Insulin inhibition of apolipoprotein B mRNA translation is mediated via the PI-3 kinase/mTOR signaling cascade but does not involve internal ribosomal entry site (IRES) initiation

ARTICLE *in* ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS · OCTOBER 2007

Impact Factor: 3.02 · DOI: 10.1016/j.abb.2007.06.029 · Source: PubMed

4 AUTHORS, INCLUDING:

Reza Meshkani
Tehran University of Medical Sciences
47 PUBLICATIONS **511** CITATIONS
SEE PROFILE

Khosrow Adeli
University of Toronto
319 PUBLICATIONS **8,946** CITATIONS
SEE PROFILE

Available online at www.sciencedirect.com
ScienceDirect
Archives of Biochemistry and Biophysics 465 (2007) 380–388
ABB
www.elsevier.com/locate/yabbi

Insulin inhibition of apolipoprotein B mRNA translation is mediated via the PI-3 kinase/mTOR signaling cascade but does not involve internal ribosomal entry site (IRES) initiation ☆

Konstantinos Gus Sidiropoulos, Reza Meshkani, Rita Avramoglu-Kohen, Khosrow Adeli *

Molecular Structure and Function, Research Institute, The Hospital for Sick Children, University of Toronto, Toronto, Ontario, Canada M5G 1X8

Received 7 April 2007, and in revised form 19 June 2007
Available online 10 July 2007

Abstract

Although insulin normally activates global mRNA translation, it has a specific inhibitory effect on translation of apolipoprotein B (apoB) mRNA. This suggests that insulin induces a unique signaling cascade that leads to specific inhibition of apoB mRNA translation despite global translational stimulation. Recent studies have revealed that insulin functions to regulate apoB mRNA translation through a mechanism involving the apoB mRNA 5′ untranslated region (5′ UTR). Here, we further investigate the role of downstream insulin signaling molecules on apoB mRNA translation, and the mechanism of apoB mRNA translation itself. Transfection studies in HepG2 cells expressing deletion constructs of the apoB 5′ UTR showed that the *cis*-acting region responding to insulin was localized within the first 64 nucleotides. Experiments using chimeric apoB UTR-luciferase constructs transfected into HepG2 cells followed by treatment with wortmannin, a PI-3K inhibitor, and rapamycin, an mTOR inhibitor, showed that signaling via PI-3K and mTOR pathways is necessary for insulin-mediated inhibition of chimeric 5′ UTR-luciferase expression. *In vitro* translation of chimeric cRNA confirmed that the effects observed were translational in nature. Furthermore, using RNA-EMSA we found that wortmannin pretreatment blocked insulin-mediated inhibition of the binding of RNA-binding factor(s), migrating near the 110 kDa marker, to the 5′ UTR. Radiolabeling studies in HepG2 cells also showed that insulin-mediated control of the synthesis of endogenously expressed full length apoB100 is mediated via the PI-3K and mTOR pathways. Finally, using dual-cistronic luciferase constructs we demonstrate that apoB 5′ UTR may have weak internal ribosomal entry (IRES) translation which is not affected by insulin stimulation, and may function to stimulate basal levels of apoB mRNA translation.

Keywords: Apolipoprotein B; mRNA; Translation; Untranslated region; Insulin signaling; PI-3 kinase; IRES

Apolipoprotein B (apoB)[1] is synthesized by the liver and intestine, and secreted by the liver in the form of very low density lipoproteins (VLDL), and from the intestine as chylomicrons. ApoB is the main structural protein of low-density lipoproteins (LDL) and also functions as a ligand for the LDL receptor, mediating the uptake and clearance of LDL from plasma [1,2]. One of the risk factors for coronary artery disease and atherosclerosis is increased plasma levels of LDL-cholesterol and LDL-apoB [3]. Acute regulation of VLDL-apoB secretion appears to

☆ This work was supported by an operating grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) to K.A. K.G.S. is a recipient of a Banting and Best Diabetes Centre Novo-Nordisk Scholarship, the Hospital for Sick Children Restracomp graduate scholarship, and the Ontario Graduate Scholarship (OGS).
* Corresponding author. Fax: +1 416 813 6257.
E-mail address: k.adeli@utoronto.ca (K. Adeli).
[1] *Abbreviations used:* ALLN, *N*-acetyl-leucyl-leucyl-norleucinal; ApoB, Apolipoprotein B; Bis-I, Bis-indolylmaleimide I (2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide; BisI); eIF-4E, eukaryotic initiation factor 4E; IRES, internal ribosomal entry site; LDL, low density lipoprotein; PCR, polymerase chain reaction; PI-3K, protein inositol 3 kinase; PMA, phorbol 12-myristate 13-acetate; mRNA, messenger RNA; mTOR, mammalian target of rapamycin; nt, nucleotides; PKC, protein kinase C; UTR, untranslated region; VLDL, very low density lipoprotein.

0003-9861/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2007.06.029

occur post-transcriptionally as levels of apoB mRNA remains stable under a variety of acute metabolic stimuli such as treatment with insulin and thyroid hormone [4–10].

We have shown previously that the apoB mRNA 5′ UTR *cis*-acting region is required for the optimal translation of apoB mRNA [11]. In addition, we have also demonstrated that insulin decreases apoB mRNA translation through a *cis*-acting element located on the apoB 5′ UTR and characterized by decreased binding to the 5′ UTR of a *trans*-acting 110 kDa protein factor (p110) [12]. More recently, we found that protein kinase C (PKC) isoforms may be involved in the activation of apoB mRNA translation since treatment with PKC activator PMA showed an increased expression of apoB reporter gene, while treatment with PKC inhibitors Bis-I and Go6976 showed a decrease in expression of apoB reporter gene and apoB protein. [13].

Interestingly, although insulin has an inhibitory effect on apoB mRNA translation [4–10], insulin is widely known to stimulate global protein synthesis in mammalian cells through general activation of the process of mRNA translation [14]. Furthermore, in insulin-resistant states such as obesity and type-2 diabetes, this regulation is lost and apoB over-production ensues [3,15–17]. Several translation factors are known to be activated in response to insulin, often as a consequence of changes in their phosphorylation states. The cap-binding factor or eukaryotic initiation factor 4E (eIF-4E) is known to play an important role in mRNA translation by binding the 5′-cap structure of mRNAs and facilitating the recruitment of other translation factors and the 40 S ribosomal subunit [18,19]. Recent biophysical studies indicate that eIF-4E phosphorylation at serine 209 actually decreases the affinity of eIF-4E for cap or capped RNA. It has been also proposed that eIF-4E phosphorylation at serine 209 may reprogram the translational machinery by releasing the translational apparatus from existing complexes, allowing other mRNAs to be translated. These mRNAs could be highly structured mRNAs, such as the apoB mRNA. PKCs are known to activate phosphorylation of eIF-4E, however in most cell types insulin does not induce phosphorylation of eIF-4E at Ser209 [18,19]. Furthermore, phosphorylation of eIF-4E has been shown to decrease the affinity of eIF-4E for the 5′ cap [19].

It has been previously shown that inhibition of PI-3 kinase is able to block insulin's effect on hepatic apoB secretion [20]. Rapamycin, a signaling inhibitor is known to act by binding to a 12 kDa cytosolic protein, FK506-binding protein, and this complex interacts with a large protein about 250 kDa in size, the FK506-binding protein rapamycin-associating protein/mammalian target of rapamycin (FRAP/mTOR). In the context of insulin and other growth factors, rapamycin is important because it inhibits their effects on translation, suggesting that mTOR is important in their signal transduction [21,22]. It is well known that mTOR phosphorylates two well-characterized downstream targets, S6K1 and eukaryotic initiation factor 4E (eIF-4E) binding protein (4E-BP1), which are positive and negative regulators of protein synthesis, respectively. Activation and phosphorylation of S6K1 kinase leads to phosphorylatation of the the downstream 40S ribosomal protein S6 and enhances the translation of mRNA containing repressive 5′-tandem oligopolypyridimine (TOP) tract containing mRNAs. Conversely, phosphorylation of 4E-BP1 induces its release from eIF-4E, thereby enhancing eIF-4E-mediated cap-dependent translation [23,24]. To our knowledge, the relationship between mTOR and apoB has not been studied.

In addition, of considerable interest is the potential role of internal ribosome entry segment or site (IRES) in regulating apoB mRNA translation. It has been previously demonstrated that translation of uncapped picornaviral mRNA is mediated by an RNA structure, termed ''IRES'', which allows the assembly of the translational machinery at a position close to or directly at the initiation codon [25,26]. Most cellular IRESs have been shown to function preferentially when cap-dependent translation is physiologically impaired. Conditions in which cap-dependent translation is physiologically impaired include proteolytic cleavage of eIF-4G during apoptosis, and dephosphorylation of 4E-BP1 during mitosis, quiescence, differentiation, and/or stress [27]. It was demonstrated that IRES elements were found to be active during many conditions of stress, including amino acid starvation [28] and hypoxia [29]. This led to the recent hypothesis that IRES-mediated translation of certain mRNAs represents a regulatory mechanism that helps the cell cope with aberrant conditions. Furthermore, *trans*-acting factors binding to IRES could mediate this regulation. IRES elements are defined exclusively on functional criteria and cannot be accurately predicted yet by the presence of characteristic RNA sequences or structural motifs [30]. Some progress has been made however for cellular IRES elements based on structure algorithms that identify a Y-shaped, double-hairpin structure followed by a small hairpin that constitute an RNA motif that can be found upstream of the start-site codon in a variety of cellular IRESs [31]. Interestingly, recent analysis of the UTR database, UTRdb [32] demonstrated that the apoB 5′ UTR may contain an IRES. Further analysis of the apoB 5′ UTR sequence in both the UTRscan [33] and Transterm [34] confirmed this. The putative IRES site within the apoB 5′ UTR region appears to be at nt 45–128.

In the present study, we attempted to investigate the insulin-signaling mechanisms involved in the inhibition of apoB mRNA translation and the role of internal ribosomal entry (IRES) in its insulin-mediated regulation. Our data suggests that the insulin inhibition of apoB mRNA translation requires PI-3K and mTOR activation. Furthermore, the apoB 5′ UTR may function as a weak IRES allowing translation of the apoB message even during insulin-stimulatory conditions.

Methods and materials

Cell culture

HepG2 cells were maintained in complete α-MEM containing 5% fetal bovine serum and 1% antibiotic/antimycotic. Cells were maintained at 37 °C under 95% air/5% CO_2. Media was replenished every 3 days and cells were subcultured once a week, usually after reaching 90% confluency.

Treatment of cells with signaling modulators

HepG2 cells were treated with 100 nM of Wortmannin, a PI-3K inhibitor (CalBiochem) or 10 nM of rapamycin, a FRAP/mTOR inhibitor (Cell Signaling) for 4 h.

Transient transfection experiments

HepG2 cells were transfected with 1 μg of each of the various UTR-Luciferase constructs and PRL-Tk *Renilla* (Promega) using Fugene-6 (Roche). The media was changed to complete α-MEM media after 4 h of transfection and treated with signaling modulators as described. After drug treatment, the cells were lysed using passive lysis buffer (Promega), and placed on an orbital shaker for 15 m.

Dual luciferase assay

The dual luciferase assay was performed essentially according to manufacturer's protocol, using 7 μl of lysate and 25 μl of Luciferase Assay Reagent II (LAR-II) and Stop-and-Glow (Dual Luciferase Kit; Promega).

Construction of KSplus-apoB5′ UTR and KSplus-apoB3′ UTR vectors

Vectors were constructed as described previously [12].

Construction of chimeric apoB UTR-luciferase vectors, apoB 5′ UTR-LUC deletion constructs and T7 apoB5′ UTR/3′ UTR-luciferase vectors

The chimeric apoB UTR-luciferase vectors, the chimeric T7 apoB UTR-luciferase vectors, and the UTR-apoB15 constructs were created as described previously [11,12]. ApoB 5′ UTR deletion constructs linked to luciferase were constructed as mentioned previously [11].

In vitro transcription of KSplus-apoB5′ UTR and KSplus-apoB3′ UTR vectors

The KSplus-ApoB5′ UTR and KSplus-ApoB3' UTR vectors were linearized by digestion with EcoRI and transcribed using RiboMAX (Promega) and biotin-16-UTP as per the manufacturer's instructions. RNA was then purified using G-50 Sephadex columns for radiolabeled RNA purificatoin (Roche) and quantified by UV spectrophotometry.

In vitro transcription and translation of T7 apoB5′ UTR/3′ UTR-luciferase vectors

The T7 SKminus-apoB-UTR luciferase vectors were linearized by digestion with BamHI and transcribed using RiboMAX (Promega) as per the manufacturer's instructions. RNA was treated with RQ1 DNase (Promega) for 15 m. The RNA was then purified by Sephadex G-50 RNA purification columns (Roche) and quantified by UV spectrophotometry. The T7 SKminus apoB-UTR luciferase RNAs (1 μg per construct) were *in vitro* translated using the Flexirabbit reticulocyte lysate kit (Promega) essentially as per the manufacturers instructions in the presence and absence of 1 μg of cytosol from HepG2 cells treated in the presence or absence of 150 nM insulin and/or signaling modulators. Reactions were incubated at 30 °C for 20 m and then frozen at −20 °C until ready for the luciferase assay.

Preparation of HepG2 liver cystosolic extracts

Cells were washed once with 1 mL of 250 mM sucrose and 3 mM of imidazole pH, 7.4 and once with 2 mL of 50 mM sucrose and 3 mM imidazole pH, 7.4. Cells were then scraped into 500 μl of 50 mM sucrose and 3 mM imidazole pH, 7.4 and homogenized using a Dounce homogenizer on ice with 20 strokes. Fifty microliters of 49% sucrose was added and the cells were homogenized with 5 more strokes. The homogenate was transferred to a clean microfuge tube and was centrifuged at 2200*g* for 10 min at 4 °C. The supernatant was placed in an ultracentrifuge tube and was centrifuged at 100,000*g* for 1 h at 4 °C in an SW-55 rotor. The supernatant was collected for dialysis. Cytosol was dialyzed using Spectra/Por molecular porous membrane tubing (Spectrum; CA) MWCO: 6–8000. Dialysis was conducted with dialysis buffer containing 20 mM Hepes, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, and 100 KIU/mL trazylol solution.

Electromobility shift assay (EMSA)

In vitro hybridization was performed with 14 μg of cytoplasmic lysate incubated with 400 ng of biotinylated-apoB UTR RNA in a 20 μl reaction consisting of 10% glycerol, 12 mM Hepes, pH 7.9, 40 mM KCl, 0.25 mM EDTA, 0.5 mM DTT, 5 mM $MgCl_2$, and yeast tRNA (200 ng/ml) for 10 m at 30 °C, essentially as described previously [12].

UV cross-linking assay

To identify the molecular weights of RNA–protein complexes, probe–protein reactions were set up similar to the EMSA protocol described above. After incubation at 30 °C, samples were exposed to UV light at 3000 mwatts/cm2 for 15 m on ice. RNase T1/A mix was added to the mix and incubated for 30 m. Laemmli sample buffer was then added, and samples were boiled for 5 m. Samples were electrophoresed on a 10% SDS–PAGE. Gels were transferred onto positively charged Nylon Membranes (Roche). The membrane was then processed according to the LightShift Chemiluminescent EMSA kit protocol (Pierce). The UV-crosslinking experiments were performed as described previously [12], however, mini-gels were used in place of slab gels and gels were run for 1–2 h instead of overnight at 70 V, thus improving gel resolution.

Metabolic radiolabeling of apoB15

HepG2 cells grown to 70% confluency and were preincubated in methionine-free α MEM at 37 °C for 1 h in the presence or absence of wortmannin or rapamycin with or without 150 nM of insulin, and in presence of the proteasome/protease inhibitor *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) which was added where indicated at a concentration of 10 μg/mL.The cells were then labeled with 75 μCi/ mL [^{35}S] methionine for 10 min. Following the labeling pulse, the cells were washed twice with PBS and harvested by lysis in solubilization buffer (phosphatebuffered saline containing 1% Nonidet P-40, 1% deoxycholate, 5 mM EDTA, 1 mM EGTA, 2 mM PMSF, 0.1 mM leupeptin, and 2 ng/mL ALLN). The lysates were centrifuged for 10 min at 4 °C in a microcentrifuge (12000 rpm), and the supernatants were collected for immunoprecipitation.

Immunoprecipitation, SDS–PAGE, and fluorography

Immunoprecipitation was performed as described previously [35,36] using goat anti-human apoB antibody. Immunoprecipitates were washed with wash buffer (10 mM Tris–HCl pH, 7.4, 2 mM EDTA, 0.1% SDS, 1% Triton X-100) and prepared for SDS–PAGE by resuspension and boiling

in 100 μL of Laemmli sample buffer. SDS–PAGE was performed essentially as described [35,36]. The gels were fixed and saturated with Amplify (Amersham Pharmacia Biotech) before being dried and exposed to Dupont autoradiographic film at −80 °C for 1–4 days. ApoB bands were excised from the gel, digested in hydrogen peroxide/perchloric acid, and associated radioactivity was quantified by liquid scintillation counting [37].

Bioinformatic prediction of putative internal ribosomal entry site (IRES) in the apoB 5′ UTR

The UTR database, UTRdb [34] demonstrated that the apoB 5′ UTR may contain an IRES spanning nucleotides 45–128 GCCGC TGAGGA GCCCG CCCAG CCAGC CAGGG CCGCG AGGC CGAGG C CAGGC C GCAGC CCAGG AGCCG CCCCACCG CAGCT GGCG. Further analysis of the apoB 5′ UTR sequence in both the UTRscan [33] and Transterm [34] confirmed this.

Cloning of the apoB 5′ UTR into the pRV dual-cistronic luciferase construct for IRES analysis

In order to clone the apoB 5′ UTR into the MCS of the pRV construct (kindly given as a gift by Anne Willis), PCR was conducted from the KS-plus apoB 5′ UTR vector using primers specific for the apoB 5′ UTR and containing SpeI and NcoI sites. PCR products were subcloned into the TOPO-4 vector (Invitrogen) and digested using SpeI and NcoI. The insert was then directionally cloned into the pRV construct. Sequencing and restriction digestion confirmed insertion. pRV-HRV is a positive control construct containing the human rhinovirus 2 5′ UTR which is known to act as an IRES.

Assessment of apolipoprotein B IRES activity

The dual-cistronic luciferase constructs were transfected along with a β-galactosidase control vector into HepG2 cells using FuGene-6 transfection reagent and lysed 2-days post-transfection. Treatment of cells using wortmanin, rapamycin, and insulin was conducted as mentioned previously 1-day post-transfection. Dual-luciferase activity was measured using the dual-luciferase assay (Promega). Luciferase values were determined and normalized to β-galactosidase which was measured as a control (Promega).

Results

Deletional analysis of the apoB 5′ UTR and effects on luciferase reporter expression and insulin responsiveness

Recent published data from our laboratory has demonstrated that insulin negatively regulates apoB mRNA translation via its 5′ UTR sequences [11] and its binding to a 110 kDa insulin sensitive protein factor [12]. To determine the sequence element within the 5' UTR required for the observed translational inhibition by insulin, expression constructs were generated, containing various 32 nucleotide deletions of the 128-nucleotide 5' UTR cloned upstream of the luciferase gene in the pGL3 control vector, and were transfected into HepG2 cells. *Renilla* luciferase was co-transfected as a transfection control. Cells were treated in the presence or absence of insulin overnight. As illustrated in Fig. 1, insulin treatment significantly reduced luciferase activity by about 50% in cells transfected with constructs

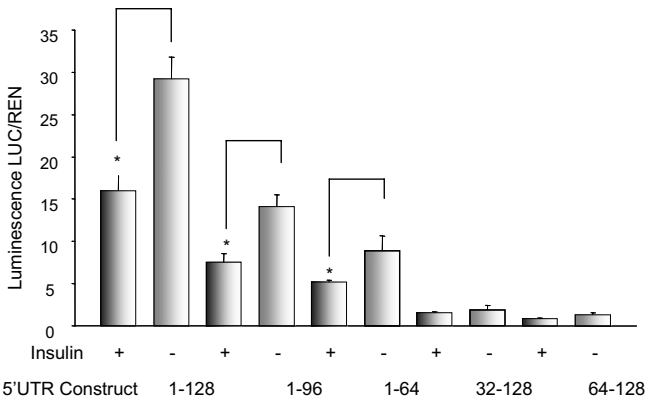

Fig. 1. Deletional analysis of the apoB 5' UTR. Luciferase reporter expression experiments were conducted in HepG2 cells transfected with apoB 5′ UTR-luciferase deletion constructs and control *Renilla* luciferase vectors. Cells were treated in the presence or absence of 150 nM of insulin over-night. Data shown are means ± SD, $^{*}p < 0.05$; n=3.

containing the full length 5′ UTR, as expected. Cells transfected with constructs containing the first 64 nucleotides of the apoB 5′ UTR retained responsiveness to insulin (constructs containing nucleotides 1–128; 1–96; 1–64 had significantly reduced luciferase expression in the presence of insulin compared to vehicle-treated controls) ($p < 0.05$; $n = 3$), whereas deletions of the first 64 nucleotides abolished insulin-mediated inhibition of luciferase expression.

Effects of the PI-3K inhibitor, wortmannin and the mTOR inhibitor, rapamycin on 5′ UTR-luciferase reporter expression and insulin responsiveness

Luciferase reporter expression experiments in HepG2 cells co-transfected with apoB 5′ UTR-luciferase constructs and control *Renilla* luciferase vectors were conducted. Four hours following transfection, cells were treated with wortmannin or rapamycin in the presence or absence of 150 nM insulin for 4 h. As illustrated in Fig. 2a, wortmannin, a PI-3 kinase inhibitor abolished the effect of insulin on the apoB 5′ UTR ($p < 0.05$). Rapamycin, an mTOR inhibitor also abolished the effect of insulin ($p < 0.05$). In contrast, wortmannin and rapamycin did not significantly alter luciferase expression in cells transfected with the negative control luciferase construct (containing no UTRs, Fig. 2b). LUC activity was assessed using the dual LUC assay system. Transfection efficiency for each dish was normalized by dividing firefly LUC activity by *Renilla* LUC activity.

In vitro translation of apoB UTR-luciferase cRNA transcripts in the presence or absence of signaling inhibitors

In order to directly assess the effect of insulin on the translational control of apoB mRNA, we conducted *in vitro* translation experiments using synthetic/purified cRNA. One microgram of RNA produced by *in vitro*

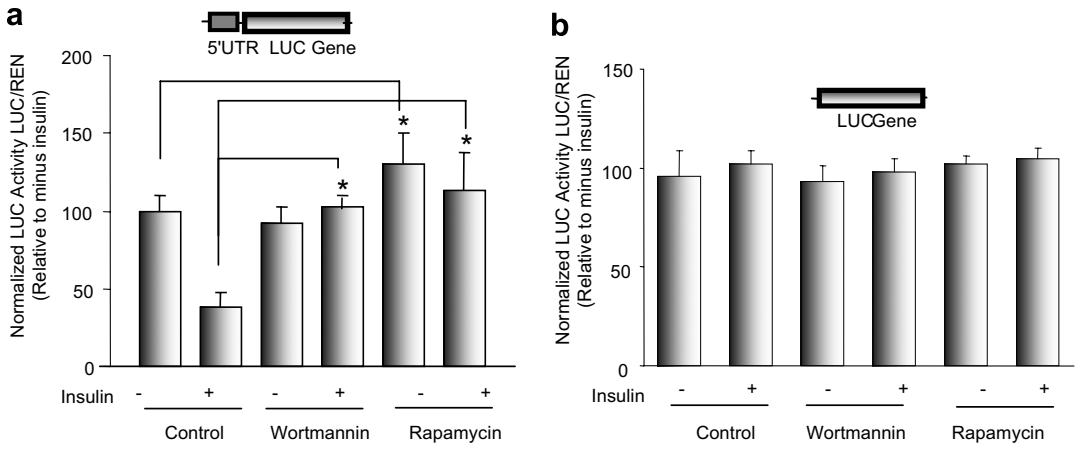

Fig. 2. Effects of the PI-3 K inhibitor, wortmannin and the mTOR inhibitor, rapamycin on 5′ UTR-luciferase reporter expression and insulin responsiveness. HepG2 cells were transiently co-transfected with the chimeric apoB UTR-LUC constructs (a,b) and the pRL-TK (*Renilla* LUC) vector. Cells were treated with wortmannin or rapamycin in the presence or absence of 150 nM insulin for 4 h, 4 h post-transfection. LUC activity was assessed using the dual LUC assay system. Transfection efficiency for each dish was normalized by dividing firefly LUC activity by *Renilla* LUC activity. Data shown are means ± SD) (three separate experiments in triplicate).

transcription of the T7 SKminus apoB UTR-luciferase vectors was added to rabbit reticulocyte lysate in the presence and absence of 1 μg of HepG2 cytosol isolated from cells treated in the presence or absence of insulin and/or wortmannin or rapamycin. As demonstrated in Fig. 3a, cytosol isolated from wortmannin and insulin-treated cells restored translation (luciferase activity) when used in an *in vitro* translation system of the apoB 5′ UTR containing cRNAs ($p < 0.05$). Similarly, cytosol isolated from rapamycin and insulin-treated cells restored translation (luciferase activity) when used in an *in vitro* translation system of the apoB 5′ UTR containing cRNAs ($p < 0.05$).

Wortmannin blocks the interaction of the 5′ UTR with the 110 kDa trans-acting factor(s)

The effects of wortmannin on binding of proteins to the apoB 5′ UTR were also assessed. Complex formation between cytoplasmic proteins and apoB UTR biotin-probe was analyzed by denaturing SDS–PAGE with cytosol isolated from HepG2 cells grown under different conditions (see Methods and materials). As shown, several bands were detected with the major band migrating near the molecular weight of 110 kDa. As shown in Fig. 3b and reported previously [12], in EMSA experiments, the 5′ UTR-biotinylated probe binds to RNA-binding proteins with approximate molecular weights of 110 kDa. Interestingly, however, cytosol isolated from cells treated with wortmannin induced significantly higher binding of the 110 kDa protein factor(s) in the presence or absence of insulin ($p < 0.05$). The insulin-mediated inhibition of this *cis–trans* interaction, as clearly observed in control cells was also abolished in the presence of the PI-3K inhibitor. Rapamycin appeared to have an effect similar to wortmannin.

Synthesis of the full-length apoB100 in the presence and absence of PI-3K and mTOR inhibitors

To confirm the *in vitro* observations made with the reporter constructs above, we also assessed the ability of both signaling modulators to alter de novo synthesis of apoB100 protein by conducting short 10 m radiolabeling pulse experiments in HepG2 cells treated with the various signaling modulators in the presence or absence of ALLN, a proteasome inhibitor which blocks apoB degradation, so that the effects observed could be attributed to synthesis alone. As shown in Fig. 4, treatment with rapamycin or wortmannin had no effect on basal (untreated) levels of apoB but blocked insulin-mediated inhibition of apoB synthesis ($p < 0.05$).

UTRdb bioinformatic prediction of possible internal ribosomal entry site in the apoB 5′ UTR

A recent search of the UTR database, UTRdb [34] demonstrated that the apoB 5′ UTR may contain an IRES between 45 and 128 (Fig. 5a). This was based on UTRdb signal manager pattern similarity from data obtained Le et al. [31].

Generation of pRV-IRES vectors and determination of apoB IRES activity

The apoB 5′ UTR was cloned into the pRV-IRES vector carrying a dual-cistronic luciferase construct (Fig. 5b). pRV-HRV containing the human rhinovirus 2 5′ UTR was used as a positive control construct. The dual-luciferase constructs were transfected into HepG2 cells under standard conditions (10% FBS and α-MEM) (Fig. 6a). The positive control IRES (human rhinovirus 2) had about a 9-fold higher IRES activity than the empty construct ($p < 0.05$). Interestingly, the apoB 5′ UTR had about a

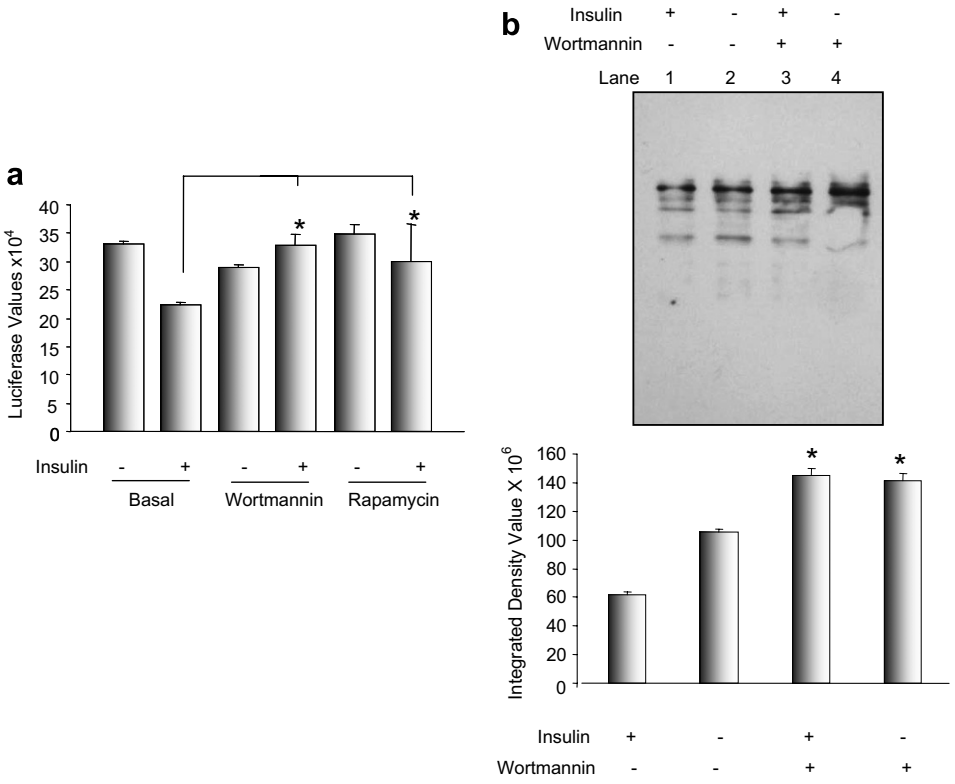

Fig. 3. *In vitro* translation of apoB UTR-luciferase cRNA transcripts and *cis–trans* interactions in the presence or absence of signaling inhibitors. (a) In order to directly assess the effect of insulin on the translational control of apoB mRNA, we conducted *in vitro* translation experiments using cRNA. One microgram of RNA produced by *in vitro* transcription of the T7 SKminus apoB 5′ UTR-Luciferase vector was added to rabbit reticulocyte lysate in the presence and absence of 1 μg of HepG2 cytosol isolated from cells treated in the presence or absence of 150 nM insulin and rapamycin or wortmannin. (Data shown are means ± SD of four separate experiments in triplicate). (b) Complex formation between cytoplasmic proteins and apoB UTR biotin-probe was analyzed by denaturing SDS–PAGE with cytosol isolated from HepG2 cells grown under different conditions (see Methods and materials). As shown, several bands were detected with the major band migrating near the molecular weight of 110 kDa. Cytosol was isolated from cells treated with wortmannin in the presence or absence of 150 nM insulin and incubated with biotinylated apoB 5′ UTR RNA. After UV-crosslinking, RNAse digestion, and SDS–PAGE, the complexes were visualized by the Pierce LightShift Chemiluminescent EMSA detection kit for which a representative experiment is shown (top panel). Quantitation of complex formation for all experiments was performed using densitometry. Data were plotted from imaging densitometric analysis of three separate experiments each performed in triplicate (shown as means ± SD).

2-fold increase over empty vector which was statistically significant ($p < 0.05$).

Effect of insulin on apoB 5′ UTR IRES activity

In order to determine the effect of IRES on the regulation of apoB mRNA translation under the PI-3 kinase signaling pathway, the dual-luciferase constructs were transfected into HepG2 cells which were treated overnight with insulin. Analysis was conducted using the dual-luciferase assay. Values are normalized to β-galactosidase (LacZ). HRV 5′ UTR enabled a high rate of IRES-mediated expression through the second cistron as expected, however IRES activity was not affected by insulin treatment (Fig. 6b). The apoB 5′ UTR allowed a 2-fold increase in IRES-mediated expression of the second cistron over the empty construct, but similarly was not affected by treatment with insulin. The empty vector had the lowest expression of the second cistron and was not significantly influenced by insulin treatment.

Discussion

Insulin is a known negative regulator of apoB synthesis and secretion despite its widely recognized stimulatory effect on global mRNA translation. The signaling mechanisms underlying this insulin-mediated inhibition are largely unknown. Early evidence showed that inhibition of PI-3 kinase can block the insulin's inhibitory effect on hepatic apoB secretion [20]. Our present data suggests that PI-3 kinase inhibition may be sufficient to block the inhibitory effect of insulin on apoB mRNA translation. Luciferase reporter expression experiments, *in vitro* translation of cRNAs, and pulse-chase experiments in HepG2 cells all confirmed that inhibition of PI-3 kinase ameliorates the negative effect of insulin on apoB mRNA. This suggests that insulin exerts its negative effect through the PI-3 kinase signaling cascade likely through changes at the level of the 5′ UTR. The EMSA experiments showed that inhibition of PI-3 kinase increases binding of the insulin-sensitive p110 protein factor(s) to the apoB 5′ UTR. We thus postulate that PI-3 kinase activation is involved both in the inhibition

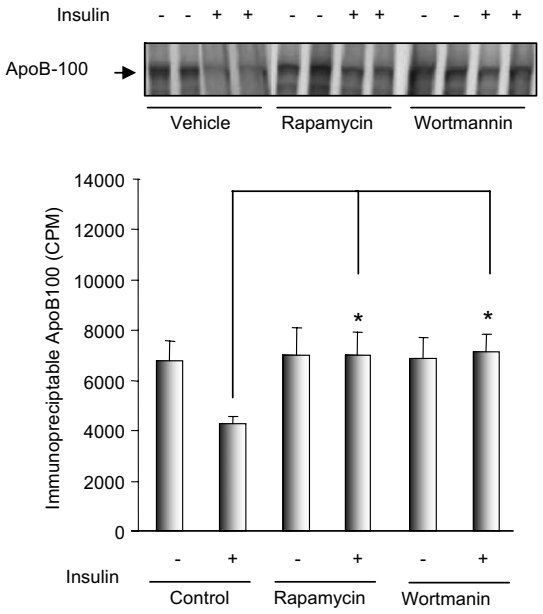

Fig. 4. Synthesis of the full-length apoB100 in HepG2 Cells in the presence and absence of inhibitors of PI-3 kinase and mTOR. HepG2 cells, treated with and without 150 nM insulin and/or signaling inhibitors for 1 h, were subjected to short 10 m radiolabeled pulse experiment in the presence of ALLN, a proteasome and protease inhibitor (see Methods and materials). Cells were preincubated with insulin, wortmannin or rapamycin, and ALLN for 1 h prior to radiolabeling with [^{35}S] for 10 min. ApoB was immunoprecipitated from cells, followed by SDS–PAGE and fluorography. The radiolabeled apoB100 protein was excised from the gel and quantified by liquid scintillation counting. Data shown are means ± SD (three separate experiments each performed in duplicate).

of *cis–trans* interactions within the apoB 5′ UTR and the inhibition of apoB mRNA translation.

We also demonstrated that mTOR, known to operate downstream of PI-3 kinase [21,22], may be involved in the inhibition of apoB mRNA translation and the inhibition of 110 kDa *trans*-acting protein factor(s) binding to the apoB 5′ UTR. Luciferase reporter expression studies and *in vitro* translation experiments confirmed that the mTOR inhibitor, rapamycin, could block the effect of insulin on 5′ UTR activity. There was no effect on the control construct (lacking the UTR sequence), suggesting that mTOR may mediate the insulin signal to inhibit luciferase-reporter expression via the apoB 5′ UTR. Short pulse radiolabeling experiments in HepG2 cells further illustrated that blocking mTOR signaling by rapamycin can exert similar stimulatory effects on the accumulation of newly-synthesized full-length apoB100. *In vitro* translation experiments confirmed that effect of rapamycin was exerted at the translational level via the 5′ UTR. Taken together, the data suggests that mTOR may be a downstream mediator of insulin-signaling via the PI-3 kinase cascade, and may play a role in inhibiting apoB mRNA translation through its interactions with known translational regulators [21,22].

Although most mRNAs are translated via a cap-dependent mechanism, the extensive secondary structure of the apoB mRNA 5′ UTR suggests that apoB message may be translated via a cap-independent or IRES pathway. The features or criteria that are not consistent with most cellular mRNAs are that the 5′ UTR of apoB is translated preferentially under conditions not favoring cap-dependent translation (i.e., not during insulin signaling), it is relatively short and very GC rich, it has extensive secondary structure, and it does not possess upstream AUGs. Since our data indicates that the presence of the 5′ UTR results in increased luciferase-reporter expression, the cap-independent or IRES hypothesis is one potential mechanism by which the apoB mRNA may be translated efficiently. The 5′ UTR of apoB is highly structured and has a stable Y-shaped secondary structure stem loop with the presence of a smaller stem loop (in most RNA structure predictions) which is shared by many IRES elements. Many of the structural features exhibited in the apoB 5′ UTR are consistent with RNA structural motifs common to other cellular mRNAs that use internal ribosomal mechanisms of translation [31]. Bioinformatic prediction programs including Transterm and the UTRdb predicted an IRES for the apoB 5′ UTR. We thus attempted to measure IRES activity of the apoB 5′ UTR. The ‘di-cistronic test’ is the standard *in vitro* assay for detecting IRES structural elements [27]. We thus cloned the apoB 5′ UTR into the pRV bicistronic vector and conducted transfection experiments in HepG2 cells under standard conditions (Fig. 6b). Expression from the second cistron in the pRV vector was 2-fold higher than that of the empty construct. The apoB 5′ UTR IRES activity was however appreciably lower than that of the human rhinovirus 2 positive control IRES, which is known viral IRES with strong IRES activity. Thus, if the apoB 5′ UTR is indeed an IRES, we propose it is a weak IRES that functions to maintain translation of the apoB mRNA during periods where it is translated the highest—when insulin signaling is absent—i.e., during periods when general cap-dependent translation is not stimulated.

To our knowledge, there are no published reports about IRES elements being regulated by signaling factors such as insulin stimulation or PKC activity. In the present study, we tested whether the activity of the apoB 5′ UTR IRES element is responsive to insulin. We transfected the pRV-empty construct, the pRV-apoB 5′ UTR and the pRV-HRV positive control IRES constructs into HepG2 cells and treated the cells with and without insulin (Fig. 6b). There was no appreciable effect of insulin treatment on the IRES activity of either the apoB 5′ UTR nor the positive control IRES, HRV. Also the basal activity of the empty vector construct remained unchanged in the presence or absence of insulin.

ApoB 5′ UTR is normally responsive to insulin when cloned at the 5′ end of the luciferase reporter or a short apoB sequence, apoB15, and the 5′ UTR stimulatory effect on translation is reduced by insulin treatment [11–13]. Interestingly, our current data show that when the 5′ UTR is placed between two cistrons as a putative IRES element, no insulin sensitivity is observed. One possible

a

Features			
Key	**Location**	**Qualifier**	**Value**
5'utr	1..128	source	EMBL::AY324608:1949..2076
		gene	APOB
		product	apolipoprotein B (including Ag(x) antigen)
ires	45..128	source	EMBL::AY324608:1993..2076
		evidence	Pattern Similarity
		standard_name	Internal Ribosome Entry Site (IRES)
		db_xref	UTRSITE:U0015

-UTRScan Analysis of 5' UTR (33): Found 1 matches in 1 sequence:
utr|BB353585|5HSA094436 :[45,128] :
GCCGC TGAGGA GCCCG CCCAG CCAGC CAGGG CCGCG AGGC CGAGG
C CAGGC C GCAGC CCAGG AGCCG CCCCACCG CAGCT GGCG
-Several results were given by Transterm (34).

b

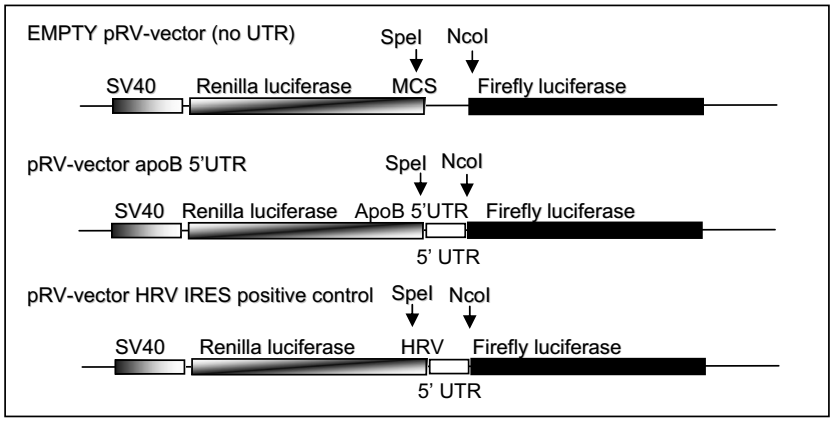

Fig. 5. Bioinformatic prediction of the IRES activity of the apoB 5′ UTR and dual-cistronic luciferase constructs. Bioinformatic analysis from the UTRdb predicted an IRES in the apoB 5′ UTR between nucleotides 45–28 based on the UTR database's Signal Manager from pattern similarity data obtained by Le et al. [31] (a). Dual-cistronic constructs used in the functional assessment of IRES activity (b).

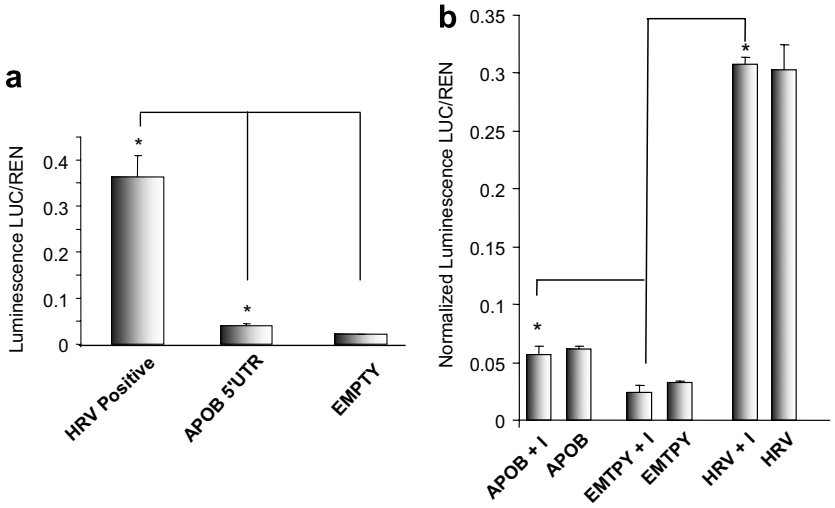

Fig. 6. Functional assessment of the IRES activity of the apoB 5′ UTR and its sensitivity to insulin. Dual-cistronic constructs were used in the functional assessment of IRES activity under standard conditions (FBS and α-MEM) (a). Constructs were transfected into HepG2 cells along with a β-galactosidase control vector. Cells were lysed 2-days post-transfection. Luciferase expression was measured and normalized to β-galactosidase activity. The dual-cistronic constructs were used to assess IRES activity in the absence or presence of insulin (b). Cells were treated 1-day post-transfection with (+I) or without insulin.

explanation may be that the structure of the IRES element may be influenced by sequences 5′ and 3′ of the multiple cloning site (MCS) where it was inserted and possibly coding sequences of the firefly and *Renilla* luciferase genes, especially considering the relatively small size of the apoB 5′ UTR (128 nt). Another possibility is that the apoB 5′ UTR IRES element only exerts its effect on basal mRNA translation, and is not subject to insulin-mediated

regulation. This would suggest that the IRES function of the apoB 5′ UTR may be distinct from the *cis*-acting element that interacts with the p110 protein factor(s) and mediates insulin regulation.

In conclusion, our data suggest that the apoB 5′ UTR has a relatively weak IRES activity and this appears to be observed only at the basal level and not influenced by insulin. Internal ribosomal entry may function to help the basal translation of the apoB mRNA during conditions where its translation may otherwise be inhibited by the lack of translational activation. In addition, we have demonstrated that the PI-3 kinase—mTOR cascade is critical to the transmission of the insulin's inhibitory signal on translation via *cis–trans* interactions at the apoB 5′ UTR. These observations may have important clinical implication in conditions such as insulin resistance. Reduced insulin signaling in the liver can potentially lead to the de-regulation of apoB mRNA translation leading to increased apoB synthesis and VLDL overproduction.

References

[1] E.A. Fisher, H.N. Ginsberg, J. Biol. Chem. 20 (2002) 17377–17380, Review.
[2] J.D. Sparks, C.E. Sparks, Adv. Lipid Res. 21 (1985) 1–46, Review.
[3] J.D. Brunzell, A.D. Sniderman, J.J. Albers, P.O. Kwiterovich Jr., Arteriosclerosis 4 (1984) 79–83.
[4] K. Adeli, C. Sinkevitch, FEBS Lett. 2 (1990) 345–348.
[5] A. Theriault, R. Cheung, K. Adeli, Clin. Biochem. 25 (1992) 321–324.
[6] K. Adeli, A. Theriault, Biochem. Cell Biol. 70 (1992) 1301–1308.
[7] A. Theriault, G. Ogbonna, K. Adeli, Biochem. Biophys. Res. Commun. 186 (1992) 617–622.
[8] A. Mohammadi, A. Theriault, K. Adeli, Biochem. Biophys. Res. Commun. 228 (1996) 852–858.
[9] N. Dashti, D.L. Williams, P. Alaupovic, J. Lipid Res. 9 (1989) 1365–1373.
[10] C.R. Pullinger, J.D. North, B.B. Teng, V.A. Rifici, A.E. Ronhild de Brito, J. Scott, J. Lipid Res. 30 (7) (1989) 1065–1077.
[11] L. Pontrelli, K.G. Sidiropoulos, K. Adeli, Biochemistry 43 (2004) 6734–6744.
[12] K.G. Sidiropoulos, L. Pontrelli, K. Adeli, Biochemistry 44 (37) (2005) 12572–12581.
[13] K.G. Sidiropoulos, A. Zastepa, K. Adeli, Arch. Biochem. Biophys. 459 (1) (2007) 10–19.
[14] C.G. Proud, Biochem. J. 328 (Pt 2) (1997) 329–341.
[15] C. Taghibiglou, A. Carpentier, S.C. Van Iderstine, B. Chen, D. Rudy, A. Aiton, G.F. Lewis, K. Adeli, J. Biol. Chem. 275 (12) (2000) 8416–8425.
[16] C. Taghibiglou, F. Rashid-Kolvear, S.C. Van Iderstine, H. Le-Tien, I.G. Fantus, G.F. Lewis, K. Adeli, J. Biol. Chem. 277 (1) (2002) 793–803.
[17] R.K. Avramoglu, H. Basciano, K. Adeli, Clin. Chim. Acta. 368 (1-2) (2006) 1–19, Epub 2006 Feb 9.
[18] G.C. Scheper, C.G. Proud, Eur. J. Biochem. 269 (22) (2002) 5350–5359.
[19] J. Zuberek, A. Wyslouch-Cieszynska, A. Niedzwiecka, M. Dadlez, J. Stepinski, W. Augustyniak, A.C. Gingras, Z. Zhang, S.K. Burley, N. Sonenberg, R. Stolarski, E. Darzynkiewicz, RNA 9 (1) (2003) 52–61.
[20] J.D. Sparks, T.L. Phung, M. Bolognino, C.E. Sparks, Biochem. J. 313 (Pt 2) (1996) 567–574.
[21] A.C. Gingras, B. Raught, N. Sonenberg, Prog. Mol. Subcell. Biol. 27 (2001) 143–174.
[22] A.C. Gingras, B. Raught, N. Sonenberg, Genes Dev. 15 (7) (2001) 807–826.
[23] D.C. Fingar, C.J. Richardson, A.R. Tee, L. Cheatham, C. Tsou, J. Blenis, Mol. Cell Biol. 24 (1) (2004) 200–216.
[24] L. Lal, Y. Li, J. Smith, A. Sassano, S. Uddin, S. Parmar, M.S. Tallman, S. Minucci, N. Hay, L.C. Platanias, Blood 105 (4) (2005) 1669–1677.
[25] S.K. Jang, H.G. Krausslich, M.J. Nicklin, G.M. Duke, A.C. Palmenberg, E. Wimmer, J. Virol. 62 (8) (1988) 2636–2643.
[26] J. Pelletier, G. Kaplan, V.R. Racaniello, N. Sonenberg, Mol. Cell Biol. 8 (3) (1988) 1103–1112.
[27] S. Vagner, B. Galy, S. Pyronnet, EMBO Rep. 2 (10) (2001) 893–898.
[28] J. Fernandez, I. Yaman, R. Mishra, W.C. Merrick, M.D. Snider, W.H. Lamers, M. Hatzoglou, J. Biol. Chem. 276 (15) (2001) 12285–12291.
[29] I. Stein, A. Itin, P. Einat, R. Skaliter, Z. Grossman, E. Keshet, Mol. Cell Biol. 18 (6) (1998) 3112–3119.
[30] M. Lopez-Lastra, A. Rivas, M.I. Barria, Biol. Res. 38 (2–3) (2005) 121–146.
[31] S.Y. Le, J.V. Maizel, Nucleic Acids Res. 25 (1997) 362–369.
[32] F. Mignone, G. Grillo, F. Licciulli, M. Iacono, S. Liuni, P.J. Kersey, J. Duarte, C. Saccone, G. Pesole, Nucleic Acids Res. 33 (Database issue) (2005) D141–D146.
[33] G.H. Jacobs, P.A. Stockwell, W.P. Tate, C.M. Brown, Nucleic Acids Res. 34 (2006) D37–D40.
[34] G. Pesole, S. Liuni, Trends Genet. 15 (9) (1999) 378.
[35] J. Macri, K. Adeli, Arterioscler. Thromb. Vasc. Biol. 11 (1997) 2982–2994.
[36] K. Adeli, J. Biol. Chem. 12 (1994) 9166–9175.
[37] B.D. Hames, D. Rickwood (Eds.), Gel Electrophoresis of Proteins: A Practical Approach, IRL Press, London, 1981.