenal hyperplasia, in which pathogenic mutations in the Star gene render the patients almost incapable of making adrenal steroids (8, 9).

The major route for physiological regulation of Star gene expression is through activation of the cAMP-protein kinase A (PKA). In active steroidogenic tissues, cholesterol metabolism depends on new synthesis and PKA phosphorylation of STAR protein. This occurs in an organized complex at the mitochondrial outer membrane (5, 10–15). PKA also activates the transcription of Star as well as other steroidogenic genes. Key participants include the transcription factors CREB, SF1 and GATA4 and the coactivator CBP/p300 (16–18).

Posttranscriptional mechanisms also regulate Star mRNA. cAMP stimulates two major transcripts (1.6 and 3.5 kb) in rodent steroidogenic cells (19). These transcripts arise from different use of polyadenylation signals in exon 7 and therefore differ only in their 3′-untranslated region (3′-UTR) (lengths 0.7 and

Abbreviations: AURE, AU-rich elements; AURE-BP, AURE-binding proteins; BAC, bovine adrenocortical cells; COX2, cyclooxygenase 2; DNAse, deoxyribonuclease; GST, glutathione-S-transferase; PKA, protein kinase A; RNP, ribonucleoprotein; siRNA, small interfering RNA; STAR, steroidogenic acute regulatory; 3′-UTR, 3′-untranslated region; VEGF, vascular endothelial growth factor.
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 retain 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 AUF1/heterogeneous nuclear ribonucleoprotein D (34–36), HuR (37), and other more specific AURE-BP (38). The four isoforms of AUF1 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 AUF1 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-
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 cotransfected with luciferase-Star chimeras (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 UAUUUAAU 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 by 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
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 StardUTR 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 \((P < 0.05, \text{Fig. 5C})\). 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 \((P < 0.05, \text{Fig. 5C})\). 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
The 1.6-kb mRNA was unaffected as indicated by the primer pair 2 responses. *TIS11b* suppression specifically increased the 3.5-kb *Star* mRNA by 50% at all times from 3–24 h. There was no effect of *TIS11b* 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 *TIS11b* 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 *TIS11b* binding elements (Fig. 5C).

Because removal of *TIS11b* stimulates 3.5-kb *Star* mRNA while halving *Star* protein synthesis (Fig. 6), the Br-cAMP-induced increase in *TIS11b* should produce the opposite effect: an increased turnover of 3.5-kb mRNA coupled to greatly increased efficiency of *Star* mRNA translation.

### *TIS11b* binds selectively to *Star* AURE in vitro and in vivo

To determine whether *TIS11b* 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)-*TIS11b* 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 *TIS11b* (Fig. 9A). An excess of unlabeled UTRL completely inhibited the interaction, confirming that UTRL harbors the *TIS11b* 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). *TIS11b*-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-*TIS11b* immunoprecipitates. These results demonstrate that *TIS11b-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 response was apparently much more efficient in providing a cDNA from the 1.6-kb transcript that includes the primer pair 2 sequences. The poly-A tail targeted by the poly-dT primers is 2 kb closer for the 1.6-kb mRNA than for the 3.5-kb mRNA. In Y-1 cells, suppression of 1.6-kb mRNA (27) and was again independent of *TIS11b* suppression. The opposing effects of *TIS11b* suppression on *Star* 3.5-kb mRNA and protein (Fig. 6) therefore apply equally to Y-1 cells.

Northern blots on the same MA-10 mRNA confirm that the 3.5-kb transcript was increased by the *TIS11b* suppression to the extent as estimated by primer pair 1 (compare Figs. 7 and 8).
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 Star 3.5-kb mRNA as the predominant transcript without affecting the 1.6-kb mRNA. Suppression of TIS11b on mRNA turnover were independent of the upstream sequence (Figs. 3 and 4). A late decline in the 3.5-kb mRNA that is most evident in Br-cAMP-stimulated MA10 cells (Figs. 1 and 7) also depended on the extended sequence but was independent of AURE and, presumably, TIS11b (Fig. 5). We have shown 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 affects 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 stalled 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 size that TIS11b enhances STAR translation by displacing AUF1 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 Tnfa through their respective AURE. The primary AU-rich region in the Tnfa 3′-UTR (1291–1329) (Tnfa-AURE) mediates rapid TTP-induced degradation (67, 68) via three adjacent UAUUUAUU octamers. This Tnfa-AURE binds a complex consisting of Fragile X mental retardation-related protein (FXR1), Argo-

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**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 Br-cAMP and scrambled siRNA. 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 3β-hydroxysteroid dehydrogenase. 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.
were transfected with siRNA and incubated with fresh medium containing 10 nM ACTH for the indicated period of time. A, BAC cells were lysed to evaluate Star protein expression in BAC, MA-10, and Y-1 cells. A, BAC cells were either lysed to evaluate TIS11b suppression or incubated with fresh medium containing 10 nM ACTH for the indicated period of time. At each time of stimulation by ACTH, total RNA was isolated, and RT-PCR analysis was performed to determine total Star mRNA expression levels. HPRT mRNA levels were used for standardization. Inset shows effects on Star mRNA 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 anisomycin 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.

Interestingly, STAR is also regulated by the PKA-binding protein AKAP 121, which like FXR1, interacts with 3′-UTR through a KH domain that may recognize TTP/TIS11b oc-

naute (AGO2), and certain micro-RNA, which can enhance Tnfα translation (69). TIS11b could stimulate STAR translation by enhancing an equivalent FXR1/AGO2/micro-RNA complex.
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 TransIT-LT1 reagent for DNA vector transfection and TransIT-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 rabbit polyclonal antibody against human TIS11b/BRF1 was a generous gift from Dr. Christoph Moroni (36). The rabbit polyclonal antibody against actin was purchased from Sigma-Aldrich (A2066). The murine polyclonal antibody against COX-2 was purchased from Cayman Chemicals, Inc. (Ann Arbor, MI; catalog no. 160126). 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. They were cultured in F12K medium (Sigma) supplemented with 15% horse serum, 50 IU penicillin, and 50 μg/ml streptomycin. Bovine adrenal cortical fasciculata-reticularis 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 day 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 TransIT-LT1 (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/well 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:TransIT-LT1 reagent:OPTI-MEMI medium is 1 μg:2.5 μl:50 μl. OPTI-MEMI media (GIBCO) were mixed with TransIT-LT1 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 Pharmingen Monolight 3010 luminometer.

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 mRNA were made as described previously. The mutant 3.5-kb mRNA vector was made by site-directed mutagenesis (Stratagene, La Jolla, CA) using the following primers: first motif, 5'-CTCTGCAAGGATCGCCGTCTCGTTATGAAACAGAAAGT-3' and 5'-AGTGTGTTTCGTCTTACAAAGACCACGTCCTGAGG-3'. 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. They were cultured in F12K medium (Sigma) supplemented with 15% horse serum, 50 IU penicillin, and 50 μg/ml streptomycin. Bovine adrenal cortical fasciculata-reticularis 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 day 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 TransIT-LT1 (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/well 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:TransIT-LT1 reagent:OPTI-MEM medium is 1 μg:2.5 μl:50 μl. OPTI-MEMI media (GIBCO) were mixed with TransIT-LT1 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 Pharmingen Monolight 3010 luminometer.

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 mRNA were made as described previously. The mutant 3.5-kb mRNA vector was made by site-directed mutagenesis (Stratagene, La Jolla, CA) using the following primers: first motif, 5'-CTCTGCAAGGATCGCCGTCTCGTTATGAAACAGAAAGT-3' and 5'-AGTGTGTTTCGTCTTACAAAGACCACGTCCTGAGG-3'. The luciferase-StAR 3'-UTR chimeric vectors, Star expression constructs, and the in vitro transcription constructs have been previously described (28).

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Data from luciferase transfections are expressed as mean ± SD calculated from the triplicate cultures.
Western blot analysis

To harvest total cellular proteins, cells were washed once in PBS and harvested with RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 1% Nonidet P-40, 0.25% deoxycholic acid, 0.05% SDS, 40 mM NaF, 10 mM sodium molybdate, 1 mM phenylmethylsulfonyl fluoride, and 1% protease inhibitors cocktail from Sigma]. Lysate was passed through a 25-gauge needle six times, centrifuged at 12,000 x 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 electrophotically 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.

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) 1 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 quantified 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'-TTTCTATCCGGACGCTGGAGCTTT-3' and 5'-ACAGCTTAAAGAGCAGAGTGGG-3', and primer pair 2, 5'-AAAGACACCGACAGCTCAGACAG-3' and 5'-GGTAAGACAAGTCCGCAGTCTT-3'. The primers used for detecting TIS11b were 5'-CCACATTGTGTTCAGTGGCCGG-3' and 5'-TGAGGATCTTGTTGCTTACCAAC-3'. The primers used for detecting β-actin were 5'-TGTGACACCTGGAAGTCTGGG-3' and 5'-TTGAGAGGGTCTGGGTTCGCAAG-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 MyQ 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 X 100 for 30 sec). The membrane was then prehybridized at 65°C for 1 h in QuickHyb hybridization solution (Strategene). Hybridization was performed at 65°C for 2 h in QuickHyb with the probes of 0.9-kb mouse StAR cDNA probe.
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 (SSC) buffer at room temperature followed by a stringent wash in 0.1× standard saline citrate (SSC)/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 pG3-LucUTRS or pG3-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–polyacrylamide gels. Quantification was performed with the ImageQuant 5.2 software. The level of 28S rRNA was measured as internal standard for the RNA loading.

RNP 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 mM glycine, and cells were lysed in RIPA buffer containing protease inhibitors. Protein A-agarose preadsorbed cell lysates were immunoprecipitated with protein A-agarose beads preincubated with 2 μg anti-Tis11b antibody (anti-NC-terminal or anti-C-terminal antibodies), 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 Superscript II (Invitrogen). A PCR amplification of Star transcripts was then carried out using Taq polymerase (QBiogen, Illkirch, France) with the primer pair 5'-CAGAAGATGCAAAAGACCGCTG-3' and 5'-AGTGAGATTTGTCCTTGAGGGC-3', under the following conditions: 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min for 40 cycles. The PCR products were analyzed by 2% agarose gel electrophoresis.

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