# Insulin-Mediated Suppression of Apolipoprotein B mRNA Translation Requires the 5′ UTR and Is Characterized by Decreased Binding of an Insulin-Sensitive 110-kDa 5′ UTR RNA-Binding Protein†

Konstantinos Gus Sidiropoulos, Louisa Pontrelli, and Khosrow Adeli*

*Clinical Biochemistry Division, Department of Laboratory Medicine and Pathobiology, Hospital for Sick Children, University of Toronto, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada*

*Received February 27, 2005; Revised Manuscript Received June 28, 2005*

ABSTRACT: Insulin has been shown to acutely regulate hepatic apolipoprotein B (apoB) secretion at both translational and post-translational levels; however, mechanisms of apoB mRNA translational control are largely unknown. Recent studies of apoB untranslated regions (UTRs) revealed a potentially important role for cis−trans interactions at the 5′ and 3′ UTRs. In the present paper, deletion constructs of the UTR regions of apoB revealed that the 5′ UTR was necessary and sufficient for insulin to inhibit synthesis of apoB15. Metabolic radiolabeling and *in vitro* translation experiments in the presence of protease inhibitors confirmed that the effect of insulin on the apoB 5′ UTR was translational in nature. Using the nondenaturing electrophoretic mobility shift assay (EMSA), protein−RNA complexes were detected binding to the apoB 5′ and 3′ UTRs. Denaturing EMSA identified a 110-kDa protein interacting at the 5′ UTR. Nondenaturing EMSA determined that insulin altered binding of large protein complexes to the 5′ UTR. Binding specificity was determined by competition with both specific and nonspecific competitors. Insulin treatment decreased binding of the 110-kDa protein to the 5′ UTR as visualized by EMSA. Absence of insulin increased binding of this trans-acting factor to the 5′ UTR by 2-fold. Analysis of the 3′ UTR showed no significant insulin-mediated changes in binding of trans-acting factors. We thus propose the existence of a novel RNA-binding insulin-sensitive factor that binds to the 5′ UTR and may regulate apoB mRNA translation. Perturbations in hepatic insulin signaling as observed in insulin-resistant states may alter cis−trans interactions at the 5′ UTR, leading to alterations in the rate of apoB mRNA translation, thus contributing to apoB−lipoprotein overproduction.

Hepatic and intestinal secretion of apolipoprotein B (apoB)[1]-containing lipoproteins is a complex process involved in regulating the plasma levels of atherogenic lipoproteins (*1*, *2*). ApoB is subject to multiple steps of co- and post-translational regulation at the levels of apoB messenger ribonucleic acid (mRNA) translation, ER translocation, proteasome-mediated degradation, and assembly with lipids to form lipoprotein particles (*1*, *2*). Previous studies have provided ample evidence for the translational control of apoB mRNA by insulin and thyroid hormone in HepG2 cells (*3*−*9*). Studies in primary rat hepatocytes have also shown that insulin suppresses apoB secretion at least in part by stimulating degradation of freshly translated apoB and also by reducing apoB protein synthesis. It was suggested that reduced apoB synthesis was due to a decrease in translational efficiency (*10*). Additional studies in streptozotocin-induced diabetic rats have provided further evidence of apoB mRNA translational control. Decreased apoB synthesis is observed in the primary hepatocyte cultures derived from these diabetic rats and was believed to be due to a reduction in translational efficiency. This was found to be a result of impaired or slowed translation rates as determined by ribosome transit studies. Levels of apoB mRNA remained constant, and ribosomal transit studies determined that the decrease in translation was a result of delayed polypeptide elongation (*11*). Studies in cultured human fetal intestinal cells have also determined that the insulin-mediated suppression of apoB secretion may be mediated by co- and post-translational regulation, including mRNA translation (*12*). A recent study in HepG2 cells found that apoB synthesis decreased in response to treatment with CP-10447, an inhibitor of microsomal triglyceride transfer protein (MTP) (*13*). The decrease was attributed to a translational effect because apoB mRNA levels remained unchanged in response to the MTP inhibitor. The authors postulated that the decrease in apoB translation was due 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 Hospital for Sick Children Restracomp graduate scholarship, the BBDC-Novo Nordisk scholarship, and the Ontario Graduate Scholarship (OGS).

* To whom correspondence should be addressed: Division of Clinical Biochemistry, DPLM, Hospital for Sick Children, University of Toronto, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada. Telephone: 416-813-8682. Fax: 416-813-6257. E-mail: khosrow.adeli@sickkids.ca.

[1] Abbreviations: ALLN, *N*-acetyl-leucyl-leucyl-norleucinal; apoB, apolipoprotein B; CPEB, cytoplasmic polyadenylation element binding protein; DNA, deoxyribonucleic acid; cRNA, copy RNA; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; FP, free probe; mRNA, messenger RNA; PTP-1B; protein tyrosine phosphatase 1B; RNA, ribonucleic acid; SD, standard deviation; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SNP, single nucleotide polymorphism; SREBP, sterol regulatory element binding protein; UTR, untranslated region.

10.1021/bi050368e CCC: $30.25 
Published on Web 08/23/2005

delayed polypeptide elongation rates as determined by synchronization studies with puromycin and by ribosome transit studies (*13*). Overall, these studies suggest that apoB synthesis may be regulated at the level of translation. However, the molecular mechanisms or factors that mediate translational control of apoB mRNA have not been elucidated.

Sequence and structural elements localized within the 5′ and 3′ untranslated regions (UTRs) of mRNAs are believed to play a significant role in translational control, and such regions may contain cis-regulatory elements. These elements are thought to regulate mRNA translational efficiency and stability and act as binding sites for trans-acting protein factors (*14*). Recent investigation of the 5′ and 3′ UTR sequences of apoB mRNA (*15*) revealed elements with the potential to form a stable secondary structure, which in turn may mediate translational control of apoB mRNA translation. ApoB mRNA is 14 121 nucleotides long and its 5′ and 3′ UTR sequences are 128 and 301 nucleotides in length, respectively. The 5′ UTR sequence is comprised of 76% G and C nucleotides. GC-rich regions are known to have a high potential to form stable secondary structures (*16*). Studies have shown that highly structured 5′ UTR sequences tend to inhibit efficient translation (*16*, *17*). Analysis of the apoB 5′ UTR revealed the presence of two GC boxes with the typical CCGCCC sequence similar to promoter sequences of some eukaryotic genes (*18*). Results obtained from analysis of the 5′ UTR using the computer program M-fold predict a free energy of formation of specific structure in the range of −50.5 to −52.8 kcal/mol (*15*). A $\Delta G$ value in this range is indicative of the potential to form very stable secondary structures and would likely result in inefficient translation because of the inability of the ribosomal complex to unwind the secondary structure and reach the AUG codon (*16*, *17*). This suggests that the 5′ UTR should confer an inhibition of translation; however, this is not what we observed in our cell culture and *in vitro* experiments. Studies with both UTR-luciferase chimeric as well as UTR-apoB15 constructs clearly showed that 5′ UTR is a potent stimulator of apoB mRNA translation, while the 3′ UTR is inhibitory (*15*). This is in contrast to published literature showing inefficient translation of most mRNAs containing highly structured 5′ UTRs. However, a recent paper by Vivinus et al. (*19*) on the human heat-shock protein 70 (Hsp70), describes the first report of a 5′ UTR with a high degree of secondary structure that confers increased translational efficiency of Hsp70 mRNA translation. It is possible that the secondary structure present in the 5′ UTR of apoB mRNA is capable of being unwound by the presence of the initiation factor, eIF4A. Initiation factor eIF4A, upon activation by eIF4B, may unwind highly structured 5′ UTRs through its ATP-dependent RNA helicase activity and thus decrease the repression imposed by secondary structure (*20*). Thus, the apoB message may be translated according to the scanning model as is suggested for human Hsp70 mRNA translation (*21*). We further postulate that the stem-loop structures noted from the secondary structure predictions may be potential binding sites for eukaryotic initiation factors or other RNA-binding proteins whose function is to alleviate or relax the secondary structure present in the 5′ UTR. These protein factors may recruit translational machinery, unwind the secondary structure, or increase mRNA stability, thus contributing to efficient translation of the apoB message as reported for the 5′ UTR of p27 mRNA (*22*).

In the present study, we have investigated the molecular factors that mediate insulin-induced down-regulation of apoB mRNA translation via cis−trans interactions at the 5′ and 3′ UTRs. In addition to defining the cis-acting untranslated region involved in the translational regulation by insulin, we have identified trans-acting RNA-binding proteins interacting specifically with the 5′ and 3′ UTRs. Evidence is provided for an insulin-sensitive trans-acting factor that responds to insulin treatment and may mediate regulation of translational activity at the 5′ UTR.

## METHODS AND MATERIALS

*Cell Culture.* HepG2 cells were maintained in complete α-MEM (CellGrow). Complete α-*MEM* medium contained 5% fetal bovine serum (FBS) and a 1% antibiotic and antimycotic mix (CellGrow). COS-7 cells were maintained in DMEM medium containing 10% FBS. Cells were maintained in a Nuaire incubator at 37 °C under 95% air/5% $CO_2$. Cells were seeded into T-75 flasks, and media was replenished every 3 days. Cells were subcultured on a weekly basis usually after reaching 90% confluency.

*Transient Transfection Experiments.* Cos-7 cells were transfected with 1 $\mu$g each of the various UTR-apoB-15 constructs and PRL-Tk renilla (Promega) using Lipofectamine (Sigma). The cells were lysed 48 h post-transfection using solubilization buffer (phosphate-buffered saline containing 1% Nonidet P40, 1% deoxycholate, 5 mM EDTA, 1 mM EGTA, 2 mM PMSF, 0.1 mM leupeptin, and 2 $\mu$g/mL *N*-acetyl-leucinyl-leucinyl-norleucinal) and were passaged through a 25 G 5/8 syringe 7 times. HepG2 cells were transfected with 1 $\mu$g each of the various UTR-luciferase constructs and PRL-Tk renilla (Promega) using Fugene-6 (Roche). The cells were lysed 24 h post-transfection using passive lysis buffer (Promega) and lysed by orbital shaking for 15 min.

*Chemiluminescent Immunoblot Analysis.* Cell samples were subjected to chemiluminescent immunoblotting for apoB using 1D1 monoclonal antibody (kindly provided by Dr. Ross Milne, University of Ottawa). Samples were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) using 8% polyacrylamide minigels (8 × 5 cm). After SDS−PAGE, the proteins were transferred electrophoretically overnight at 4 °C onto poly(vinylidene difluoride) membranes using a Bio-Rad wet transfer system. The membranes were blocked with a 5% solution of fatfree dry milk powder, incubated with apoB antiserum, washed, and incubated with a secondary antibody conjugated to peroxidase (Amersham). Membranes were then incubated in an enhanced chemiluminescence detection reagent (Amersham Pharmacia Biotech) for 60 s and exposed to Kodak Hyperfilm (Eastman Kodak). Films were developed, and quantitative analysis was performed using an Imaging Densitometer. Blots were stripped and reprobed with $\beta$-actin antiserum, which was used as a loading control.

*Metabolic Radiolabeling of ApoB15.* COS-7 cells transfected with the UTR-apoB15 constructs were preincubated in methionine-free Dulbecco's minimum essential medium at 37 °C for 30 min or 1 h and labeled with 100 $\mu$Ci/mL [$^{35}$S]-methionine for 20 min, 1 or 2 days post-transfection.

Table 1: Primers Used in Cloning the ApoB 5′ and 3′ UTRs

| primer name | primer sequence |
|---|---|
| *Bam*HI-5′ UTR forward | GCGGAATTCCGCCAGCTGCGGTGGGGCGGCTCCTGGGCTGCGGC |
| *Eco*RI-5′ UTR reverse | GCGGAATTCCGCCAGCTGCGGTGGGGCGGCTCCTGGGCTGCGGC |
| 3′ UTR forward | GAATTCTTTTTTAAAAGAAATCTTCAT |
| 3′ UTR reverse | GGTACCTATGATACACAATAAAGACT |

After the labeling pulse, the cells were washed twice and lysed in solubilization buffer. The lysates were centrifuged for 10 min at 4 °C in a microcentrifuge (12 000 rpm), and supernatants were collected for immunoprecipitation.

*Immunoprecipitation, SDS−PAGE, and Fluorography.* Immunoprecipitation was performed as described previously (*23*, *24*) using goat anti-human apoB antibody. Immunoprecipitates were washed with wash buffer (10 mM Tris-HCl at pH 7.4, 2 mM EDTA, 0.1% SDS, and 1% Triton X-100) and prepared for SDS−PAGE by resuspension and boiling in 100 μL of Laemmli electrophoresis sample buffer. SDS−PAGE was performed essentially as described (*23*, *24*). 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. ApoB15 bands were excised from the gel and digested in hydrogen peroxide/perchloric acid, and associated radioactivity was quantified by liquid scintillation counting.

*Construction of KSplus-ApoB 5′ UTR and KSplus-ApoB 3′ UTR Vectors.* PCR of the 5′ and 3′ UTRs was conducted using the primers in Table 1. The PCR products were run on a gel to assess the PCR specificity. The 5′ UTR PCR product was digested with *Bam*HI and *Eco*RI, and the 3′ UTR PCR product was digested with *Xba*I. The restriction digest products were then run on a gel, and the 128-bp 5′ UTR band and the 301-bp 3′ UTR band were gel-purified using the Qiaquick Gel Extraction Kit (Qiagen). These were then ligated into digested and purifed pBluescript-II KSplus and T7 vectors (Stratagene) using their respective restriction sites.

*Construction of Chimeric ApoB UTR-Luciferase Vectors, UTR-ApoB15 Vectors, and T7 ApoB 5′ UTR/3′ UTR-Luciferase Vectors.* The chimeric apoB UTR-luciferase vectors and the UTR-apoB15 constructs were created as described previously (*15*). The apoB15 copy deoxyribonucleic acid (cDNA) was 2070 kb in size, and the protein contained 690 amino acids with an approximate molecular weight of 76 kDa. The T7 apoB 5′ UTR/3′ UTR-luciferase vectors were created by *Hin*dIII and *Bam*HI restriction digestion of the apoB UTR-luciferase vectors and subsequent directional ligation into the pBluescript-II SKminus − T7 vector (Stratagene).

*In Vitro Transcription of KSplus-ApoB 5′ UTR and KSplus-ApoB 3′ UTR Vectors.* The KSplus-apoB 5′ UTR and KSplus-apoB 3′ UTR vectors were linearized by digestion with *Eco*RI and transcribed using RiboProbe (Promega) and $^{32}$P-UTP for radiolabeled probe and RiboMAX (Promega) kit for the cold (unlabeled) probe as per the instructions of the manufacturer.

*In Vitro Transcription and Translation of ApoB 5′ UTR/3′ UTR-Luciferase Vectors.* The T7 SKminus apoB-UTR luciferase vectors were linearized by digestion with *Bam*HI and transcribed using RiboMAX (Promega) as per the instructions of the manufacturer. RNA was treated with RQ1 DNase (Promega) for 15 min. 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/construct) were *in vitro* translated using the Flexirabbit reticulocyte lysate kit (Promega) as per the instructions of the manufacturer in the presence and absence of 1 μg of HepG2 cytosol treated in the presence or absence of insulin. *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) was also added where indicated at a concentration of 10 μg/mL.

*Insulin Treatment of COS-7/HepG2 Cells.* COS-7 cells were transfected with the various UTR-apoB15 constructs. After 1 day of transfection, the cells were serum-depleted for 3 h and incubated in the presence (1 μg/mL) or absence of insulin for 16 h. Control and treated cells were harvested and used in the total protein measurements. HepG2 cells used in the preparation of cytosol were grown to 70% confluency using the standard culturing conditions mentioned. The cells were serum-depleted for 3 h and then treated in the presence (1 μg/mL) or absence of insulin for 16 h. HepG2 cells used in the luciferase transient transfections were treated in the presence (1 μg/mL) or absence of insulin for 4 and/or 16 h.

*Preparation of HepG2 Liver Cystosolic Extracts.* Cells were washed once with 1 mL of 250 mM sucrose and 3 mM of imidazole at pH 7.4 and once with 2 mL of 50 mM sucrose and 3 mM imidazole at pH 7.4. Cells were then scraped into 500 μL of 50 mM sucrose and 3 mM imidazole at pH 7.4 and homogenized using a Dounce homogenizer on ice with 20 strokes. A total of 50 μL 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-45 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 at pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, and 100 KIU/mL of trazylol solution.

*Electromobility Shift Assay (EMSA). In vitro* hybridization was performed with 20 μg of cytoplasmic lysate incubated with $5 \times 10^3-10^4$ cpm of apoB UTR RNA in a 20 μL of reaction consisting of 10% glycerol, 12 mM HEPES at 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 min at 30 °C, essentially as described (*25*). A total of 50 units of RNase T1 was added, and reaction mixtures were incubated for 30 min at 37 °C prior to electrophoresis in a 6% native polyacrylamide gel with 0.5× Tris borate-EDTA running buffer. After electrophoresis, gels were dried and exposed to X-ray film for identification of probe−protein complexes. In some experiments, a nonspecific competitor such as yeast

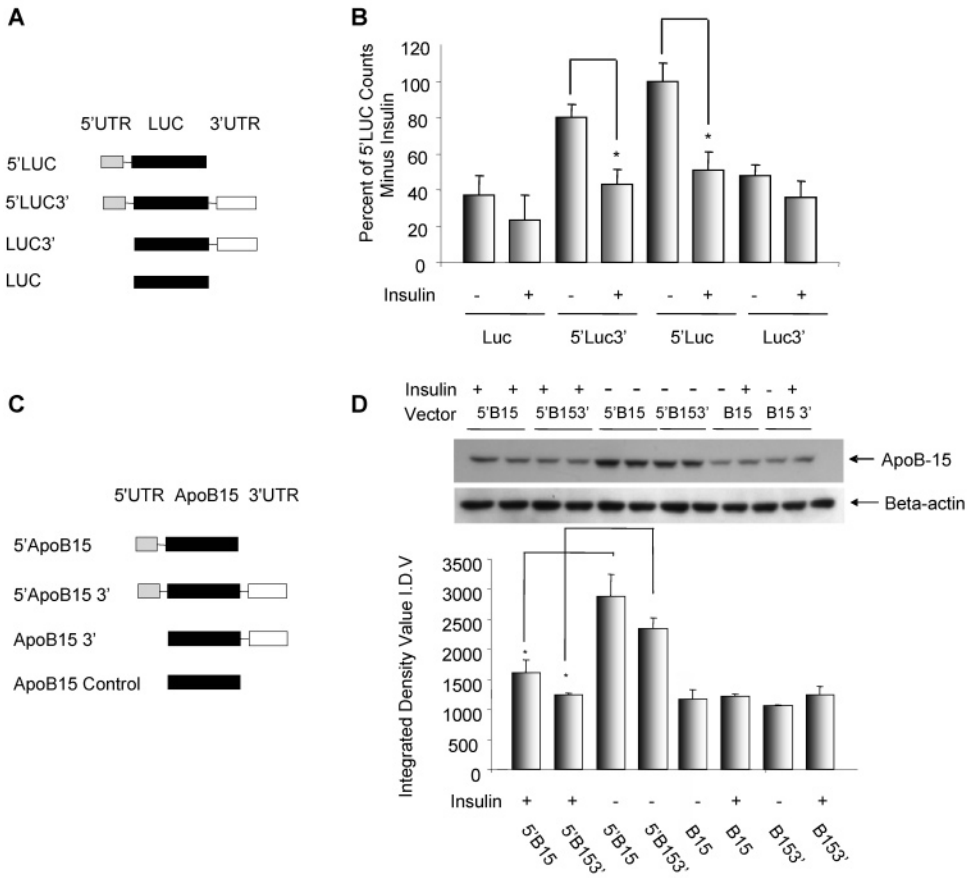


FIGURE 1: Effect of the apoB 5′ and 3′ UTR sequences on expression of luciferase reporter constructs or constructs carrying apoB15, in the presence and absence of insulin. HepG2 cells were transiently cotransfected with the chimeric apoB UTR-LUC constructs (A) and the pRL-TK (renilla LUC) vector (see the Materials and Methods). Cells were treated in the presence or absence of insulin for 16 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 mean ± standard deviation (SD) (four separate experiments in triplicate). UTR-apoB15 constructs carrying the 5′ and/or 3′ UTR of apoB mRNA were constructed by cloning the 5′ and 3′ UTR sequences into the pGL3 control vector (with the LUC gene removed (C). Cos-7 cells were transfected with the UTR-apoB15 constructs as described (see the Methods and Materials) and treated in the absence or presence of insulin for 16 h. Total apoB15 protein mass and $\beta$-actin were assessed by immunoblotting and densitometric analysis (D). Data shown are mean ± SD (three separate experiments in triplicate). $\beta$-Actin was employed as a loading control.

tRNA (4 $\mu$g) was added to the protein extract and incubated for 10 min before the addition of labeled apoB UTR probe. For specific competition experiments, a large molar excess of unlabeled apoB UTR RNA was preincubated for 10 min with protein before addition of the labeled apoB UTR probe.

*UV Cross-Linking Assay.* To identify the molecular weights of RNA−protein complexes, probe−protein reactions were set up as for the electromobility assay. After incubation at 30 °C, samples were exposed to UV light at 3000 mW/cm$^2$ for 15 min on ice. RNase T1 and/or RNase A digestion was then performed for 30 min; Laemmli sample buffer was added; samples were boiled for 5 min and were electrophoresed on 10% SDS−PAGE. Gels were dried and processed for autoradiography as with EMSA experiments.

## RESULTS

*Effect of 5′ and 3′ UTRs on Luciferase Reporter Expression in the Presence and Absence of Insulin.* We conducted luciferase reporter expression experiments in HepG2 cells transfected with apoB UTR-luciferase constructs and control renilla luciferase vectors (Figure 1A). Cells were treated in the presence or absence of insulin for 16 h, 4 h post-transfection. As illustrated in Figure 1B, insulin treatment significantly reduced luciferase activity in cells tranfected with constructs containing the 5′ UTR (either the 5′ UTR-apoB15 or 5′ UTR-apoB15-3′ UTR constructs). Cells transfected with apoB 5′ UTR luciferase constructs showed approximately 50% lower levels of luciferase activity in the presence of insulin, which was statistically significant (Figure 1B, $p < 0.05$). Insulin treatment did not affect synthesis of luciferase in cells transfected with either the control luciferase construct (containing no UTRs) or the apoB 3′ UTR-luciferase construct in the presence or absence of insulin. Luciferase activity was normalized to renilla activity as a transfection control.

*Effect of 5′ and 3′ UTRs on ApoB15 Protein Expression in the Presence and Absence of Insulin Changes in the Total ApoB Protein Mass.* To further assess the effect of insulin on the function of 5′ and 3′ UTRs in modulating translational control of the apoB mRNA, we employed a series of constructs containing apoB15 (N terminal 15% of full-length apoB) linked to the apoB 5′ and/or 3′ UTRs (Figure 1C) transfected into Cos-7 cells. The expressed protein level of apoB15 was examined by immunoblot analysis. Figure 1D shows the relative expressed protein levels of apoB15 in Cos-7 cells transfected with various constructs and treated

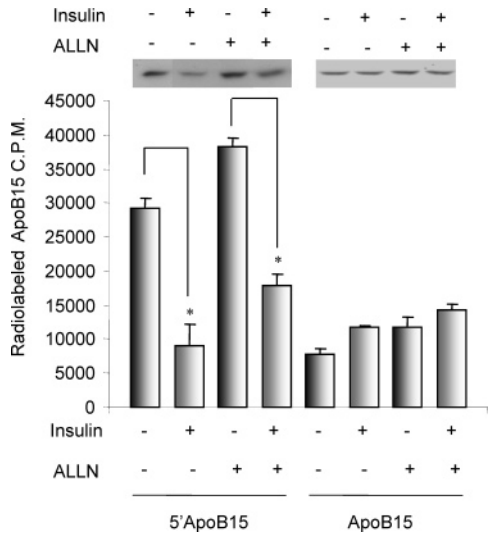


FIGURE 2: Effect of the apoB 5′ and 3′ UTR sequences on apoB15 protein synthesis in the presence and absence of insulin and protease inhibitors. Cos-7 cells were cotransfected with the pRL-TK (renilla LUC) vector and either the 5′ UTR-apoB15 or the apoB15 control construct (see the Materials and Methods). Cells were preincubated with insulin for 4 h and ALLN for 2 h prior to radiolabeling with [$^{35}$S] for 20 min. ApoB15 was immunoprecipitated from cells, and the protein was analyzed by SDS−PAGE. The radiolabeled apoB15 protein was excised from the gel and quantified by liquid scintillation counting. Data shown are mean ± SD (three separate experiments in triplicate).

with or without insulin after 16 h of treatment. A significantly higher level of apoB15 protein was expressed in cells transfected with constructs containing the 5′ UTR, while the 3′ UTR was found to be inhibitory in the absence of insulin. Insulin treatment of Cos-7 cells caused a significant (2-fold, $p < 0.05$) decrease in the protein mass of apoB15 in cells transfected with the 5′ UTR-apoB15 construct. Expression of the 5′ UTR-apoB15-3′ UTR construct was also sensitive to insulin, such that there was a 2-fold decrease in expressed apoB15 protein mass in the presence of insulin ($p < 0.05$). The 5′ UTR-apoB15 construct did not have a significantly higher protein mass than the 5′ UTR-apoB15-3′ UTR construct in the presence or absence of insulin; however, there was a trend toward higher protein mass in the 5′ UTR only construct. Expression of constructs carrying only the 3′ UTR or no UTR was insensitive to insulin treatment because no significant change in apoB15 protein mass was observed in the presence or absence of insulin. These data suggest that insulin-mediated modulation of apoB mRNA expression may be mediated by the apoB 5′ UTR. Membranes were also stripped and reprobbed for $\beta$-actin, which acted as a loading control.

*Effect of the 5′ UTRs on ApoB15 Protein Expression in the Presence and Absence of Insulin Changes in Radiolabeled ApoB Protein.* To assess the effect of insulin on the synthetic rate of apoB protein, we conducted [$^{35}$S]-radiolabeling experiments in cells transfected with the 5′ UTR-apoB15 and apoB15 control constructs and treated in the presence or absence of insulin for 4 h. Cells were then pulsed for 20 min in the presence or absence of insulin. As illustrated in Figure 2, insulin treatment significantly ($p < 0.05$) reduced radiolabeled apoB15 protein levels in cells tranfected with the 5′ UTR-apoB15 construct. Insulin treatment did not affect synthesis of apoB15 control construct (containing no UTR) in the presence or absence of insulin. Addition of the protease inhibitor, ALLN (10 $\mu$g/mL), 2 h prior to the pulse, did not alter the effect of insulin on apoB15 synthesis, although it increased the basal level of labeled apoB15. Cotransfection with renilla luciferase was used to control for transfection efficiency.

*Insulin-Mediated Inhibition of in Vitro Translation of 5′ UTR-Luciferase Copy RNA (cRNA).* To more directly assess the effect of insulin on the translational control of apoB mRNA, we conducted *in vitro* translation experiments using synthetic/purified cRNA. A total of 1 $\mu$g of RNA produced by *in vitro* transcription of each of the T7 SKminus apoB UTR-luciferase vectors was added to rabbit reticulocyte lysate in the presence and absence of 1 $\mu$g of HepG2 cytosol (isolated from cells treated in the presence or absence of insulin for 13 h). The luciferase activity for each cRNA translated in the presence of insulin-treated cytosol was normalized to its untreated (minus insulin) control (which was taken as 100%). Addition of insulin-treated HepG2 cytosol reduced the rate of translation of the apoB 5′ UTR-luciferase-3′ UTR construct by approximately 40%, $p < 0.05$ (Figure 3). Similarly, translation of the apoB 5′ UTR-luciferase construct was significantly reduced in the presence of insulin, by approximately 45% ($p < 0.05$). However, treatment with insulin did not significantly alter translation of the control luciferase construct lacking apoB UTRs or the luciferase apoB 3′ UTR construct.

*Nondenaturing EMSA: The ApoB 5′ UTR.* EMSA assays were performed to explore potential cis−trans interactions that mediate the translational regulation of apoB mRNA via the UTR sequences. Using nondenaturing EMSA and HepG2 cytosol, two protein complexes were found to bind to the 5′ UTR RNA as shown in Figure 4A (lane 2). Increasing probe and protein concentrations increased the amount of 5′ UTR RNA−protein complexes formed (lanes 2−5). Lane 1, which contained the probe incubated in the absence of cytosol, did not show the two larger protein complexes resolved in the presence of cytosol.

*Nondenaturing EMSA: The ApoB 3′ UTR.* EMSA was also carried out using HepG2 cytosol to identify potential trans-acting protein factors binding to the 3′ UTR sequences of apoB mRNA. As shown in Figure 4B, nondenaturing EMSA showed the presence of two protein complexes binding to the 3′ UTR RNA (lane 2). Increasing probe and protein concentrations increased the amount of 3′ UTR RNA−protein complexes formed (lanes 2−5). Lane 1, which contained the probe incubated in the absence of cytosol, did not show the two larger complexes resolved in the presence of cytosol. A very low molecular-weight band was visualized that may be the riboprobe bound to enzymes that were added to the experiment such as RNaseT1 and RNase inhibitor. Another possibility is that this represents a dimeric form of the riboprobe or an alternate secondary structure.

*Effect of Insulin on Nondenaturing EMSA of the ApoB 5′ UTR.* To determine the effect of insulin on protein complex formation at the apoB 5′ UTR, we used cytosolic extracts from HepG2 cells that were either treated in the presence or absence of insulin for 16 h in DMEM media or treated with complete medium consisting of DMEM supplemented with 10% FBS (F). Nondenaturing EMSA (Figure 5A) showed that there were two protein complexes binding to the 5′ UTR in the presence and absence of insulin. Formation of these

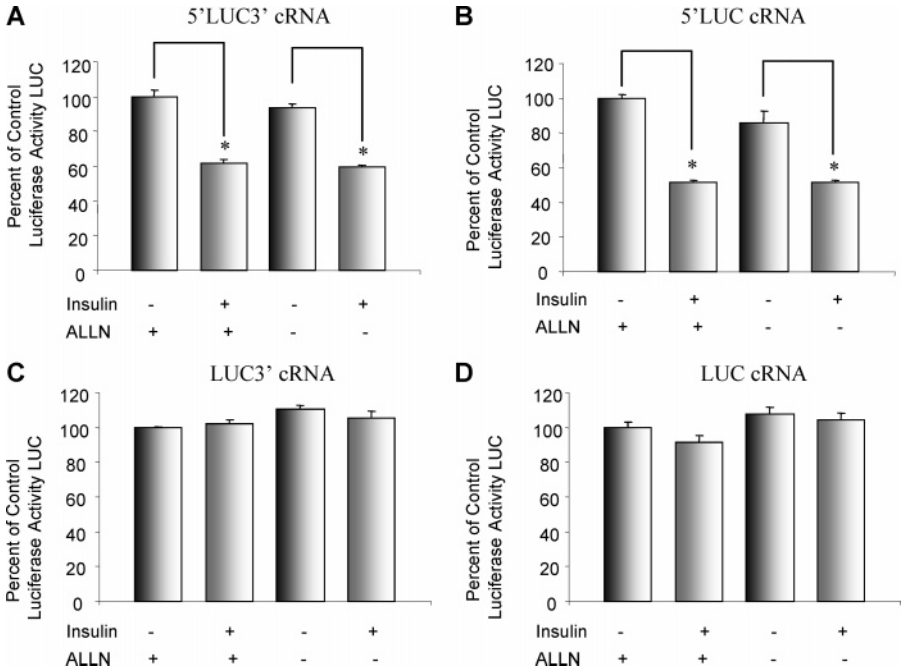


FIGURE 3: *In vitro* translation of cRNAs carrying apoB-5′ and/or 3′ UTRs in the presence and absence of insulin-treated HepG2 cytosol. T7 UTR-luciferase constructs carrying the 5′ and/or 3′ UTR of apoB mRNA were constructed by cloning the 5′ and 3′ UTR sequences with the luciferase gene into the pBluescript II-SK- vector, which contains the T7 promoter. RNA was *in vitro* transcribed from linearized T7 UTR-luciferase constructs. A total of 1 $\mu$g of each Sephadex G-50 purified RNA construct was *in vitro* translated in the rabbit reticulocyte lysate incubated in the presence or absence of 1 $\mu$g of HepG2 cytosol treated with or without insulin (see the Materials and Methods). The protease inhibitor, ALLN was added to some of the *in vitro* translation reactions. The luciferase activity for each cRNA translated in the presence of insulin-treated cytosol was normalized to its untreated (minus insulin) control (which was taken as 100%). (A−D). Data shown are mean ± SD (four separate experiments in triplicate).

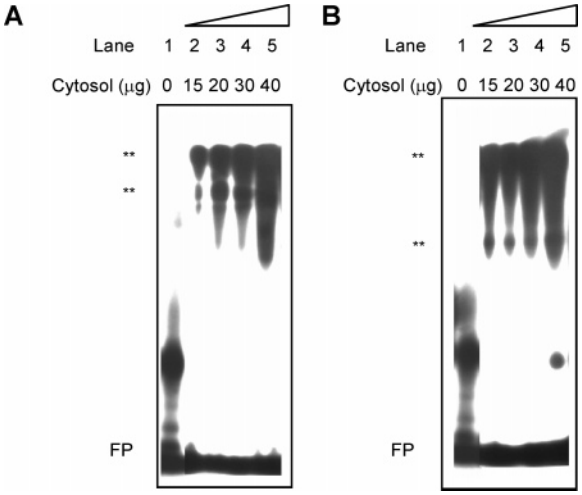


FIGURE 4: Analysis of cis−trans interactions at the 5′ and 3′ UTRs of apoB mRNA. HepG2 cytosolic S100 extracts were incubated in the presence of $^{32}$P-labeled apoB riboprobes (cRNAs) containing the 5′ or 3′ UTR and then run under nondenaturing conditions. Protein complexes were visualized as binding to the 5′ UTR (A) and the 3′ UTR (B) under nondenaturing conditions. Complex formation was monitored with the apoB UTR riboprobes without protein extract (lane 1) or in the presence of 15 $\mu$g (lane 1), 20 $\mu$g (lane 2), 30 $\mu$g (lane 3), or 40 $\mu$g (lane 4) of cytosol. ** indicates RNA with complexed proteins (A and B). FP denotes the free probe.

complexes was not prevented by preincubation with nonspecific yeast tRNA competitor (lanes 1−3) but was inhibited by preincubation with a 100-fold molar excess of unlabeled 5′ UTR cold specific competitor: standard conditions (F) with 100× cold competitor (lane 4), the presence of insulin with 100× cold competitor (lane 5), and the absence of insulin with 100× cold competitor (lane 6).

*Effect of Insulin on Denaturing EMSA of the ApoB 5′ UTR.* To assess modulation of protein complex formation at apoB 5′ UTR RNA, we used denaturing EMSA conditions and incubated the 5′ UTR riboprobe with cytosolic extracts from HepG2 cells treated in the presence or absence of insulin for 16 h, illustrated in Figure 5B.

The specific apoB UTR RNA−protein complexes identified in the previous experiments were resolved on denaturing PAGE, to determine the number of proteins directly binding to the UTR and their molecular weights. We performed binding reactions under similar conditions as before but followed the *in vitro* hybridization by exposure of the probe−protein reactions to UV light. After RNase A/T1 digestion, the complexes were resolved on denaturing SDS polyacrylamide gels. Nonspecific competitor (tRNA) was not included in the reactions. With riboprobe incubated without cytosol (lane 1), only the free probe was resolved and did not yield any shift to slower migrating bands. In the presence of cytosol, there was a band visualized that ran at approximately 110 kDa. This protein appeared to bind at a higher efficiency in the absence of insulin treatment (lanes 3 and 5) and at a lower amount in the presence of insulin stimulation (lanes 2 and 4). Other bands were visualized running lower than the 110-kDa protein. These were determined to be approximately 35, 55, and 65 kDa in size, respectively. As shown in lane 6, no bands were visible without cytosol or riboprobe being present.

To test for the specificity of the 110-kDa interaction, we repeated the experiment with 5′ UTR cold competitor and nonspecific competitor (Figure 5C). Results were similar to the previous experiment, with increased binding of the 110-kDa protein in the absence of insulin (lane 1). Similarly, the

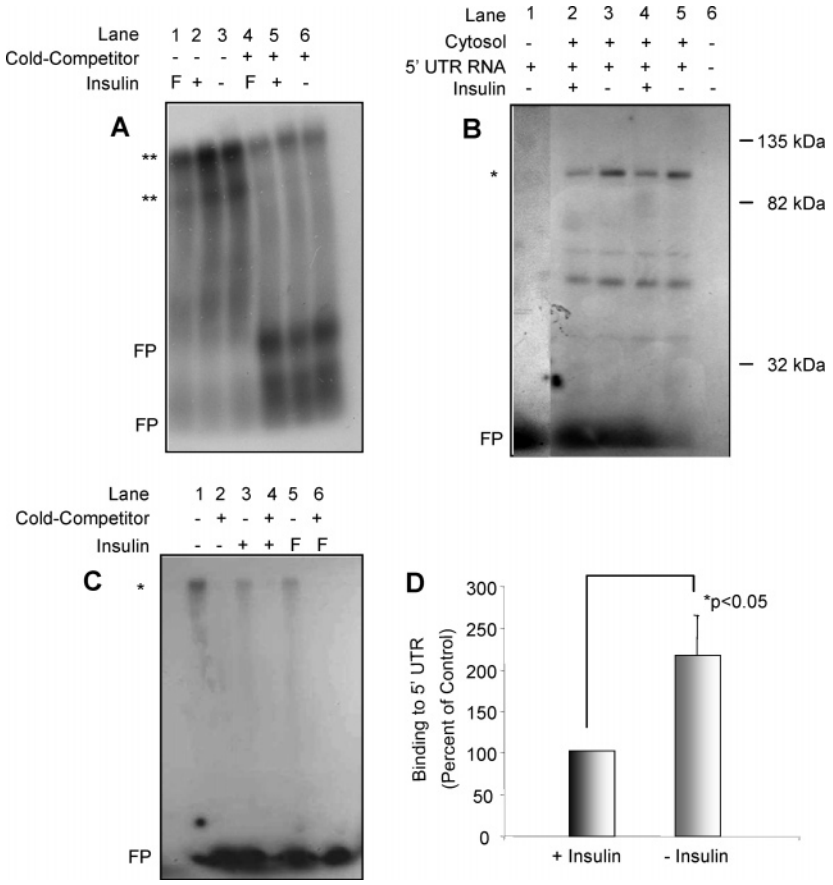


FIGURE 5: Effect of insulin on binding of trans-acting proteins to the 5′ UTR. Binding of protein complexes to the 5′ UTR and their sensitivity to insulin treatment were assessed. (A) Complex formation between cytoplasmic proteins and apoB UTR riboprobe was analyzed by native nondenaturing PAGE with cytosol isolated from HepG2 cells grown with FBS (F, cells treated with FBS) for 16 h post-transfection (lane 1), HepG2 cytosol treated with insulin for 16 h (+) (lane 2), or HepG2 cytosol untreated (absence of insulin) (−) for 16 h (lane 3). To assess the specificity of binding, cytosol from each reaction was preincubated with 100-fold excess cold competitor before the addition of riboprobe. Lane 4, cytosol from cells grown in complete serum (F); Lane 5, cytosol from cells treated with insulin for 16 h; Lane 6, cytosol from cells starved of insulin for 16 h. (B) Denaturing EMSA of the 5′ UTR riboprobe. Complex formation between cytoplasmic protein extract and apoB UTR riboprobe was monitored by SDS−PAGE after RNase A/T1 digestion and exposure to UV light. Riboprobe was treated without cytosol (lane 1), incubated with cytosol isolated from HepG2 cells treated with insulin (lanes 2 and 4), or incubated with cytosol isolated from HepG2 cells starved of insulin (lanes 3 and 5). Lane 6 contained no riboprobe or cytosol. (C) Specificity of the insulin-sensitive binding of the 110-kDa RNA-binding protein to the 5′ UTR was tested in the presence of the cold competitor. Binding reactions with untreated cytosol (lane 1), cytosol from cells treated with insulin for 16 h (lane 3), or cytosol treated with FBS (F) for 16 h (lane 5). To test for specificity of binding, reactions were preincubated with 1000-fold excess of specific 5′ UTR apoB cold competitor (lanes 2, 4, and 6). (D) Quantitative analysis was performed using an imaging densitometer to assess for differences in binding activity of the 110-kDa factor in the presence or absence of insulin. Data plotted from imaging densitometric analysis of three separate experiments in triplicate (shown as mean ± SD; $p < 0.05$). * indicates RNA−protein binding observed in denaturing gels; ** indicates RNA−protein complexes observed in nondenaturing gels; FP indicates the free RNA probe.

110-kDa protein showed decreased binding in the presence of insulin (lane 3). In the presence of complete media (F), there was a moderate amount of binding of the 110-kDa RNA-binding protein factor to the 5′ UTR (lane 5). Binding of the 110-kDa protein was not prevented by preincubation with nonspecific yeast tRNA competitor (4 $\mu$g; lanes 1, 3, and 5) but was inhibited by preincubation with a 1000-fold molar excess of unlabeled 5′ UTR cold specific competitor (lanes 2, 4, and 6). Furthermore, the lower molecular mass proteins visualized in the previous experiment (Figure 5B) were not detected.

To quantify the change in binding in the presence or absence of insulin, we conducted densitometric analysis of the bands from several experiments (Figure 5D). In the absence of insulin, there was a greater than 2-fold increase in the binding of the 110-kDa protein over insulin-treated conditions.

*Effect of Insulin on cis−trans Interactions at the 3′ UTR.* Using denaturing EMSA conditions, we incubated the 3′ UTR riboprobe with HepG2 cytosol treated or depleted of insulin for 16 h, illustrated in Figure 6A. Three major proteins were visualized to bind to the 3′ UTR RNA. The largest band had a size of approximately 75 kDa, with two other bands at 40 and 30 kDa. To quantify the change in binding in the presence or absence of insulin, we conducted densitometric analysis of the bands. There was no significant difference in band intensity of the 75-kDa (Figure 6B), 45-kDa (Figure 6C), or 30-kDa (Figure 6D) proteins in the presence or absence of insulin.

## DISCUSSION

Previous studies indicate that hepatic apoB secretion is decreased in response to insulin treatment; however, mRNA levels remain stable in response to insulin (3−11), suggesting

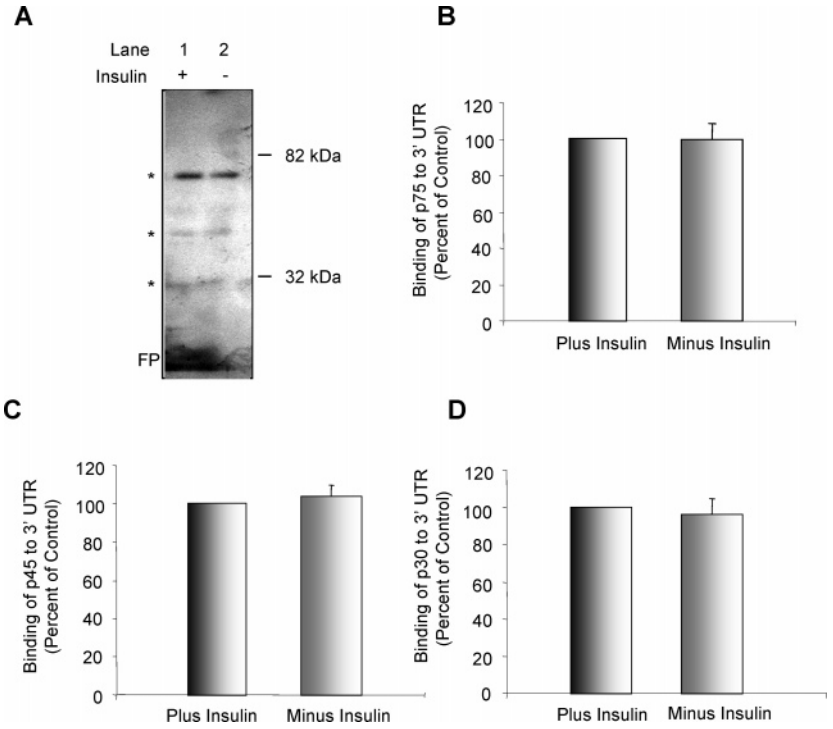


FIGURE 6: Effect of insulin on binding of trans-acting proteins to the 3′ UTR. The 3′ UTR riboprobe was incubated with HepG2 cytosol from cells treated with insulin for 16 h (lane 1) or cytosol from untreated, control cells (lanes 2). Denaturing SDS−PAGE, UV cross-linking and RNase A/T1 digestion visualized three bands binding at 75, 40, and 30 kDa (A). Quantitative analysis was performed using an imaging densitometer to assess for differences in binding activity. Using the insulin-treated condition as a control, we quantified the amount of increase in binding, following starvation for insulin. Data plotted from imaging densitometric analysis of three separate experiments performed in triplicate (shown as mean ± SD). * indicates an RNA−protein complex, and FP indicates the free RNA probe.

translational and/or post-translational control mechanisms. Recent studies using luciferase reporter constructs carrying apoB 5′ and 3′ UTR sequences have shown that the 5′ UTR is important for efficient translation of the apoB message, whereas the 3′ UTR is inhibitory (*15*). In addition, *in vitro* translation studies using cellular mRNA further confirmed that UTR sequences exert their effects at the level of mRNA translation/protein synthesis (*15*).

In the studies described here, we investigated which untranslated region was necessary and sufficient for the insulin-mediated repression of apoB mRNA translation and whether differences in binding of trans-acting factors to the 5′ and 3′ cis-acting UTR elements during insulin stimulation may be responsible for these effects. Using immunoblot and pulse-radiolabeling experiments, we found that the 5′ UTR is necessary and sufficient to transmit insulin's effect on apoB mRNA translation in Cos-7 cells because only constructs containing the 5′ UTR showed a decrease in apoB15 synthesis in response to insulin treatment. Furthermore, inhibition of protein degradation by ALLN (or MG132, data not shown) did not alter the effect of insulin on newly synthesized apoB15 (pulse-labeling experiments) or apoB15 mass (immunoblot experiments). Lack of an appreciable effect of these protease inhibitors on apoB15 accumulation may be related to the considerably higher stability of this shorter apoB construct compared to the full-length apoB100 (*26*). Similar observations were made in HepG2 cells. Insulin suppressed the translation of 5′ UTR-containing luciferase constructs transfected in HepG2 cells. Further confirmation for the inhibitory effect of insulin on 5′ UTR activity came from the use of UTR-LUC cRNAs synthesized *in vitro* and translated in rabbit reticulocyte lysate. Addition of insulin-treated cytosol significantly inhibited the translation of 5′ UTR-containing cRNAs (5′ LUC RNA and 5′ 3′ LUC RNA). Under these conditions, control LUC RNA and LUC 3′ RNA were found to be insensitive to translational inhibition with insulin-treated cytosol.

Using nondenaturing EMSAs, we were able to identify two large complexes of proteins binding to the 5′ UTR. Also, two relatively smaller complexes bound to the 3′ UTR. These trans-acting complexes of proteins may be responsible for the cis-acting effects of the UTRs on translation, specifically the stimulatory effect of the 5′ UTR and the inhibitory effect of the 3′ UTR. The denaturing UV-cross-linking EMSA experiments revealed the number and size of the trans-acting factors binding directly to the apoB UTR-RNA. UV-cross-linking experiments conducted with the 5′ UTR identified a trans-acting factor that ran between the 85- and 135-kDa markers with an approximate molecular mass of 110 kDa. Binding of this factor was specific because it could not be out-competed by excess nonspecific cold tRNA competitor but was out-competed by excess specific cold competitor. Further UV-cross-linking experiments with the 3′ UTR indicated the presence of three trans-acting factors binding to the 3′ UTR with sizes of approximately 75, 45, and 30 kDa. These factors were out-competed by specific competitor, and later experiments indicated that the binding of these factors was not affected by the nonspecific competitor. Proteinase K digestion of cytosol prior to *in vitro* hybridization and EMSA abolished the 110-kDa band, suggesting that the factor binding to the 5′ UTR was indeed a protein factor (data not shown).

The effect of insulin on such cis−trans interactions involving the 5′ and 3′ UTRs was then investigated by

conducting RNA-EMSA with cytosol from control and insulin-treated HepG2 cells. Binding of the 110-kDa 5′ UTR trans-acting factor (p110) was significantly increased (greater than 2-fold; $p < 0.05$) in the absence of insulin. In contrast, the binding of the 75-, 45-, and 30-kDa trans-acting proteins to the 3′ UTR was unaltered in the presence or absence of insulin. These data correspond with our UTR-deletion construct studies and confirm that the 5′ UTR is necessary for the effect of insulin on apoB mRNA translation. The p110 RNA-binding protein may thus be an insulin-sensitive factor mediating the suppressive effect of insulin on apoB mRNA translation. Another possibility is that the apoB UTR may act as an internal ribosomal entry site (IRES), because it is translated at a higher rate in the absence of the general translation activator, insulin.

The data suggests that the apoB 5′ UTR is both necessary and sufficient for the insulin-mediated repression of apoB mRNA translation via a mechanism involving the decreased binding of an insulin-sensitive 110-kDa RNA-binding protein to the 5′ UTR. In certain pathologic states, this mechanism may play an important role in regulating apoB synthesis and thus the production of atherogenic, apoB-containing VLDL particles by the liver. For instance, alteration in the sensitivity and signaling of insulin as observed in insulin-resistant states such as obesity and type-2 diabetes may potentially lead to chronic binding of p110 and thus increased activation of mRNA translation, resulting in VLDL overproduction, a metabolic change commonly observed in diabetic/metabolic dyslipidemia. Furthermore, single nucleotide polymorphisms (SNPs) in the UTR regions are known to affect binding of the UTR trans-acting factors leading to altered cis−trans interactions with important pathological consequences (*27*). A 5′ UTR SNP in sterol response element binding protein (SREBP) was shown to increase the risk of atherosclerosis, and a 3′ UTR SNP in protein tyrosine phosphatase 1-B (PTP-1B) was found to enhance susceptibility to insulin resistance (*28*, *29*). We also attempted a computerized SNP analysis of apoB UTR sequences using the Pharmacogenetics and Risk for Cardiovascular Disease (PARC) website (*30*). SNP analysis revealed the presence of a SNP at residue 14 of the apoB 5′ UTR, which is near stem loop I. A SNP was also present 180 nucleotides from the beginning of the 3′ UTR. There may be other SNPs/polymorphisms of the 5′ and/or 3′ UTRs that have not been determined yet may affect binding of UTR-binding proteins and alter apoB mRNA translation.

We also found that the 3′ UTR was inhibitory to translation of the apoB mRNA and that the identified trans-acting 3′ UTR-specific binding factors may be the source of this constitutive inhibition. Although translation of apoB mRNA was decreased in the presence of insulin, we did not observe any change in the binding of these factors to the 3′ UTR. Therefore, we hypothesize that the 3′ UTR factor(s) may bind to the 3′ UTR at a constant rate and function to generally down-regulate its translation, causing an inhibition of translational initiation. Cytoplasmic polyadenylation element binding proteins (CPEBs) have been shown to be linked to translational repression (*31*). It has been shown previously that CPEs may repress translation before directing polyadenylation (*32*, *33*). Furthermore, the size (∼70 kDa) of one of our 3′ UTR binding proteins corresponds closely with known CPEBs.

In conclusion, our studies have revealed novel mechanisms that appear to mediate the translational repression of apoB mRNA induced by insulin. Further investigations are currently underway to positively identify the nature of the 110-kDa RNA-binding protein interacting with the 5′ UTR. Identification of this protein factor and its function may lead to a greater understanding of co-translational regulation of apoB and eventually to the development of novel drug targets in the treatment of dyslipidemia and its cardiovascular complications.

## REFERENCES


1. Fisher, E. A., and Ginsberg, H. N. (2002) Complexity in the secretory pathway: The assembly and secretion of apolipoprotein B-containing lipoproteins, *J. Biol. Chem. 20*, 17377−17380.
2. Sparks, J. D., and Sparks, C. E. (1985) Apolipoprotein B and lipoprotein metabolism, *Adv. Lipid Res. 21*, 1−46.
3. Adeli, K., and Sinkevitch, C. (1990) Secretion of apolipoprotein B in serum-free cultures of human hepatoma cell line, HepG2, *FEBS Lett. 2*, 345−348.
4. Theriault, A., Cheung, R., and Adeli, K. (1992) Expression of apolipoprotein B *in vitro* in cell-free lysates of HepG2 cells: Evidence that insulin modulates apoB synthesis at the translational level, *Clin. Biochem. 25*, 321−324.
5. Adeli, K., and Theriault, A. (1992) Insulin modulation of human apolipoprotein B mRNA translation: Studies in an *in vitro* cell-free system from HepG2 cells, *Biochem. Cell Biol. 70*, 1301−1308.
6. Theriault, A., Ogbonna, G., and Adeli, K. (1992) Thyroid hormone modulates apolipoprotein B gene expression in HepG2 cells, *Biochem. Biophys. Res. Commun. 186*, 617−622.
7. Mohammadi, A., Theriault, A., and Adeli, K. (1996) *In vitro* translation and translocation of apolipoprotein B in a cell-free system from HepG2 cells, *Biochem. Biophys. Res. Commun. 228*, 852−858.
8. Dashti, N., Williams, D. L., and Alaupovic, P. (1989) Effects of oleate and insulin on the production rates and cellular mRNA concentrations of apolipoproteins in HepG2 cells, *J. Lipid Res. 9*, 1365−1373.
9. Pullinger, C. R., North, J. D., Teng, B. B., Rifici, V. A., Ronhild de Brito, A. E., and Scott, J. (1989) The apolipoprotein B gene is constitutively expressed in HepG2 cells: Regulation of secretion by oleic acid, albumin, and insulin, and measurement of the mRNA half-life, *J. Lipid Res. 7*, 1065−1077.
10. Sparks, J. D., and Sparks, C. E. (1990) Insulin modulation of hepatic synthesis and secretion of apolipoprotein B by rat hepatocytes, *J. Biol. Chem. 265*, 8854−8860.
11. Sparks, J. D., Zolfaghari, R., Sparks, C. E., Smith, H. C., Fisher, E. A. (1992) Impaired hepatic apolipoprotein B and E translation in streptozotocin diabetic rats, *J. Clin. Invest. 89*, 1418−1430.
12. Levy, E., Sinnett, D., Thibault, L., Nguyen, T. D., Delvin, E., and Menard, D. (1996) Insulin modulation of newly synthesized apolipoproteins B-100 and B-48 in human fetal intestine: Gene expression and mRNA editing are not involved, *FEBS Lett. 393*, 253−258.
13. Pan, M., Liang, J., Fisher, E. A., and Ginsberg, H. N. (2000) Inhibition of translocation of nascent apolipoprotein B across the endoplasmic reticulum membrane is associated with selective inhibition of the synthesis of apolipoprotein B, *J. Biol. Chem. 275*, 27399−27405.
14. McCarthy, J. E., and Kollmus, H. Cytoplasmic (1995) mRNA−protein interactions in eukaryotic gene expression, *Trends Biochem. Sci. 20*, 191−197.
15. Pontrelli, L., Sidiropoulos, K. G., and Adeli, K. (2004) Translational control of apolipoprotein B mRNA: Regulation via cis elements in the 5′ and 3′ untranslated regions, *Biochemistry 43*, 6734−6744.
16. Kozak, M. (1991) Structural features in eukaryotic mRNAs that modulate the initiation of translation, *J. Biol. Chem. 266*, 19867−19870.
17. Kozak, M. (1991) An analysis of vertebrate mRNA sequences: Intimations of translational control, *J. Cell Biol. 4*, 887−903.
18. Blackhart, B. D., Ludwig, E. M., Pierotti, V. R., Caiati, L., Onasch, M. A., Wallis, S. C., Powell, L., Pease, R., Knott, T. J., Chu, M.

L., et al. (1986) Structure of the human apolipoprotein B gene, *J. Biol. Chem. 261*, 15364−15367.
19. Vivinus, S., Baulande, S., van Zanten, M., Campbell, F., Topley, P., Ellis, J. H., Dessen, P., and Coste, H. (2001) An element within the 5′ untranslated region of human Hsp70 mRNA which acts as a general enhancer of mRNA translation, *Eur. J. Biochem. 7*, 1908−1917.
20. Jaramillo, M., Browning, K., Dever, T. E., Blum, S., Trachsel, H., Merrick, W. C., Ravel, J. M., and Sonenberg, N. (1990) Translation initiation factors that function as RNA helicases from mammals, plants, and yeast, *Biochim. Biophys. Acta 1−3*, 134−139.
21. Kozak, M. (1989) The scanning model for translation: An update, *J. Cell Biol. 108*, 229−241.
22. Millard, S. S., Vidal, A., Markus, M., and Koff, A. (2000) A U-rich element in the 5′ untranslated region is necessary for the translation of p27 mRNA, *Mol. Cell Biol. 20*, 5947−5959.
23. Adeli, K. (1994) Regulated intracellular degradation of apolipoprotein B in semipermeable HepG2 cells, *J. Biol. Chem. 12*, 9166−9175.
24. Macri, J., and Adeli, K. (1997) Conformational changes in apolipoprotein B modulate intracellular assembly and degradation of apoB-containing lipoprotein particles in HepG2 cells, *Arterioscler. Thromb. Vasc. Biol. 11*, 2982−2994.
25. Rondon, I. J., MacMillan, L. A., Beckman, B. S., Goldberg, M. A., Schneider, T., Bunn, H. F., and Malter, J. S. (1991) Hypoxia up-regulates the activity of a novel erythropoietin mRNA binding protein, *J. Biol. Chem. 25*, 16594−16598.
26. Cavallo, D., McLeod, R. S., Rudy, D., Aiton, A., Yao, Z., and Adeli, K. (1998) Intracellular translocation and stability of apolipoprotein B are inversely proportional to the length of the nascent polypeptide, *J. Biol. Chem. 50*, 33397−33405.
27. Mendell, J. T., and Dietz, H. C. (2001) When the message goes awry: Disease-producing mutations that influence mRNA content and performance, *Cell. 4*, 411−414.
28. Vedie, B., Jeunemaitre, X., Megnien, J. L., Atger, V., Simon, A., and Moatti, N. (2001) A new DNA polymorphism in the 5′ untranslated region of the human SREBP-1a is related to development of atherosclerosis in high cardiovascular risk population, *Atherosclerosis 3*, 589−597.
29. Di Paola, R., Frittitta, L., Miscio, G., Bozzali, M., Baratta, R., Centra, M., Spampinato, D., Santagati, M. G., Ercolino, T., Cisternino, C., Soccio, T., Mastroianno, S., Tassi, V., Almgren, P., Pizzuti, A., Vigneri, R., and Trischitta, V. (2002) A variation in 3′ UTR of hPTP1B increases specific gene expression and associates with insulin resistance, *Am. J. Hum. Genet. 3*, 806−812.
30. Located on the World Wide Web at http://droog.gs.washington.edu/parc/data/apob/welcome.html.
31. Mendez, R., and Richter, J. D. (2001) Translational control by CPEB: A means to the end, *Nat. Rev. Mol. Cell Biol. 7*, 521−529.
32. de Moor, C. H., and Richter, J. D. (1999) Cytoplasmic polyadenylation elements mediate masking and unmasking of cyclin B1 mRNA, *EMBO J. 8*, 2294−2303.
33. Minshall, N., Walker, J., Dale, M., and Standart, N. (1999) Dual roles of p82, the clam CPEB homolog, in cytoplasmic polyadenylation and translational masking, *RNA 1*, 27−38.

BI050368E