Inhibition of Corticotropin Releasing Hormone Type-1 Receptor Translation by an Upstream AUG Triplet in the 5′ Untranslated Region

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ABSTRACT

The influence of an upstream open reading frame (ORF) in the 5′-untranslated region (UTR) of the mRNA on corticotropin-releasing hormone receptor type 1 (CRHR1) translation was studied in constructs containing the 5′-UTR of CRHR1, with or without an ATG-to-ATA mutation in the upstream ORF, and the main ORF of luciferase or CRHR1. Upstream mutation in luciferase constructs increased luciferase activity when transfected into COS-7 or AtT20 cells compared with the native 5′-UTR. Transfection of CRHR1 constructs containing the upstream mutation into AtT20 or LVIP2.0zc reporter cells, resulted in higher 125I-Tyr-oCRH binding and corticotropin-releasing hormone-stimulated cAMP production, without changes in CRHR1 mRNA levels (measured by RNase protection assay). In vitro translation of luciferase or CRHR constructs with or without mutation of the upstream ATG, and Western blot analysis with anti-luciferase and anti-CRHR1 antibodies confirmed that mutation of the upstream ATG increases translation of the main ORF. The mechanism by which the upstream ORF inhibits translation may involve translation of the upstream peptide, because in vitro translation, or transfection into LVIP2.0zc cells of a fusion construct of the upstream ORF and green fluorescent protein (GFP) yielded a band consistent with the molecular size of GFP protein. The study shows that the upstream AUG in 5′-UTR of CRHR1 mRNA inhibits receptor expression by inhibiting mRNA translation and suggests the short open reading frame in the 5′-UTR plays a role in regulating translation of the CRH receptor.

The 41-amino-acid hypothalamic peptide corticotropin-releasing hormone (CRH), produced by parvocellular neurons of the hypothalamic paraventricular nucleus is a major regulator of ACTH secretion during activation of the hypothalamic-pituitary-adrenal (HPA) axis (Vale et al., 1983; Antoni, 1986). CRH initiates its biological effects by binding to specific, high-affinity plasma membrane receptors located in the pituitary corticotroph and other target tissues (DeSouza and Kuhar, 1986; Aguilera et al., 1987). Two major CRH receptor subtypes have been identified by molecular cloning: type 1 (CRHR1), which is widely distributed in the brain and is the only subtype in the pituitary corticotroph, and type 2 (CRHR2), which is present in discrete areas of the brain and in the periphery (Lovenberg et al., 1995; Perrin et al., 1995). Both CRH receptor subtypes belong to the 7 transmembrane domain, G protein receptor family coupled to adenylate cyclase (Aguilera, 1994).

CRH receptors in the pituitary undergo changes during manipulation of the HPA axis, but it should be noted that binding down-regulation occurs regardless of whether there is stimulation (chronic stress, adrenalectomy) or inhibition (glucocorticoid administration) of the HPA axis (Aguilera, 1994). Moreover, analysis of the changes in CRHR1 mRNA has shown no correlation between steady-state mRNA levels and receptor content in the pituitary (Aguilera, 1998). For example, CRH receptor loss in all the above conditions is associated with normal or elevated CRHR1 mRNA levels (Rabadan-Diehl et al., 1996, 1997). This indicates that CRH receptor levels are mainly regulated at the translational and post-translational levels and that mRNA levels are not the determining factor.

It has been shown that the presence of short open reading frames (ORF) in the 5′-UTR of the mRNA can affect translation of the main ORF in a number of transcripts (Kozak, 1986).
1991; Geballe and Morris, 1994). Although this occurs in less than 10% of transcripts in vertebrates, it is more frequent in low abundance proteins, such as some members of the G protein-coupled receptor family, including muscarinic, adrenergic, serotoninergic, substance P, substance K, and angiotensin II receptors (Curnow et al., 1995; Koblika et al., 1987; Kozak, 1991). For β₂-adrenergic and type 1 angiotensin II receptors, it has been reported that AUG triplets that are part of upstream open reading frames (uORFs) in the 5'-UTR of the transcript inhibit translation of the receptor protein (Parola and Kobilka, 1994; Mori et al., 1996). The 5'-UTR of the CRHR1 mRNA contains about 220 bp, and sequence analysis reveals a short ORF located between bp −50 and −16 upstream of the main ORF initiating methionine, potentially encoding a 10-amino-acid peptide. The structure of the 5'-UTR of the CRHR1, and especially the 33-bp uORF, is highly conserved between species, including human, rat, and mouse, suggesting that it is an important regulatory feature of the mRNA (Chen et al., 1993; Perrin et al., 1993; Vita et al., 1993). Because uORFs in the 5'-UTR can influence translation of the main ORF, it is possible that the uORF of the CRHR1 inhibits translation of CRHR1 mRNA and contributes to CRH receptor down-regulation in the presence of high CRHR1 mRNA levels. In this study, we investigated whether mutational inactivation of the upstream AUG of the CRHR1 5'-UTR affects translation of CRHR1 and examined possible mechanisms by which the uORF can influence this event.

Materials and Methods

CRHR1 5'-UTR Mutants and Fusion Protein Constructs. A 2.5-kilobase full cDNA clone of the rat CRHR1, kindly provided by Dr. W. Vale (Salk Institute, La Jolla, CA) was used as template for PCR amplification of DNA fragments used for all constructs. First, a fragment corresponding to the 5'-UTR of the CRHR1 was generated by PCR using primers F1 (5’-GAG CTG GTA CGG GGC AGA GGT ACC AG -3’) and R1 (5’-CTG CAC CGG CGG GCC TCC TGG ATG CC -3’), and subcloned into pcDNA3.1(+)(Invitrogen, Carlsbad, CA). The resulting construct was named p5UTR. A second construct, p5UTRm, was generated by a similar approach, but the ATG codon of the upstream open reading frame (uORF), located 50 bp upstream from the initiating methionine, was mutated into an ATA using the sequencing Kit; PE Applied Biosystems, Foster City, CA.

The accuracy of all constructs shown in Fig. 1 was confirmed by nucleotide sequencing using a dyeoxy DNA sequence analysis (DNA sequencing Kit; PE Applied Biosystems, Foster City, CA). Cell Culture and Transfections. COS-7 and A9T-20 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 U/ml penicillin G, and 100 mg/l streptomycin, at 37°C under 95% air/5% CO₂. COS-7 and A9T-20 cells were cultured in the same medium containing an additional 25 mg/l hygromycin B. Cells were grown to 80% confluence and transfected using Lipofectamine PLUS reagent (Life Technologies, Gaithersburg, MD). All cell culture reagents were also purchased from Life Technologies (Gaithersburg, MD).

Luciferase Activity Assay. COS-7 and A9T-20 cells cultured in 24-well plates to 80% confluence were transfected with 0.4 μg per well of p5UTR-luc or p5UTRm-luc plasmid DNA. Cotransfection with the renilla luciferase vector, pRLsv40, (Promega) at a 1:50 ratio was performed to correct for transfection efficiency. Twenty four hours after transfection, cells were harvested for measurement of firefly and renilla luciferase activities using a dual-luciferase assay system (Promega) in a Monolight 20101 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI).

Measurement of Functional CRHR1. The content of biologically active CRHR1 was quantified by binding of 125I-Tyr-oCRH.

![Fig. 1. Schematic depiction of the CRHR1 cDNA (A) showing boxes corresponding to the uORF in the 5'-UTR, the main ORF encoding the CRHR1 (CRHR1 ORF). The sequence of the 5'-UTR below highlights the uORF and the initiation codon of the main ORF. The diagrams in B show the structure of the different wild-type and mutant (m) constructs used in the experiments described under Materials and Methods and Results. The mutations are shown by the arrows, and the two fusion constructs at the bottom are indicated by -F at the end.](image)
RNA Single-Stranded Conformational Polymorphism. To determine whether modifications in the ATG of the uORF in the 5'-UTR of the CRH receptor influences mRNA structure, p5UTR and p5UTRm constructs were transcribed using [32P]UTP in an in vitro transcription system (Promega). The transcription reaction was performed as described by Sarkar et al. (1992), in a total volume of 20 μl of 40 mM Tris-HCl, pH 7.9, containing 10 mM NaCl; 6 mM MgCl2; 10 mM DTT; 2 mM spermidine; 0.05% Tween 20; 20 U RNasin; 0.5 mM each ATP, GTP, and CTP; 12 μM UTP; 0.5 μg of linearized template, 50 μCi of [32P]UTP and 20 U of T7 RNA polymerase. After 1 h incubation at 37°C, the reaction was terminated by placing the tube on ice. One microliter of the [32P]-labeled RNA was mixed with 4 μl of 50% formamide, 2 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue. The mixture was heated at 70°C for 5 min, cooled on ice for 5 min, and 1 μl was electrophoresed through a 5.6% nondenaturing polyacrylamide gel at 4°C with precooled 1× Tris/borate/EDTA buffer. After 4 to 6 h of electrophoresis at 1200 V, the gel was dried and exposed to Kodak X-Omat AR X-ray film (Eastman Kodak, Rochester, NY) for 2 or 5 h.

Western Blot Analysis of CRHR1, Luciferase, and GFP. The relative amounts of CRHR1 protein produced after transfection of the cells with p5UTR-CRHR1 or p5UTRm-CRHR1 were measured by immunoblot. LVIP2.0zc cells were cultured in 75-cm² flasks, transfected with 10 μg of either p5UTR-CRHR1 or p5UTRm-CRHR1 and 48 h later harvested and pelleted at 1,000 g for 5 min. Membrane-rich fractions were prepared by homogenization in 1 ml of 50 mM Tris-HCl, pH 7.4, containing 2 mM EDTA, 5 mM MgCl2, 1 mM DTT, and 1 μg/ml aprotinin, by 12 strokes in a Dounce glass/glass homogenizer. After centrifugation for 5 min at 800 g to discard nuclei and unbroken cells, the supernatant was centrifuged at 30,000 g for 30 min. Membrane pellets were resuspended in homogenization buffer containing 1% Triton X-100, gently shaken for 90 min at 4°C, and centrifuged at 14,000 rpm in a microcentrifuge for 30 min at 4°C. Thirty micrograms of protein were loaded on a SDS-polyacrylamide gel, transferred to a nylon membrane, blocked for 1 h in PBS containing 0.1% Tween 20 and 5% nonfat milk, and incubated for 1 h with a rabbit polyclonal anti-rat CRHR1 antibody (1:500 dilution) in PBS containing 0.1% Tween 20 and 1% nonfat milk. The antibody was prepared at the Max Planck Institute (Göttingen, Germany), as described previously (Sydow et al., 1997). CRHR1 protein bands bound to the antibody were detected using donkey anti-rabbit γ-globulin coupled to horseradish peroxidase and the enhanced chemiluminescence Western blotting analysis system (Amersham Pharmacia, Piscataway, NJ) according to the manufacturer's instructions.

For measurement of luciferase protein by immunoblot, LVIP2.0zc cells cultured in 6-well plates were transfected with either p5UTR-luc or p5UTRm-luc. Forty-eight hours after transfection, cells were washed in PBS and lysed in 500 μl of 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% Nonidet P-40 for 30 min on ice. Twenty micrograms of protein were separated by SDS-polyacrylamide gel electrophoresis, transferred to a nylon membrane, blocked in PBS containing 1% Tween 20 and 5% fat-free milk, and incubated with a rabbit anti-luciferase polyclonal antibody (Contex Biochem, Inc., San Leandro, CA) at a 1:500 dilution for 1 h. Visualization of the luciferase bands was performed using chemiluminescence kit reagents as described for CRHR1.

For determination of the fusion upstream-GFP protein, LVIP2.0zc cells cultured in 6-well plates were transfected with either p5UTRgfp-luc or p5UTRm-luc. Forty-eight hours after transfection, cells were lysed and in 500 μl of 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% Nonidet P-40 for 30 min on ice. Twenty micrograms of protein were separated by SDS-polyacrylamide gel electrophoresis, transferred to a nylon membrane, blocked in PBS containing 1% Tween 20 and 5% fat-free milk, and incubated with a mouse anti-GFP polyclonal antibody (CLONTECH) at a 1:500 dilution, followed by incubation with goat anti-mouse IgG (1:2000) conjugated with alkaline phosphatase.

In Vitro Translation. One microgram of XbaI-digested pcDNA3.1(+), p5UTR-luc, p5UTRgfp-F, or p5UTRm-gfp-F was transcribed from the T7 promoter and translated in a 50-μl volume containing 2 μl of [35S]methionine (1,000 μCi/mmol; Amersham) and
Results

Effect of Upstream ATG Mutation on Functional Protein Expression. The influence of the upstream ATG in the 5′-UTR of the CRHR1 mRNA on protein synthesis was first studied by examining the effect of the ATG mutation on luciferase activity after transfection of the constructs p5UTR-luc and p5UTRm-luc into COS-7 or AtT-20 cells. As shown in Fig. 2A, mutation of the upstream ATG caused marked 2.1 ± 0.29-fold and 2.4 ± 0.46-fold increases in luciferase activity in COS-7 and AtT-20 cells, respectively, compared with cells transfected with the unaltered 5′-UTR construct, p5UTR-luc (p < 0.01, n = 5).

To determine the influence of the upstream ATG on the expression of functional CRH receptors, binding of 125I-Tyr-CRH to membranes and CRH-stimulated cAMP production was studied in LV1P2.0zc cells transfected with unmodified or mutant CRHR1 cDNA (p5UTR-CRHR or p5UTRm-CRHR, respectively). A radioligand binding assay showed a significant 2.4-fold increase in the number of CRH binding sites in membranes from cells transfected with the mutant upstream ATG (1254 ± 388 fmol/mg of protein), compared with membranes from cells transfected with the native CRHR1 cDNA sequence (565 ± 49 fmol/mg of protein). As shown by the parallel Scatchard plots in Fig. 3A, the binding affinity was similar in membranes from cells transfected with p5UTRm-CRHR1 or p5UTR-CRHR1 (Kd, 4.3 ± 0.7 nM versus 4.1 ± 1.6 nM, respectively, n = 3).

The effect of the upstream ATG mutation increasing CRH binding in LV1P2.0zc cells was also reflected in an increased ability of CRH to stimulate β-galactosidase activity, which reflects cAMP production, in this cell line expressing the β-galactosidase reporter gene driven by a cAMP-sensitive promoter. Incubation of cells with CRH for 6 and 18 h after transfection with the CRHR1 constructs, resulted in a dose-dependent increase in βγ-galactosidase activity. As shown in Fig. 3B, there was a significant increase in maximal βγ-galactosidase activity (134 ± 9%, p < 0.05, n = 5), without change in the half-maximal effective CRH concentration (EC50) in cells transfected with the upstream ATG mutant receptor compared with cells transfected with the native CRHR1 cDNA. The mean EC50 values for the five experiments are 0.31 ± 0.08 nM and 0.32 ± 0.07 nM, for the mutant and unaltered CRHR1 constructs, respectively.

Effect of Upstream ATG Mutation on Luciferase and CRHR1 Protein Content. To further determine whether the increases in luciferase and CRH receptor activity in cells
transfected with the mutant upstream ATG was the result of increased protein synthesis, luciferase, and CRHR protein content in the cells was quantified by Western blot. As shown in Fig. 2B, immunoblot analysis of solubilized proteins from LVIP2.0xc cells transfected with the luciferase constructs, p5UTR-luc, or p5UTRm-luc, revealed a 61-kDa luciferase band, of size similar to that of recombinant luciferase used as control. Consistent with the luciferase activity experiments, the intensity of the band was higher in the cells transfected with the mutant 5'-UTR construct p5UTRm-luc than in cells transfected with the native construct p5UTR-luc. The band was not detectable from the cell transfected with the empty vector, pcDNA3.1(+).

Using an affinity-purified specific CRHR1 antibody, immunoblot analysis of solubilized membranes from cells transfected with the native CRHR1 cDNA revealed major bands of 70, 72, and 76 kDa, which are in the range of the molecular size expected for the CRHR1 (Aguilera et al., 1990; DeSouza and Grigoriadis, 1990; Sydow et al., 1997). These bands were not apparent in cells transfected with the vector alone. In three separate transfections, the intensity of the CRHR1 specific bands was higher with the construct containing the mutant upstream ATG compared with the unaltered sequence (Fig. 4, A and B).

To rule out the possibility that the changes in protein synthesis after mutation of the upstream ATG were caused by changes in transcription, CRHR1 mRNA levels were measured by RNase protection assay in LVIP2.0xc cells transfected with native or mutant CRHR1 cDNA. RNA from cells transfected with the CRHR1 constructs displayed a clear specific bands was higher with the construct containing the mutant upstream ATG compared with the unaltered sequence (Fig. 4, A and B).

Consistent with the luciferase activity experiments, the intensity of the band was higher in the cells transfected with the mutant upstream ATG construct p5UTRm-luc than in cells transfected with the native construct p5UTR-luc. The band was not detectable from the cell transfected with the empty vector, pcDNA3.1(+).

Using an affinity-purified specific CRHR1 antibody, immunoblot analysis of solubilized membranes from cells transfected with the native CRHR1 cDNA revealed major bands of 70, 72, and 76 kDa, which are in the range of the molecular size expected for the CRHR1 (Aguilera et al., 1990; DeSouza and Grigoriadis, 1990; Sydow et al., 1997). These bands were not apparent in cells transfected with the vector alone. In three separate transfections, the intensity of the CRHR1 specific bands was higher with the construct containing the mutant upstream ATG compared with the unaltered sequence (Fig. 4, A and B).

To rule out the possibility that the changes in protein synthesis after mutation of the upstream ATG were caused by changes in transcription, CRHR1 mRNA levels were measured by RNase protection assay in LVIP2.0xc cells transfected with native or mutant CRHR1 cDNA. RNA from cells transfected with the CRHR1 constructs displayed a clear band of ~600 kb, as expected from the size of the probe. As shown in Fig. 5, no difference in CRHR1 mRNA content was observed in cells transfected with p5UTR-CRHR or p5UTRm-CRHR, indicating that mutation of the upstream ATG has no effect on transcription (p < 0.84; n = 4). On the other hand, no protected band was observed in nontransfected cells or in cells transfected with vector alone.

**Translation of the Upstream ORF.** To determine whether synthesis of the putative peptide is involved in the mechanism by which the upstream ORF inhibits translation of the main ORF, the ability of the upstream ATG to initiate translation was studied using the uORF-GFP fusion constructs p5UTRgfp-F and p5UTRmGFP-F. As shown in Fig. 6-A, lines 3 and 4, in vitro translation of p5UTRgfp-F resulted in a major band of about 28 kDa labeled with [35S]methionine corresponding to the molecular size of the fusion protein, whereas no product was obtained with the fusion construct, p5UTRmGFP-F (Fig. 6-A, lanes 1 and 2).

Consistent with the in vitro translation, Western blot analysis of extracts from cells transfected with p5UTRgfp-F revealed a 28-kDa band corresponding to the size of the fusion protein (Fig. 6B). The positive control, pEGFP-N3 (CLONTECH) yielded the expected 27-kDa band corresponding to native GFP. The construct containing the ATG mutation, p5UTRmGFP-F, yielded a faint band of molecular size similar to the control GFP. This band could be caused by minor initiation of translation at an unidentified alternative codon, as reported by Kozak (1996).

**Effect of Upstream Peptide on In Vitro Translation.** The experiments above show that the uORF is potentially translated. To determine whether the putative peptide has any effect on the translation of the main ORF, the effect of the synthetic peptide was tested in an in vitro translation system using p5UTR-luc. In vitro translation of p5UTR-luc yielded two bands of 65 and 61 kDa (Fig. 7), whereas the control luciferase construct provided by the kit, showed a single 61-kDa band (not shown). As shown in Fig. 7A, addition of 1 to 100 μM the upstream peptide caused a dose-dependent inhibition of [35S]methionine-labeled luciferase, whereas the highest concentration (100 μM) of the unrelated decapeptides angiotensin I and gonadotropin-releasing hormone were without effect. However, the scrambled sequence of the upstream peptide inhibited the in vitro translation of

![Fig. 4. Western blot analysis of CRHR1 protein after transfection of LVIP2.0xc with p5UTR-CRHR1 or p5UTRm-CRHR1. Lanes 1 and 2 in A show the lack of CRHR1 stained bands in untransfected cells or cells transfected with empty pcDNA3.1 vector. Transfection with p5UTR-CRHR1 or p5UTRm-CRHR1 reveals three protein bands labeled with the CRHR1 antibody. The pooled semiquantitation data from three experiments in B shows the intensity of all CRHR1 bands is significantly higher in cells transfected with the construct with the mutant 5’-UTR.](image-url)

![Fig. 5. CRHR1 mRNA measured by RNase protection assay in LVIP2.0xc cells transfected with p5UTR-CRHR1 or p5UTRm-CRHR1. A, ~600 bp CRHR1 protected band is evident only in cells transfected with CRHR1 constructs. Ribosomal 18S RNA was used to correct for gel loading.](image-url)
luciferase with equal potency as the native sequence (Fig. 7B). Also, the upstream peptide (100 μM) inhibited the translation of vasopressin V1b receptor (Fig. 7C).

Effect of Upstream ATG Mutation on mRNA Conformation. To determine whether mutation of the upstream ATG influences the secondary structure of the mRNA, single-stranded radiolabeled RNA transcribed in vitro from p5UTR and p5UTRm was analyzed by gel electrophoresis under non-denaturing conditions. As shown in Fig. 8, RNA produced from the native 5′-UTR of CRHR1 migrated as a single band. A similar mobility was observed for RNA from the mutant 5′-UTR, but in contrast with the native sequence, the band corresponding to mRNA transcribed from the mutant 5′-UTR appeared as a doublet.

Discussion

Previous in vivo studies showing a lack of correlation between CRH binding and CRHR-1 mRNA levels have suggested that receptor levels in the pituitary may be regulated at the translational level (Rabadan-Diehl et al., 1996, 1997). Modulation of translation rate is determined not only by the stability of the mRNA, but also by structural features of the mRNA, such as a secondary structure or initiation codons in the 5′-UTR (Kozak, 1991; Geballe and Morris, 1994). Because it has been shown that the presence of ORFs in the 5′-UTR of the mRNA can influence the translation efficiency of the main ORF, this study was conducted to determine whether the 5′-UTR could be involved in the regulation of the CRHR-1 receptor translation. The present experiments provide evidence that an upstream ORF, potentially encoding 10 amino acids, in the 5′-UTR of the CRHR1, decreases translation of the CRHR or a reporter gene. Mutation of the upstream ATG in constructs with the main ORF of CRHR1 or luciferase resulted in elevation in luciferase or CRHR1 protein in transfected cells, as well as increased protein translation from these constructs in vitro. These increases in protein levels in the Western blot and in vitro translation assay occurred in the absence of any change in CRHR mRNA levels (shown by the RNase protection assay), indicating that mutation of the uORF leads to improved translation efficiency of the mRNA without changes in transcription.

The position and sequence of the uORF in the CRHR1 are well conserved in human, rat, and mouse receptor mRNA. A computer search of the GenBank database for nucleotide sequences related to uORFs failed to reveal homology with
other leader peptides suggesting that it is a structure unique to CRH receptor transcripts. The fact that the 5′-UTR of the CRHR1 can regulate translation of an open reading frame other than CRHR1 (e.g., luciferase) indicates that the inhibitory effect depends on structural features of the 5′-UTR itself without interacting with distal sequences of the CRHR1 mRNA. Because polymorphism analysis of mutant and native CRHR1 mRNA 5′ UTR showed minor differences in migration pattern, it is possible that conformational changes of the mRNA are responsible for the increase in translation efficiency.

The upstream ORFs could affect translation of the main ORF of the mRNA through several mechanisms including interaction of the upstream AUG with the translation preinitiation complex, or translation into an active polypeptide (Geballe and Morris, 1994; Parola and Kobilka, 1994; Reynolds et al., 1996; Hinnebusch, 1997). Translation of most mRNAs involves binding of a 43S preinitiation complex near the capped 5′ end, followed by scanning downstream until the first AUG codon is reached. This induces recruitment of the 60S subunit resulting in formation of an 80S complex followed by initiation of translation (Kozak, 1987; Kozak, 1991). Thus, the presence of upstream AUGs could restrict the progression of scanning ribosomes through the 5′-UTR to the start codon of the main ORF and inhibit translation without producing a functional polypeptide product (Hunt, 1985). Such a mechanism has been described in yeast in which the presence of four ORFs in the 5′-UTR of the mRNA represses translation of the transcription factor, GCN4, by interacting with the translation initiation complex and reducing its association with the AUG of the main ORF (Hinnebusch, 1997).

In other hormone receptor systems such as retinoic acid, AT1 angiotensin II, and β-adrenergic receptors, there is evidence that upstream ORFs could be translated into potentially functional peptides (Geballe and Morris, 1994; Parola and Kobilka, 1994; Mori et al., 1996; Reynolds et al., 1996). In contrast to the main ORF of the CRHR1, computer analysis of the sequence reveals that the uORF is in a poor context for translation (Kozak, 1987). As reported for other mRNAs containing short upstream ORFs (Parola and Kobilka, 1994), in this study it was not possible to detect the peptide in the in vitro translation assay (not shown). However, the present demonstration that the fusion construct of the upstream ORF with luciferase is translated in vitro and in transfected cells, indicates that the upstream AUG can initiate translation, hence the uORF has the potential of being translated.

Attempts to determine whether the putative peptide has an effect on the translation of the main ORF yielded inconclusive results. Although the experiments clearly show that the peptide is inhibitory in the vitro translation assay, the concentrations required (10 and 100 μM) are unlikely to be reached in the cell, considering the low abundance of CRHR1 mRNA and the fact that such a small peptide would be rapidly degraded. However, it is not possible to rule out that peptide produced locally could reach concentrations sufficient to inhibit translation. Unexpectedly, the scrambled peptide inhibited in vitro translation as effectively as the native sequence, and the peptides also inhibited translation of a nonrelated mRNA, the V1b receptor. Although this suggests that the observed effects of the peptides are nonspecific, it should be noted that the CRHR1 upstream decapptide peptide (as well as other reported putative upstream peptides) contains a large proportion of charged amino acids. In the CRHR1 upstream peptide, eight of the 10 amino acids are polar and five of them charged, whereas the nonrelated peptides tested (angiotensin I and gonadotropin-releasing hormone) with no inhibitory effect contained only four or five polar amino acids, of which three are charged. Because high polarity and charge will facilitate binding to RNA, it is conceivable that the upstream peptide as well as the scrambled sequence could inhibit translation by binding to RNA, regardless of the amino acid order. However, the fact that the peptide inhibited translation of a nonrelated mRNA (V1b receptor) suggests that specificity for a particular mRNA would require functional compartmentalization (for example, that the nascent peptide would immediately bind to the mRNA strand of origin). Similar problems with the interpretation of the findings are evident in other reports showing inhibition of translation of hormone receptor mRNAs with micromolar concentrations of synthetic peptides, or some cross-specificity of the effects (Parola and Kobilka, 1994; Mori et al., 1996). In general, the present results show that the uORF has the potential of being translated into a peptide capable to inhibit translation, but there is no compelling evidence suggesting that the peptide mediates the regulatory effects of the 5′-UTR on CRHR1 translation.

The role of the uORF on the physiological regulation of the CRHR-1 translation in vivo remains unknown. As other plasma membrane receptors, CRHR1 are low-abundance proteins, and receptor number undergoes rapid regulatory changes during functional alterations of the pituitary corticotroph (Aguilera, 1994). In most alterations of HPA axis activity, CRHR1 down-regulation occurs despite elevated or normal CRHR1 mRNA levels (Aguilera, 1998). Thus, translation of the mRNA represses translation of the transcription factor, GCN4, by interacting with the translation initiation complex and reducing its association with the AUG of the main ORF (Hinnebusch, 1997).

Acknowledgments

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References


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