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Fabrizio Damiano, Simone Alemanno, Gabriele V Gnoni, Luisa Siculella. Translational Control of the sterol regulatory transcription factor SREBP-1 mRNA in response to serum starvation or ER stress is mediated by an internal ribosome entry site. *Biochemical Journal*, 2010, 429 (3), pp.603-612. 10.1042/BJ20091827 . hal-00502384

HAL Id: hal-00502384

<https://hal.science/hal-00502384>

Submitted on 14 Jul 2010

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TRANSLATIONAL CONTROL OF THE STEROL REGULATORY TRANSCRIPTION FACTOR SREBP-1 mRNA IN RESPONSE TO SERUM STARVATION OR ER STRESS IS MEDIATED BY AN INTERNAL RIBOSOME ENTRY SITE

Fabrizio Damiano, Simone Alemanno, Gabriele V. Gnoni, and Luisa Siculella
Laboratory of Biochemistry and Molecular Biology, Department of Biological and Environmental Science and Technologies, University of Salento
Via Prov.le Lecce-Monteroni, Lecce 73100, Italy

***Address correspondence to:** Gabriele V. Gnoni, Laboratorio di Biochimica e Biologia Molecolare, Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali, Università del Salento, Via Prov.le Lecce-Monteroni, Lecce 73100, Italy. E-mail: gabriele.gnoni@unisalento.it; Telephone: +39-0832298678; Fax: +39-0832298678

Short Title: SREBP-1a 5' UTR contains an IRES.

Sterol regulatory element binding proteins (SREBPs) are a family of transcription factors that modulate the expression of several enzymes implicated in endogenous cholesterol, fatty acid, triacylglycerol and phospholipid synthesis. In this study evidences for SREBP-1 regulation at translational level have been reported. By several experimental approaches, we demonstrated that 5' UTR of the SREBP-1a mRNA contains an internal ribosome entry site (IRES). Transfection experiments with SREBP-1a UTR inserted in a dicistronic reporter vector showed a remarkable increase of the downstream cistron translation, through a cap-independent mechanism. Insertion of the SREBP-1c 5' UTR in the same vector also stimulated the translation of the downstream cistron, but the observed effect can be ascribed, at least in part, to a cryptic promoter activity. Cellular stress conditions, such as serum starvation, caused in both Hep G2 and HeLa cells an increase in the level of SREBP-1 precursor and mature form, despite the overall reduction of protein synthesis, whereas mRNA levels for SREBP-1 were unaffected by serum starvation. Transfection experiments carried out with a dicistronic construct demonstrated that the cap-dependent translation was more affected than IRES-mediated translation by serum starvation. The thapsigargin- and tunicamycin-induced unfolded protein response also increased SREBP-1 expression in Hep G2 cells, through the cap-independent translation mediated by IRES. Overall, these data indicate that the presence of IRES in the SREBP-1a 5' UTR allows translation to be maintained under conditions that are inhibitory to cap-dependent translation.

Key words: Gene expression, IRES, SREBP-1, translational regulation, UPR, 5' UTR.

INTRODUCTION

Lipid homeostasis in vertebrate cells is regulated by a family of membrane-bound transcription factors designated as sterol regulatory element-binding proteins (SREBPs). SREBPs directly activate the expression of more than 30 genes implicated in the synthesis and uptake of cholesterol, fatty acids, tryglicerides, and phospholipids [1-3]. The SREBP family of basic-helix-loop-helix-leucine zipper (bHLH-LZ) transcription factors consists of SREBP-1a, SREBP-1c and SREBP-2, encoded in mammalian genome by two genes, *Srebf1* and *Srebf2*. SREBPs differ in their tissue-specific expression, target-gene selectivity and the relative potency of their trans-activation domains [4-6]. SREBP-1a is constitutively expressed at low levels in liver and in most tissues of adult animals and is the predominant isoform in most cultured cell lines. SREBP-1c expression is finely and strictly regulated in experimental

animals and humans in response to diet and hormones [4-9]. SREBPs are synthesized as inactive precursors bound to the endoplasmic reticulum (ER), where their regulatory domain co-localizes with an ER-embedded protein, the sterol cleavage activating protein (SCAP). SCAP functions as a sensor of membrane cholesterol level and as an escort protein that promotes clustering of SREBPs in the coat protein complex II (COPII)-coated membrane vesicles. When cells become depleted in cholesterol, the SREBP/SCAP complex binds to COPII proteins and translocates from the ER to the Golgi where a two-step proteolytic cleavage releases the N-terminal half of SREBP, allowing its entry into the nucleus [10]. SREBPs bind to sterol regulatory element (SRE) and E box sequences in the promoter region of genes involved in cholesterol and fatty acid biosynthesis.

SREBP-1a is a potent activator of all SREBP-responsive genes including those mediating the synthesis of cholesterol, fatty acids and triglycerides. The role of SREBP-1c is more restricted than that of SREBP-1a. SREBP-1c preferentially enhances transcription of genes required for fatty acid, but not cholesterol synthesis. SREBP-1c, a major mediator of insulin lipogenic action in liver, activates also the expression of target genes involved in glycolysis [11-12]. *In vitro* and *in vivo* studies suggested that SREBP-1c may also contribute to the regulation of glucose uptake and glucose synthesis [13-14]. When overexpressed in hepatocytes, SREBP-1c induces expression of glucokinases, a key enzyme in glucose utilization, and suppresses phosphoenolpyruvate carboxykinase, key enzyme of gluconeogenesis [13-14]. SREBP-1c expression is shown to be activated by liver X receptor (LXR), which forms heterodimers with retinoid X receptor (RXR) [15-16]. Polyunsaturated fatty acids (PUFA) suppress SREBP-1c transcription through a LXR-mediated mechanism [17]. Rodents fed on diets enriched with PUFA manifest reduced SREBP-1c mRNA expression and low rates of hepatic lipogenesis [18]. *In vitro*, PUFA inhibit SREBP-1c expression competitively blocking LXR activation by its endogenous ligands. In addition to LXR-mediated transcriptional inhibition, PUFA lower SREBP-1c levels by accelerating degradation of its mRNA [19]. Several evidences show that insulin stimulatory effect on fatty acid synthesis is mediated by increment of SREBP-1c. In isolated rat hepatocytes, insulin treatment increases the amount of mRNA for SREBP-1c in parallel with the mRNAs of its target genes. Conversely, incubating primary hepatocytes with glucagon decreases the mRNAs for SREBP-1c and its lipogenic target genes [20-21]. SREBP-1 is targeted by various posttranslational modifications, including phosphorylation, acetylation, sumoylation and ubiquitination [1].

It is well known that SREBP-1 expression is regulated by transcriptional, post-transcriptional (i.e. the turnover of SREBP-1 mRNA) and post-translational mechanisms. Translational regulation of SREBP-1 has not yet been studied. Therefore, the aim of this work was to investigate the role of human SREBP-1a and SREBP-1c 5' UTR on translation efficiency of the SREBP-1 transcript. Results here reported demonstrated that in Hep G2 and in HeLa cells SREBP-1 5' UTR strongly promotes the cap-independent translation of the downstream ORF through an internal ribosome entry site (IRES).

Cellular stress such as serum starvation enhanced the precursor and the nuclear form of SREBP-1 in Hep G2 and in HeLa cells. Therefore, an IRES mediated translation may account for the increase of the SREBP-1 protein level observed in serum starved cells.

The Unfolded Protein Response (UPR) pathway, a signaling cascade initiated by three endoplasmic reticulum (ER) membrane bound transducers, either facilitates the restoration of balance between ER load and capacity or promotes cell death. It has been shown that UPR activation triggers the proteolytic cleavage of SREBP-1c and SREBP-2 [22,23]. Here, we demonstrated that in Hep G2 cells the thapsigargin- and tunicamycin-induced UPR pathway increased both the precursor and the nuclear form of SREBP-1, through the cap-independent translation of SREBP-1, mediated by IRES.

EXPERIMENTAL

Cell culture and transient transfection assay

Hep G2 and HeLa cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin G (100 units/ml) and streptomycin (100 µg/ml). Cells were kept at 37°C in a humidified atmosphere containing 5% CO₂. For transient transfections, 5 × 10⁵ cells were seeded into 12-well plates 48 h before transfection. Cells were transfected using FuGENE 6 (Roche Diagnostics) following the manufacturer's recommendations. After an 8-h transfection period, the medium was changed to fresh DMEM supplemented with 10% (v/v) of FBS and cells were incubated for 24 h. After cells lysis, *Renilla* and firefly luciferase activities were measured using Dual Luciferase Reporter Assay System (Promega). The β-galactosidase activity was determined by using a β-galactosidase assay. To study the effect of serum starvation, cells were maintained in DMEM supplemented with 0.5% of FBS and incubated for 24h.

[³⁵S]Methionine Incorporation

Hep G2 cells were plated at a density of 5 × 10⁴ cells per 12-well dish and incubated for 48 h. 30 µCi/ml L-[³⁵S]methionine/cysteine (Perkin Elmer) was then added to fresh medium and cells were incubated for further 24 h in either serum starved-medium (0.5% FBS) or FBS-supplemented medium (10% FBS). [³⁵S]Methionine/cysteine incorporation was determined as reported [24].

Mono- and Dicistronic Constructs

The 5' UTR of the human SREBP-1a mRNA (GenBankTM accession number NM001005291) and SREBP-1c mRNA (GenBankTM accession number AK293795) (Figure 1A) were amplified from total RNA by RT-PCR. Primers used in PCR are listed in Table 1. The identity of the amplimers was checked by DNA sequencing. The amplimers SREBP-1a 5' UTR and SREBP-1c 5' UTR were then digested with *Hind*III and *Nco*I and inserted into the pGL3prom vector (Promega) to obtain pGL3S1a and pGL3S1c, respectively (Figure 1B). For engineering the pBKLuc construct, DNA fragment containing the firefly luciferase coding region with its 5' leader was excised from the pGL3prom by digestion with *Hind*III and *Xba*I, and then cloned into the *pBluescript*II plasmid. Analogously, DNA fragments containing the SREBP-1a 5' UTR and SREBP-1c 5' UTR, followed by firefly luciferase coding region, were excised from pGLS1a and pGLS1c by digestion with *Hind*III and *Xba*I, and then cloned into the same sites of *pBluescript*II to obtain the pBKS1aLuc and pBKS1cLuc, respectively. The plasmids pGL3c-myc, pRF (formerly pGL3R), phpRF, pRc-mycF, and pHpRc-mycF (formerly pGL3utrH) have been described in [25] and kindly provided by Dr. A. Willis. The SREBP-1a 5' UTR and SREBP-1c 5' UTR amplimers were digested with *Eco*RI and *Nco*I and then inserted either into the intercistronic region of pRF or into the intercistronic region of pHpRF, in order to produce the dicistronic constructs pRS1aF and pRS1cF, or pHpRS1aF and pHpRS1cF, respectively (Figure 1B).

To obtain promoterless dicistronic constructs, the simian virus 40 (SV40) promoter sequence, including the chimeric intron between *Sma*I and *Eco*RV sites, was removed by restriction digestion from pRF, pRS1aF, and pRS1cF, resulting in pRF(-P), pRS1aF(-P), and pRS1cF(-P), respectively (Figure 1B).

In vitro run-off transcription and translation

pBKLuc, pBKS1aLuc and pBKS1cLuc were linearized at a *Xba*I site downstream the firefly luciferase ORF. Capped transcripts were synthesized in a reaction mix containing 1 µg of DNA template and 20 units of T3 RNA polymerase (Promega), 1 mM ATP, 1 mM UTP, 1 mM CTP, 0.5 mM GTP, 2 mM m7G(5')ppp(5')G (Promega), and 20 units of RNasin in a final volume of 20 µl. After incubation for 2 h at 37 °C, the RNA was isolated and used to

prime a 12.5 μ l *in vitro* translation reaction mix containing 8.25 μ l of rabbit reticulocyte lysate (Promega) and up to 50 ng of RNA as recommended in the manufacturer's instructions.

Isolation of RNA, quantitative RT-PCR and Northern blotting analysis

Total RNA extraction from cultured cells and Real Time qPCR analysis were carried out as in [26]. The amount of SREBP-1, fatty acid synthase (FASN) and acetyl-CoA carboxylase (ACACA) mRNA was normalized to the internal control rRNA 18S. The sequence of primers used in real time PCR analysis is reported in Table 1. Northern blotting analysis was carried out as in [27]. DNA probe used for the detection of firefly luciferase mRNA was as described [25]. For analysis of the human X-box Binding Protein-1 (XBP1) mRNA splicing, cDNA was amplified with specific primers for XBP1 gene (Table 1). PCR products were separated by electrophoresis on 2.5% agarose gels and visualized by ethidium bromide staining.

Immunoelectrophoretic analysis

Western blot analysis was carried out as reported in [28]. After electrophoretic transfer to nitrocellulose, blots were probed with antibody directed against SREBP-1 (Santa Cruz Biotechnology). The detection system employed was the ECL Plus™ Western Blotting Reagents (GE Healthcare).

SREBP-1 half life analysis

Hep G2 cells were plated at a density of 1×10^6 cells into 25 cm² flask and incubated for 48 h. Cells were incubated for further 24 h in either serum starved-medium (0.5% FBS) or FBS-supplemented medium (10% FBS). Then, 100 μ g/ml cycloheximide, inhibitor of protein synthesis, was added to the medium and cells were incubated for the times indicated. At different times, cells from a flask were harvested and Western blot analysis was performed as described above. Autoradiograms were quantified by densitometric scanning.

RESULTS

Human SREBP-1a and SREBP-1c 5' UTRs inhibit the *in vitro* translation of the luciferase reporter gene

Human SREBP-1a and SREBP-1c 5' UTRs (185 bp and 94 bp respectively, Figure 1A) were inserted into the *pBluescriptII* vector upstream the firefly luciferase (FL) start codon to obtain pBKS1aLuc and pBKS1cLuc, respectively (Figure 1B). The control pBKLuc construct was generated by inserting the FL ORF with its 5' leader sequence, excised from the pGL3prom vector, into the *pBluescriptII* vector. Capped mRNAs generated from these constructs by T3 RNA polymerase were then used to prime rabbit reticulocyte lysates and the FL activity was measured. We found that SREBP-1a and SREBP-1c 5' UTRs strongly inhibited the translation of the downstream FL ORF. In fact, with pBKS1aLuc and pBKS1cLuc the FL activity dropped by as much as 10-fold and 3-fold, respectively, when compared to that measured by using the control pBKLuc (Figure 2A).

SREBP-1a and SREBP-1c 5' UTRs activate translation in Hep G2 cells

On the basis of the results reported above, we hypothesized that the SREBP-1a and SREBP-1c 5' UTR could affect the translation of their respective mRNAs *in vivo*. To address this question, human SREBP-1a and SREBP-1c 5' UTRs were inserted upstream the FL coding region of pGL3prom plasmid to create the pGL3S1a and pGL3S1c constructs (Figure 1B). The pGL3c-myc construct containing a 396 bp fragment of human c-Myc 5' UTR was used as a positive control, as c-Myc 5' UTR fragment contains an IRES [25]. The pGL3S1a, pGL3S1c, pGL3c-myc constructs and control plasmid pGL3prom were co-transfected with the control plasmid pcDNA3.1/HisB/LacZ, for the normalization of transfection efficiency, into Hep G2 cells. The FL activity produced with pGL3S1a and pGL3S1c was respectively 1.5- and 1.3-fold higher than that produced with the control empty vector, suggesting that both SREBP-1a 5' UTR and SREBP-1c 5' UTR did not inhibit, but augmented the expression of the downstream FL (Figure 2B). The FL activity produced by pGL3c-myc was 1.6-fold

higher than that produced from the control vector pGL3prom, in agreement with previous report [25].

Does SREBP-1a or SREBP-1c 5' UTR contain an IRES?

The discrepancy between data on the translation obtained in Hep G2 cells and those obtained in *in vitro* experiments, led us to speculate that the SREBP-1a and -1c 5' UTRs could contain an IRES. To test this hypothesis, SREBP-1a and SREBP-1c 5' UTRs were inserted into the dicistronic vector pRF [25]. This plasmid contains two reporter genes. The first cistron (*Renilla* luciferase, RL) is under the control of the SV40 promoter and it is translated via a cap-dependent mechanism, whereas the second FL cistron is translated independently from the cap structure [25,29]. The SREBP-1a and SREBP-1c 5' UTRs were cloned upstream the FL cistron to obtain pRS1aF and pRS1cF, respectively (Figure 1B). Hep G2 cells were co-transfected with either pRF, pRS1aF or pRS1cF, together with the pcDNA3.1/HisB/lacZ control plasmid. 24 h after transfection, both RL and FL activities were measured and normalized to the transfection control β -galactosidase. The presence of the SREBP-1 5' UTR between the two reporter genes did not alter RL activity (Figure 3A). Results reported in figure 3B showed that in the Hep G2 cells transfected with the pRS1cF, the FL activity was approximately 13-fold higher than that determined with the pRF control plasmid, whereas with the pRS1aF the FL activity was about 20-fold higher than that measured with the control pRF. The c-Myc 5' UTR, inserted upstream the FL cistron to obtain pRc-mycF, used as a positive control, stimulated expression of the downstream cistron by approximately 50-fold, when compared to the pRF control plasmid, in agreement with previous report [25]. Several control assays were performed to determine whether this result might be ascribed to mechanisms alternative to IRES, such as enhanced ribosomal reinitiation at the FL start codon, and/or generation of FL mRNA either by differential splicing or from a cryptic promoter. In order to investigate on the ribosomal reinitiation mechanism, SREBP-1a and SREBP-1c 5' UTRs were cloned into the pHpRF vector, to obtain the pHpRS1aF and pHpRS1cF constructs, respectively (Figure 1B). These constructs contain upstream the RL coding region an inverted repeat sequence, which produces a stable hairpin structure in the mRNA (-55 kcal/mol). Cap-dependent translation of the upstream RL cistron should be greatly reduced, whereas cap-independent translation of the downstream FL cistron should not be affected. As expected, the pHpRS1aF and pHpRS1cF constructs containing the hairpin structure showed a reduction of RL activity in Hep G2 cells by approx 80%, when compared to pRF control (Figure 3C). On the contrary, with both the constructs a FL activity higher by ~20-fold and ~12-fold, respectively, when compared with the pRF plasmid control, was observed (Figure 3D).

The enhanced expression of the downstream FL ORF might be ascribed to the translation of a shorter monocistronic transcript derived from a cryptic promoter in the 5' UTR of pRS1aF or pRS1cF constructs. To assess this hypothesis, the promoterless pRF(-p), pRS1aF(-p) and pRS1cF(-p) constructs were obtained by removing the SV40 promoter from pRF [30], pRS1aF and pRS1cF (Figure 1B). As shown in figure 3E, transfection with the promoterless pRS1aF(-p) resulted in minimal luciferase activity in Hep G2 cells, indicating that the FL expression from this construct did not depend on a cryptic promoter activity. On the other hand, after transfection of pRS1cF(-p), FL activity was about 5-fold higher than that determined with pRF(-p) control. This result suggested that a cryptic promoter has been created when the SREBP-1c UTR has been inserted upstream the FL cistron.

To investigate whether a FL monocistronic transcript originated by alternative splicing from dicistronic RNA, Northern blot analysis was carried out, using the FL ORF as a probe. In Hep G2 cells transfected with pRF or pRS1aF a dicistronic transcript was detected (Figure 3F, lanes 2 and 3).

Notably, two strong autoradiographic signals were observed in Hep G2 cells transfected with pRS1cF, the first corresponding to a dicistronic RNA, and the second corresponding to a

shorter transcript, likely containing only the FL cistron (Figure 3F, lane 4). These data are indicative of presence of a cryptic promoter sequence in the SREBP-1c 5' UTR. The autoradiographic signals of the two transcripts were similar, indicating that the cryptic promoter exhibits quite strong activity.

IRES Activity During Serum Deprivation

Cellular stress such as serum, amino acid, and glucose starvation, heat shock, oxygen deprivation, apoptosis and mitosis is known to inhibit cap-dependent but not cap-independent translation [31]. As consequence, translation of mRNAs harbouring IRES in their 5' UTR ensures adequate protein synthesis, through the cap-independent mechanism, even when the global translation is reduced. To investigate whether serum starvation could affect SREBP-1 translation, precursor (pSREBP-1) and nuclear (nSREBP-1) form of SREBP-1 protein level was quantified in control and in serum starved Hep G2 and HeLa cells. Western blotting experiments showed that pSREBP-1 and nSREBP-1 level increased about 2-fold in Hep G2 cells after 24 h of serum deprivation when compared to the control cells (Figure 4A). By contrast, serum deprivation did not cause significant variation in SREBP-1 mRNA levels in starved versus control Hep G2 cells (Figure 4B). Similar results were obtained in HeLa cells (Figures 4A and 4B). To address the question whether serum deprivation affects protein synthesis, incorporation of [³⁵S]methionine was measured in starved and in control Hep G2 cells. As shown in Figure 4(C), the incorporation of [³⁵S]methionine decreased by approx 60 % in starved with respect to control cells.

To investigate whether serum deprivation enhances protein stability, the half-life of pSREBP-1 and nSREBP-1 was determined in starved and in control Hep G2 cells. The log of pSREBP-1 and nSREBP-1 content was reported as a function of time (Figure 4D). Results show an increase in the turnover of pSREBP-1 in starved cells with respect to control cells. The apparent half-life of pSREBP-1 protein was ~2.5 h in starved vs ~6.5 h in control cells, respectively. By contrast, the apparent half life of nSREBP-1 was slightly lower in starved with respect to control cells (~5.2 h vs ~6.5 h, respectively).

The turnover of pSREBP-1 and nSREBP-1 was also evaluated in Hep G2 cells cultured in the presence of 10 µg/ml cholesterol and 1 µg/ml 25-hydroxycholesterol which block the proteolytic cleavage of pSREBP-1. Sterols were added to the medium five minutes prior the addition of cycloheximide. In this condition, the half life of nSREBP-1 strongly diminished in starved when compared to control cells (~1.6 h in starved vs ~5.1 h in control cells). By contrast, the decay curve of pSREBP-1 was similar in starved and in control cells (Figure 4D).

The increase of SREBP-1 protein level observed in Hep G2 or in HeLa starved cells could be ascribed to an efficient translation of its mRNA through IRES-mediated mechanism. Since the insertion of SREBP-1c 5' UTR in the dicistronic pRF vector originated a cryptic promoter activity (Figures 3C and 3F), the observed increase of the FL activity might be ascribed, at least in part, to an augmentation of the FL transcript abundance. However, we cannot rule out that an IRES is present also in SREBP-1c 5' UTR and that it could contribute to raise SREBP-1 protein level.

IRES in the SREBP-1a 5' UTR might allow efficient translation of its mRNA during cellular stress, such as serum starvation. If this were the case, translation of FL from the dicistronic pRS1aF mRNA would be relatively unaffected by serum starvation. To test this hypothesis, Hep G2 cells transfected with the pRS1aF were subjected to serum starvation for 24 h and then the luciferase activity was measured (Figure 5). Upon serum starvation, RL activity was reduced by approx. 70 % with respect to the RL activity measured in cells cultured in serum-complemented medium (Figure 5A). This is consistent with the effect of serum starvation on total translation rate measured by [³⁵S]methionine incorporation in cultured cells (Figure 4C). In serum-starved cells FL activity was reduced by 33 % with respect to that measured in

control cells (Figure 5A). Similar results were obtained transfecting the same cells with pRc-mycF, containing the c-myc 5' UTR upstream the FL ORF (Figure 5B).

Translation from the SREBP-1a IRES is stimulated upon ER stress

Next, we investigated whether UPR response also stimulates translation from the SREBP-1a IRES. ER stress in Hep G2 cells was induced by thapsigargin or tunicamycin. Thapsigargin modifies Ca^{2+} concentration in the ER lumen by inhibiting Ca^{2+} -ATPase. Tunicamycin leads to accumulation of proteins into the lumen of the ER by inhibiting protein glycosylation. In agreement with previous studies [22,32], 1-h incubation in the presence of 300 nM thapsigargin or 1 $\mu\text{g/ml}$ tunicamycin induced the splicing of XBP1 mRNA, a classical index of the UPR (data not shown).

To evaluate the time dependent effect of thapsigargin (300 nM) or tunicamycin (1 $\mu\text{g/ml}$) on the expression of SREBP-1 in HepG2 cells, Western-blot analysis was carried out. Treatment of Hep G2 cells with thapsigargin for 15 min caused a decrease of pSREBP-1 and an increase of nSREBP-1 (Figure 6A). However, after 1 h of incubation with thapsigargin, an increase of both pSREBP-1 and nSREBP-1 was observed, rising up to the maximum level at 3 h (Fig. 6A). Addition of tunicamycin to Hep G2 cells caused a stronger activation of both pSREBP-1 and nSREBP-1 expression than that observed in thapsigargin-treated cells, reaching the maximum level at 6 h treatment (Figure 6A). The mRNA level for SREBP-1 was unchanged in tunicamycin-treated cells when compared to control cells (Figure 6B).

The effect of SREBP-1 activation on the expression of its target lipogenic genes, i.e. fatty acid synthase (FASN), and acetyl-CoA carboxylase (ACACA) was also analyzed. Hep G2 cells were cultured for 1, 3, 6, 12, and 24 h in DMEM in the presence of tunicamycin, which induced an increase in the expression of FASN and ACACA (Figure 6B). Similar results were obtained in thapsigargin-treated cells (data not shown). To test the effect of ER stress on translation from the SREBP-1a IRES, Hep G2 cells, transiently transfected with the pRS1aF, were treated with thapsigargin or tunicamycin (Figure 6C). Both ER stressors decreased RL activity already after 15 min. This effect was consistent with the inhibition of cap-dependent translation by these agents. By contrast, both treatments caused an increase in FL activity, reaching the maximum level at 6 h of treatment (Figure 6C).

To determine if ER stress affects the protein stability, the half-life of pSREBP-1 and nSREBP-1 was determined in tunicamycin-treated Hep G2 cells. Results show that tunicamycin reduced the half life of pSREBP-1 (Figure 6D) with respect to control cells (Figure 4D) (~1.9 h vs ~6.5 h in control cells). No significant change in the half life of nSREBP-1 has been observed in tunicamycin-treated cells (Figure 6D) when compared to control cells (Figure 4D) (~6.3 h vs ~6.5 h in control cells). Addition of sterols to the medium reduced the turnover of pSREBP-1 in tunicamycin-treated cells when compared to the same cells without sterols (Figure 6D) (half life ~5.9 h in cells with sterols vs ~1.9 h in cells without sterols). By contrast, the half life of nSREBP-1 strongly diminished in tunicamycin-treated cells incubated with sterols, when compared to that observed in the same cells without sterols (~2.3 h in cells with sterols vs ~6.3 h in cells without sterols).

DISCUSSION

Translational control is a final regulatory step in gene expression. In the ribosome scanning model of translation [33], the 5'-end m⁷G structure of mRNA is recognized by the cap-binding protein complex eIF4F. The binding of eIF4F complex to mRNA further recruits other initiation factors as well as the 40 S ribosomal subunit. This complex proceeds in the 3' direction until an AUG start codon in a favorable context is encountered, and protein synthesis is initiated.

A broad range of cellular stress leads to the inhibition of translation. This event is accomplished by the phosphorylation of some initiation factors and/or their regulators [34] or by the proteolytic cleavage of several initiation factors [35].

Under conditions of reduced translation, mRNAs encoding for several oncoproteins, survival factors, and proteins critically involved in apoptosis are translated by a poorly understood cap-independent mechanism [36]. This mechanism is mediated by IRES elements found in the 5' UTR of a limited but growing number of mRNAs, preferentially involved in the control of cellular proliferation, survival, and death (for reviews see [37-39]).

There is a growing interest among researchers about SREBP-1, due to the important role that this protein exerts on lipid homeostasis in the organism. Regulation of SREBP-1 expression is very complex and involves several steps at transcriptional, post-transcriptional and post-translational levels. Among post-transcriptional mechanism, no data are available for SREBP-1 regulation at translational level.

Here, the characterization of the SREBP-1a and SREBP-1c 5' UTRs has been reported, showing that an IRES element is present in the 5' UTR of SREBP-1 mRNA.

In vitro translation experiments carried out by using rabbit reticulocyte lysates demonstrated that SREBP-1a 5' UTR and, to a lesser extent, SREBP-1c 5' UTR, inhibited the translation of the downstream FL ORF (Figure 2A). This finding suggests that a stable secondary structure could obstruct the scanning translation initiation complex or that non-canonical factors, absent in rabbit reticulocyte lysates, were required for *in vitro* translation initiation of SREBP-1 mRNAs [40]. On the basis of structure-prediction algorithms mfold [41], both the human SREBP-1a and SREBP-1c 5' UTRs, characterized by a high GC percentage (about 79% and 71% respectively), contain extensively RNA secondary structures, being those of SREBP-1a 5' UTR more complex than those of SREBP-1c 5' UTR (data not shown).

Experiments performed either with monocistronic pGLS1a and pGLS1c or with dicistronic pRS1aF and pRS1cF constructs demonstrated that both SREBP-1a and SREBP-1c 5' UTRs did not inhibit, but augmented the FL expression when compared to the control (Figures 2B and 3B). Moreover, the palindromic sequence inserted into pHpRS1aF and pHpRS1cF constructs forms a stable RNA hairpin, which reduced the RL activity by 80%, but did not affect the FL activity (Figures 3C and 3D). If a ribosomal readthrough mechanism, caused by the 5' UTR insertion, were responsible for the translation of FL, then this activity should be reduced by a similar value. Moreover, transfection experiments performed with the promoterless pRS1aF(-P) demonstrated that the enhanced expression of FL driven by SREBP-1a 5' UTR cannot be ascribed to a cryptic promoter activity, as the FL activity drastically dropped down (Figure 3E). By contrast, after transfection with the promoterless pRS1cF(-P) the expression of FL was 5-fold higher than that measured in cells transfected with pRF(-P) control plasmid (Figure 3E). Therefore, the enhanced FL expression promoted by SREBP-1c 5' UTR could be due, at least in part, to a cryptic promoter activity. The presence of a cryptic promoter has been also reported by Han and Zhang [30] in 5' UTR of eIF4G mRNA. Northern blotting analysis confirmed this finding as a short transcript (Figure 3F, lane 4), likely containing only the FL ORF, was observed in the cells transfected with pRS1cF besides the dicistronic RNA, whereas in cells transfected with pRS1aF, only the long transcript was detected (Figure 3F, lane 3). Taken together, these data for the first time support the notion that the SREBP-1a 5' UTR is capable of internal translation initiation. However, we cannot rule out that an IRES element is also present in SREBP-1c 5' UTR. Indeed, FL activity measured in Hep G2 cells transfected with the dicistronic pRS1cF was 12-fold higher than that determined with pRF control vector (Figure 3B) and, thus, approx. 2.4-fold higher than that observed in transfection experiments carried out with the promoterless construct pRS1cF(-P) (Figure 3E).

Here, we showed that cellular stress condition, such as serum starvation, caused an increase in the level of SREBP-1 precursor and mature form both in Hep G2 and in HeLa cells (Figure 4A), despite the overall reduction of protein synthesis, as demonstrated by the decrease of [³⁵S]methionine incorporation (Figure 4C). Furthermore, no significant increment of SREBP-1 mRNA amount was observed in starved Hep G2 or HeLa cells (Figure 4B).

On this basis, we speculated that, in cellular stress condition, the increase of SREBP-1 content could be due to an enhanced SREBP-1a mRNA translation through an IRES-mediated mechanism.

This hypothesis is supported by data on the RL and FL activities measured in control and in starved Hep G2 cells transfected with the dicistronic construct pRS1aF (Figure 5). Indeed, while serum-starvation reduced both RL and FL activities, the former was the more affected, suggesting that IRES-dependent was less sensitive to the serum-starvation than cap-dependent translation (Figure 5). The change in SREBP-1 content observed in starved Hep G2 cells might be ascribed to an increase of protein stability rather than to the IRES mechanism proposed. To address this question, the half-life of pSREBP-1 and nSREBP-1 was evaluated in starved and in control Hep G2 cells. Results showed that the turnover of pSREBP-1 strongly increased in starved with respect to control cells (Figure 4D). Note that the half life of nSREBP-1 was affected to a lesser extent than that of pSREBP-1 by serum starvation (Figure 4D). The reduction of pSREBP-1 content observed in starved Hep G2 cells could be due to an increase of its proteolytic cleavage rather than to its degradation. Therefore, we evaluated the turnover of pSREBP-1 and nSREBP-1 in starved and in control Hep G2 cells cultured in the presence of cholesterol and 25-hydroxycholesterol which block the proteolytic cleavage of pSREBP-1. In starved Hep G2 cells, the turnover of pSREBP-1 decreased in the presence of sterols with respect to that observed in starved cells cultured without sterols. By contrast, the half life of nSREBP-1 strongly diminished in starved cells cultured in the medium added with sterols (Figure 4D) when compared to the same cells cultured in the absence of sterols. In control Hep G2 cells, the turnover of nSREBP-1 and pSREBP-1 was unaffected by the addition of sterols, ruling out the hypothesis of a direct role of sterols in altering the SREBP-1 stability. Taken together, these results show that serum starvation: i) induces the proteolytic cleavage of SREBP-1 and ii) enhances SREBP-1 turnover.

A number of recent reports have highlighted the link between the unfolded protein response (UPR) and the hepatic lipid metabolism [42,43]. It has been shown that the homocysteine- or thapsigargin-induced UPR was able to activate SREBP-1c and to induce lipogenic gene expression by promoting the proteolytic cleavage of pSREBP-1 [22,44]. We observed a similar effect on ER-stressed Hep G2, after incubation in thapsigargin- or tunicamycin-supplemented medium (Figure 6A). A strong increase of both pSREBP-1 and nSREBP-1 content was observed after 1 h of treatment with ER-stressors (Fig. 6A). The augmented content of SREBP-1 observed in ER-stressed Hep G2 cells could be ascribed to an enhanced SREBP-1 mRNA translation through a cap-independent mechanism, as demonstrated by transfection experiments with the dicistronic construct pRS1aF (Figure 6C). As the half life of both pSREBP-1 and nSREBP-1 decreased in ER-stressed Hep G2 cells, when compared to control cells, it could be ruled out that an altered protein stability caused the increment of SREBP-1 content observed in ER-stressed cells (Figure 6D).

The physiological role of the IRES in the SREBP-1a 5'-UTR could be to allow minimal lipogenic and cholesterologenic activities, even when cap-dependent translation is inhibited. Protein synthesis, via internal ribosome entry, may be required in several conditions such as mitosis, where cap-dependent translation is reduced [45-46]. It has been reported that the mature forms of SREBP-1a and SREBP-1c are hyperphosphorylated in mitotic cells [47], and the Cdk1/cyclin B-mediated phosphorylation is important for their stabilization during mitosis [48]. Based on our data, we speculated that enhanced translation of SREBP-1 through the cap-independent mechanism could work synergically with the Cdk1/cyclin B-mediated phosphorylation and stabilization of SREBP-1, in order to promote the lipid and cholesterol synthesis during mitosis. This hypothesis is in agreement with the observation that siRNA-mediated knockdown of SREBP-1 resulted in an increase of cells in G1 phase accompanied by a decrease of cells in S phase, caused by an impaired ability to proliferate [47].

Deletion or site-specific mutation analysis of SREBP-1a 5' UTR will be performed to characterize the minimal region containing the IRES, together with the IRES trans-activating factors (ITAF) mediating the cap-independent translation of SREBP-1 upon serum starvation or ER-stress.

Abbreviation used: FL, Firefly luciferase; IRES, Internal ribosome entry site; ORF, open reading frame; RL, *Renilla* luciferase; UPR, unfolded protein response; UTR, untranslated region.

ACKNOWLEDGMENTS

We thank Dr. Anne Willis for providing the plasmid pGL3c-Myc, pRF, phpRF, and pHpRc-mycF and Dr. Maurizio Crestani for helpful discussions and critical reviewing of the manuscript.

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Table 1 Oligonucleotides used for qRT-PCR analysis and for construction of monocistronic and dicistronic vectors.

Primer	Oligonucleotide sequence
SREBP-1RTFor	5' -ACACCATGGGGAAGCACAC-3'
SREBP-1RTRev	5' -CTTCACTCTCAATGCGCC-3'
hFASN RTFor	5' -GAAGGAGGGTGTGTTTGCC-3'
hFASN RTRev	5' -GGATAGAGGTGCTGAGCC-3'
hACACA RTFor	5' -GCAACCAAGTAGTGAGGATG-3'
hACACA RTRev	5' -CTGTTTGGATGAGATGTGGG-3'
rRNA18SFor	5' -GTTGGTTTTTCGGAAGTGGG-3'
rRNA18SRev	5' -CGGTCGGCATCGTTTATGGT-3'
S1a For1	5' -AAGCTTGAATTCGGCCGGGGGAACCCAGTT-3'
S1a For2	5' -GAATTCAGCTTCGGCCGGGGGAACCCAGTT-3'
S1a Rev	5' -GAATTCATGGCGCAGCCGCCTCC-3'
S1c For1	5' -AAGCTTGAATTCAAAAATCCGCCGCGCCTTGAC-3'
S1c For2	5' -GAATTCAGCTTAAAAATCCGCCGCGCCTTGAC-3'
S1c Rev	5' -GAATTCATGGCTCCGCGATCTGCG-3'
hXBP1For	5' -AGTGGCCGGGTCTGCTGAGT-3'
hXBP1Rev	5' -CAAGTTGTCCAGAATGCCCA-3'

FIGURE LEGENDS

Figure 1 pBluescriptII, pGL3prom, pRF and pHpRF constructs

A) The sequence of human SREBP-1a 5'UTR (185 bp) and SREBP-1c 5'UTR (94 bp) are shown. In both sequences, the ATG translation start codon is in bold.

B) pBKLuc construct contains the firefly luciferase (FL) cistron with its 5' leader, excised from the pGL3prom plasmid and inserted downstream the T3 promoter in the *pBluescriptII* vector. pBKS1aLuc and pBKS1cLuc constructs contain the SREBP-1a and SREBP-1c 5' UTR, respectively, inserted into pBKLuc upstream the FL cistron. pGL3S1a and pGL3S1c contain SREBP-1a and SREBP-1c 5' UTR, respectively, inserted into pGL3prom upstream the FL cistron. The control dicistronic vector pRF contains the SV40 early promoter, a chimeric introne, and the cDNAs encoding for *Renilla* luciferase (RL) and FL separated by a short linker sequence. pHpRF is similar to pRF, but with the addition of an inverted repeat upstream the first cistron, in order to form a stem-loop at the 5' end of the transcript. pRS1aF and pRS1cF constructs are similar to the control vector pRF but they contain the SREBP-1a and SREBP-1c 5' UTR, respectively, inserted between the two cistrons RL and FL. pRS1aF(-P) and pRS1cF(-P) constructs are identical to their corresponding pRF constructs described above, except that they do not contain the SV40 early promoter and the chimeric intron. pHpRS1aF and pHpRS1cF constructs are derived from pHpRF control vector by the insertion of SREBP-1a and SREBP-1c 5' UTR, respectively, between the two cistrons RL and FL.

Figure 2 SREBP-1a and SREBP-1c 5' UTRs inhibit the *in vitro*, but not the *in vivo* translation

A) Rabbit reticulocyte lysates were primed with capped RNA from the control pBKLuc, or from pBKS1aLuc or pBKS1cLuc constructs. After 1 h, FL activity was measured as described under experimental procedures. Values are means \pm SD, $n=4$.

B) Hep G2 cells were co-transfected with pGL3prom, pGL3c-myc, pGL3S1a, or pGL3S1c constructs together with the control plasmid pcDNA3.1/HisB/LacZ. 24h after transfection, FL activity was measured and normalized with respect to β -galactosidase activity. Values were reported as percentage of FL activity determined in cells transfected with the empty vector pGL3prom. Values are means \pm SD, $n=6$.

Figure 3 SREBP-1a and SREBP-1c activities

A) Hep G2 cells were co-transfected with pRc-mycF, pRS1aF or pRS1cF constructs together with the pcDNA3.1/HisB/*lacZ* used for the normalization of transfection efficiency. *Renilla* luciferase (RL) activity, normalized to the β -galactosidase activity, was reported in histograms as fold change relative to that determined in cells transfected with the control vector pRF.

B) Hep G2 cells were transfected as described above and firefly luciferase (FL) activity, after normalization, was reported in histograms as fold change relative to that determined in cells transfected with the control vector pRF.

C) Hep G2 cells were co-transfected with pRF, pHpRS1aF, and pHpRS1cF together with the pcDNA3.1/HisB/*lacZ* used for the normalization of transfection efficiency. RL activity, normalized to the β -galactosidase activity, was reported in histograms as fold change relative to that determined in cells transfected with the control vector pRF.

D) Hep G2 cells were transfected as described above and FL activity, after normalization, was reported in histograms as fold change relative to that determined in cells transfected with the control vector pRF.

E) The promoterless pRF(-P), pRS1aF(-P) and pRS1cF(-P) constructs were co-transfected together with pcDNA3.1/HisB/*lacZ* into Hep G2 cells; 24 h after transfection, cells were harvested for determination of FL activity, which was normalized to the β -galactosidase

activity. Values were reported as fold change relative to the FL activity measured in Hep G2 cells transfected with pRF(-P).

F) Poly(A)⁺ RNA was isolated from Hep G2 cells transiently transfected with either pcDNA3.1 empty vector (lane 1), pRF (lane 2), pRS1aF (lane 3), or pRS1cF (lane 4). Northern blot analysis was performed using ³²P-labeled DNA probe for the FL cistron. The size of RNA standards is indicated. The autoradiographic signal in lane 4, marked by an asterisk, likely corresponds to FL transcript derived from the transcriptional activity of a cryptic promoter in SREBP-1c 5' UTR.

Figure 4 Effect of serum starvation on the SREBP-1 protein level

A) Hep G2 cells were incubated for 24 h in DMEM medium with FBS 10% or 0.5%. Cells were then harvested for preparation of a crude nuclear fraction. Proteins (50 µg) were separated by SDS-PAGE and immunodecorated with antisera against SREBP-1. The content of pSREBP-1 and nSREBP-1 in cells cultured in either serum starved-medium (FBS 0.5%) or in serum-supplemented medium (FBS 10%) was analyzed by Western blotting, quantified by densitometric analysis and expressed as fold change relative to SREBP-1 content in control cells cultured in serum-supplemented medium. Values are means ± SD, *n*=4.

B) Total RNA was extracted from Hep G2 cells cultured in either serum starved-medium or in serum-supplemented medium for 24h. SREBP-1 mRNA level was determined by using quantitative RT-PCR and normalized with 18S rRNA. Values were reported as fold change relative to control.

C) 48 h after cell plating, L-[³⁵S]methionine/cysteine was added to fresh medium and cells were incubated for further 24 h in either serum starved-medium or FBS-supplemented medium. [³⁵S]methionine/cysteine incorporation was normalized with respect to total protein determined by Bradford assay. Values were reported in histograms as fold change relative to control cells cultured in FBS-supplemented medium.

D) Hep G2 cells, incubated for 24 h in DMEM medium with FBS 10% or 0.5%, were then treated with 100 µg/ml cycloheximide. At different times, cells were harvested and the content of either pSREBP-1 or nSREBP-1 protein was measured by Western blotting analysis. The semi-log plot represents the decay curve of pSREBP-1 (squares) or nSREBP-1 (circles) protein in control (filled) and in serum starved (open) Hep G2 cells, incubated in the absence (left panel) or in the presence (right panel) of sterols. The data are from a representative experiment. Similar results were obtained in four independent experiments.

Figure 5 IRES activity is maintained upon serum starvation

Hep G2 cells were transiently transfected with pRS1aF and pRc-mycF. After transfection, cells were subjected to serum starvation or were left under control conditions for a further 24 h before harvesting and performing assays for *Renilla* luciferase (RL) and firefly luciferase (FL) activities. Values of RL and FL activities measured in cells transfected with pRS1aF (panel A) or pRc-mycF (panel B) and cultured in serum starvation condition (FBS 0.5 %) were reported in histograms as percentage of the corresponding luciferase activities determined in cells transfected with the same constructs and cultured in serum complemented (FBS 10%) medium. The data shown are means (±SD) of triplicate samples from each of five independent experiments.

Figure 6 Effect of ER stress on the expression of SREBP-1 and SREBP-1 target genes

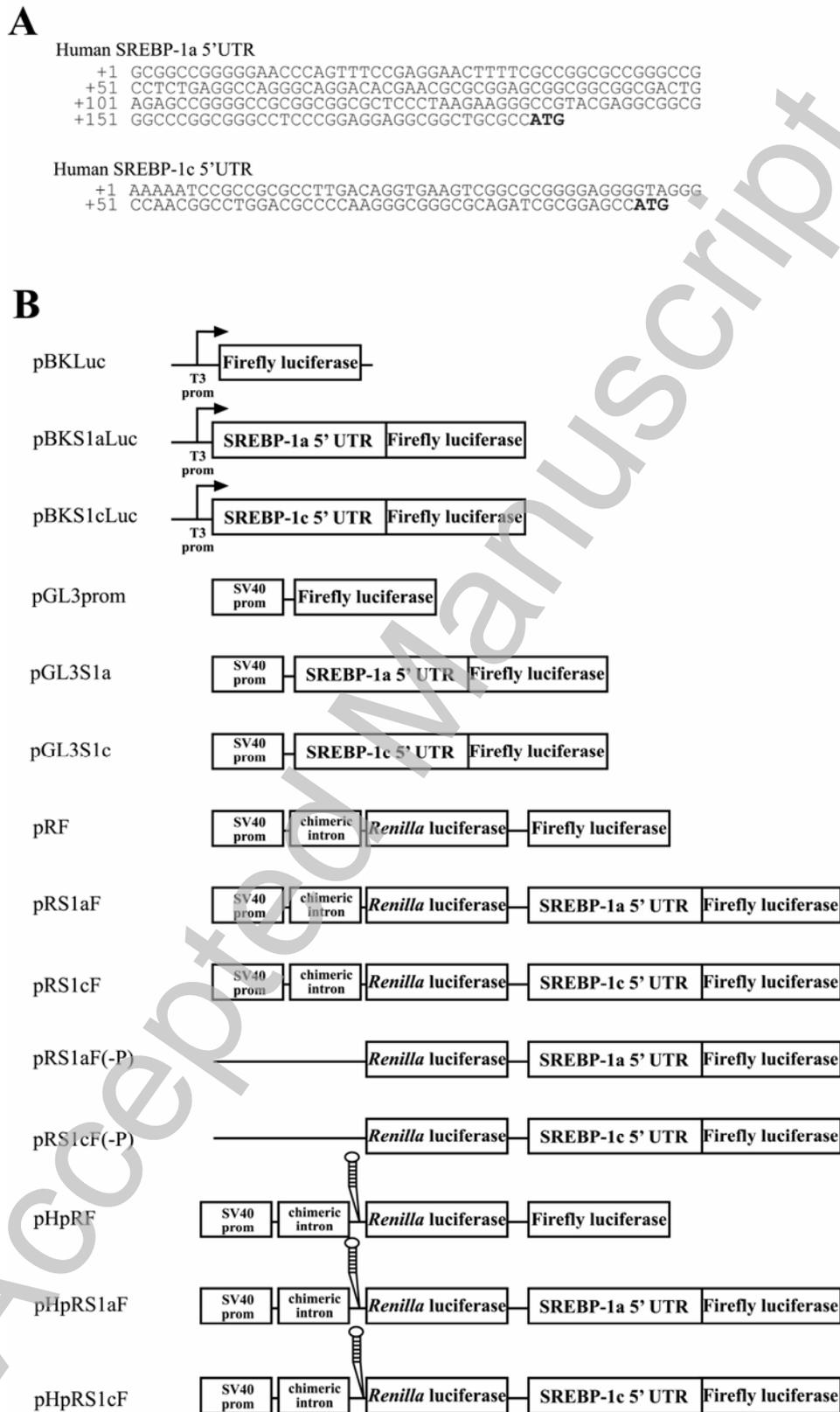
A) Hep G2 cells were treated with 300 nM thapsigargin or 1µg/ml tunicamycin. Western blotting analysis of pSREBP-1 and nSREBP-1 was carried out as described above. The data are from a representative experiment. Similar results were obtained in three independent experiments.

B) Hep G2 cells were treated for the indicated times with 1 µg/ml tunicamycin. SREBP-1, FASN and ACACA mRNA abundance was quantified by real time PCR. The data shown are means (\pm SD) of triplicate samples from each of four independent experiments.

C) Hep G2 cells were transiently transfected with pRS1aF. 24 h after transfection, cells were subjected to ER stress with thapsigargin (300nM) or tunicamycin (1 µg/ml) for the times indicated. Then, the cells were harvested and RL and FL activities were evaluated and reported in histograms as percentage of the corresponding luciferase activities determined in cells harvested at time 0. Data are means (\pm SD) of triplicate samples from each of five independent experiments.

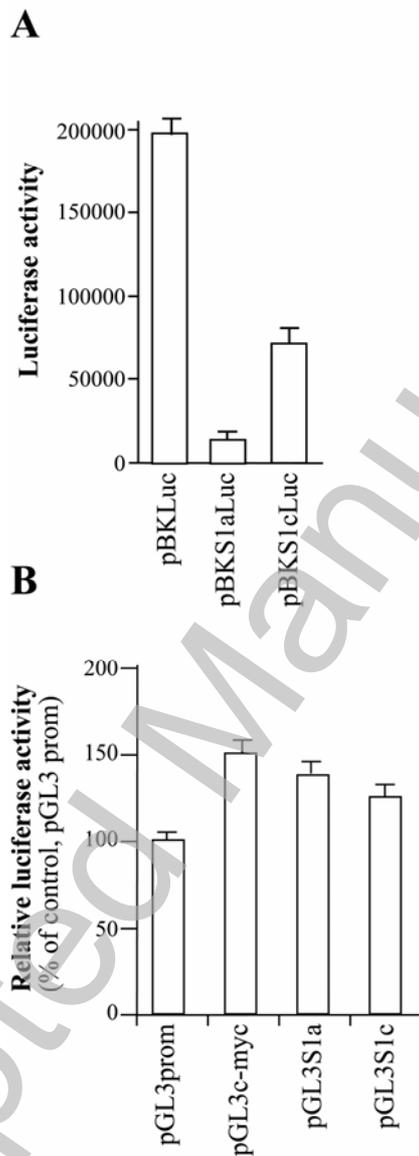
D) Hep G2 cells were incubated for 24 h in DMEM medium with FBS 10%. ER-stress was induced by adding 1 µg/ml tunicamycin to the medium for 4 h, then protein synthesis was blocked by addition of 100 µg/ml cycloheximide. Cells were harvested and the content of either pSREBP-1 or nSREBP-1 protein was analyzed by Western blotting as described above. The semi-log plot represents the decay curve of pSREBP-1 (squares) or nSREBP-1 (circles) protein measured in tunicamycin-treated cells, incubated in the presence (filled) or in the absence (open) of sterols. The data are from a representative experiment. Similar results were obtained in four independent experiments.

Figure 1



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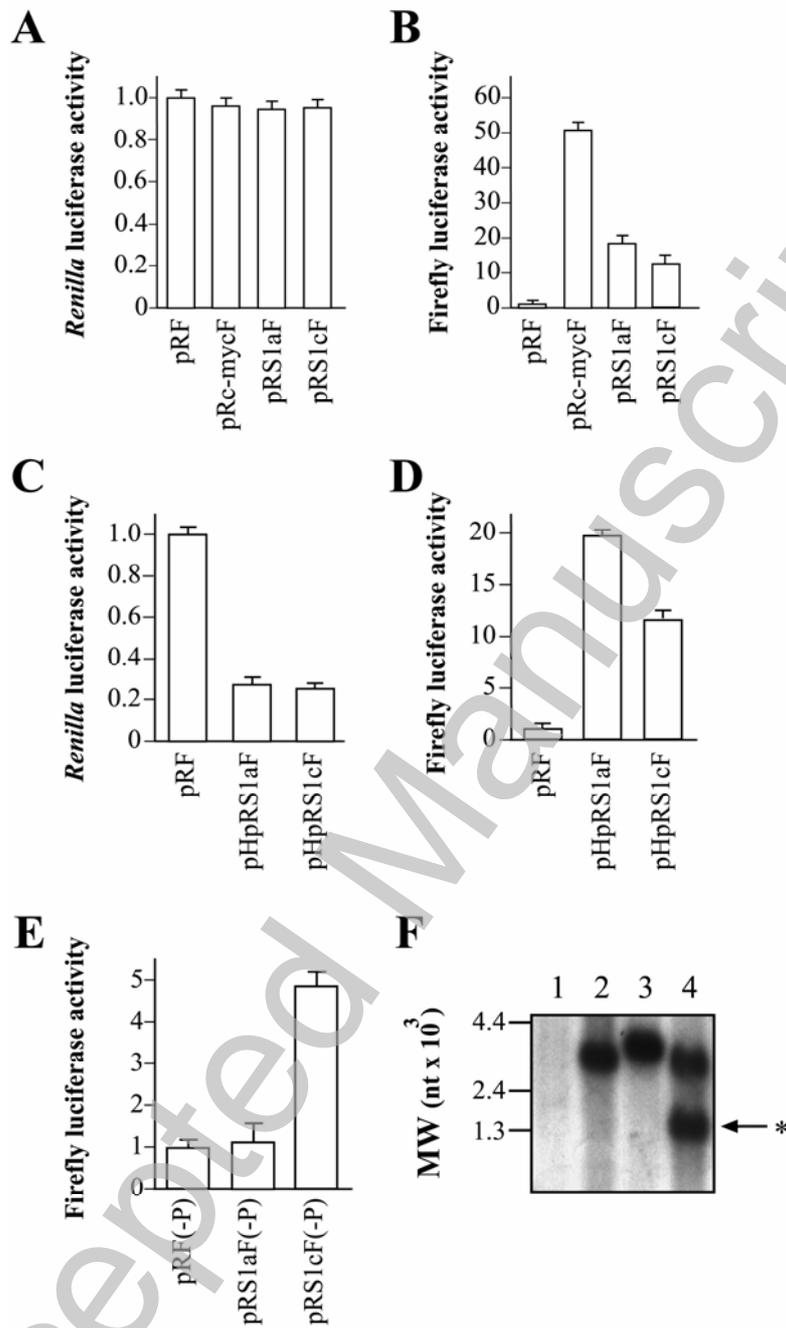
Figure 2



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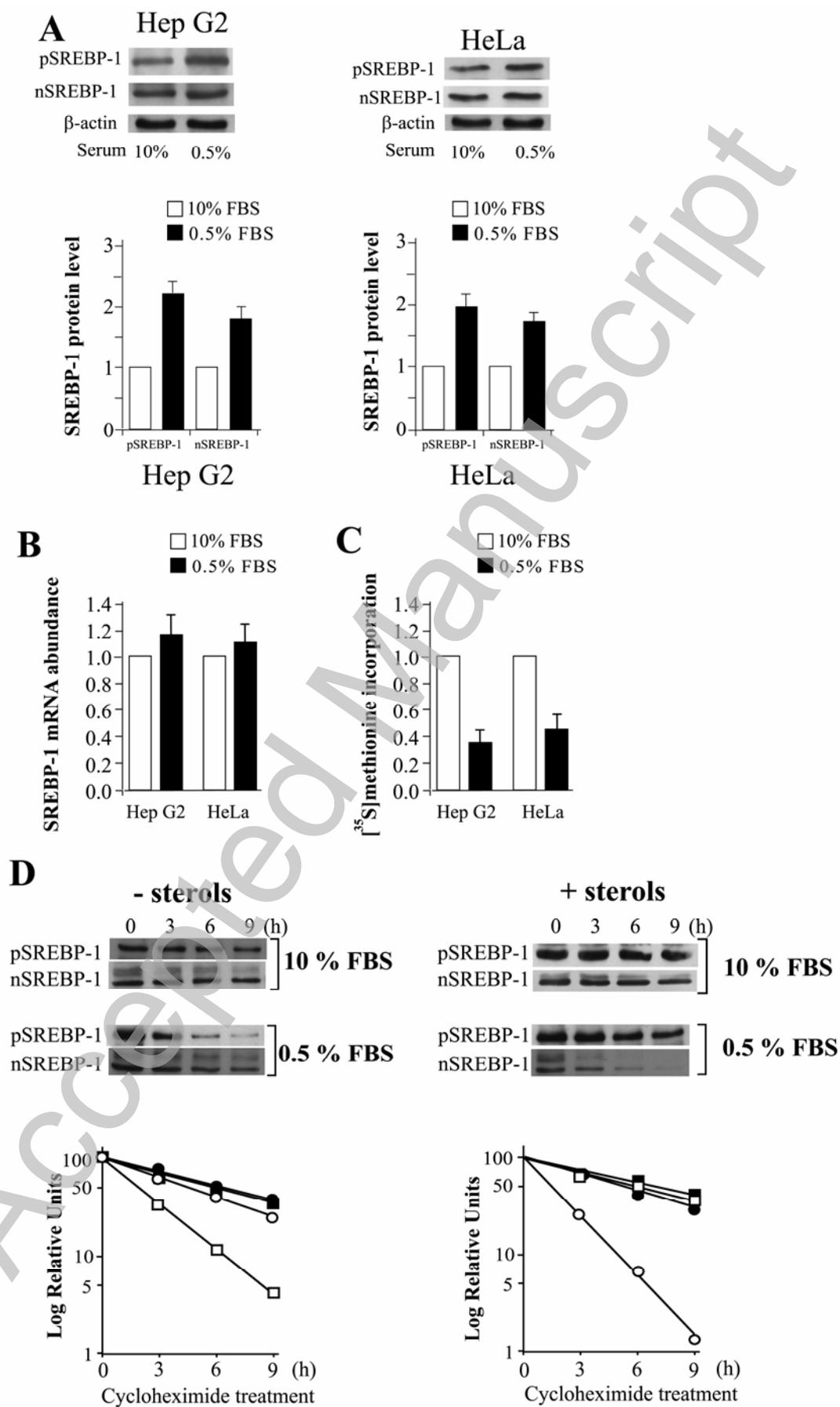
Figure 3



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Figure 4



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Figure 5

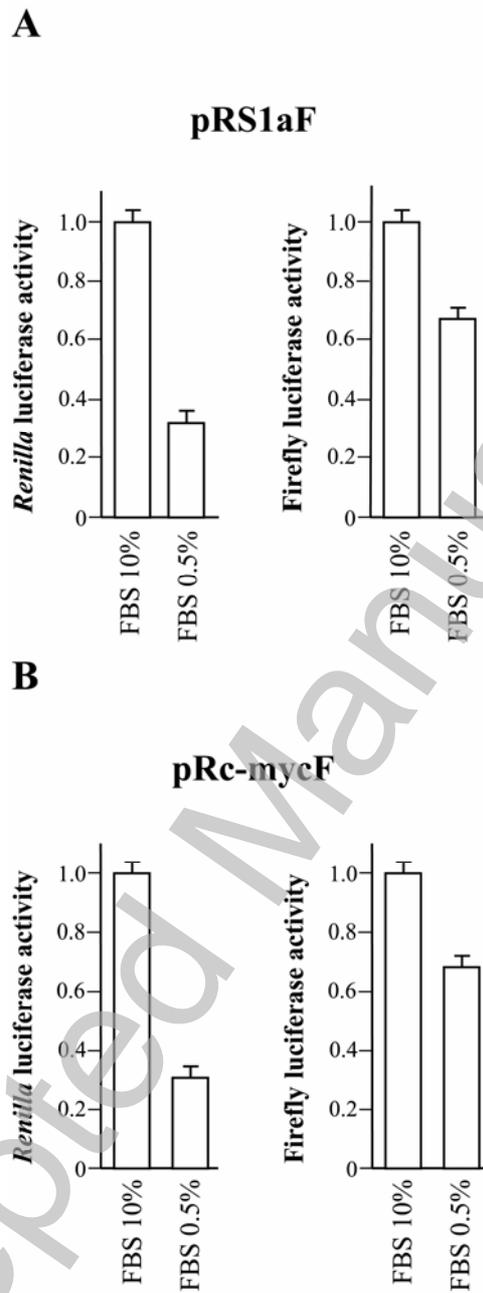
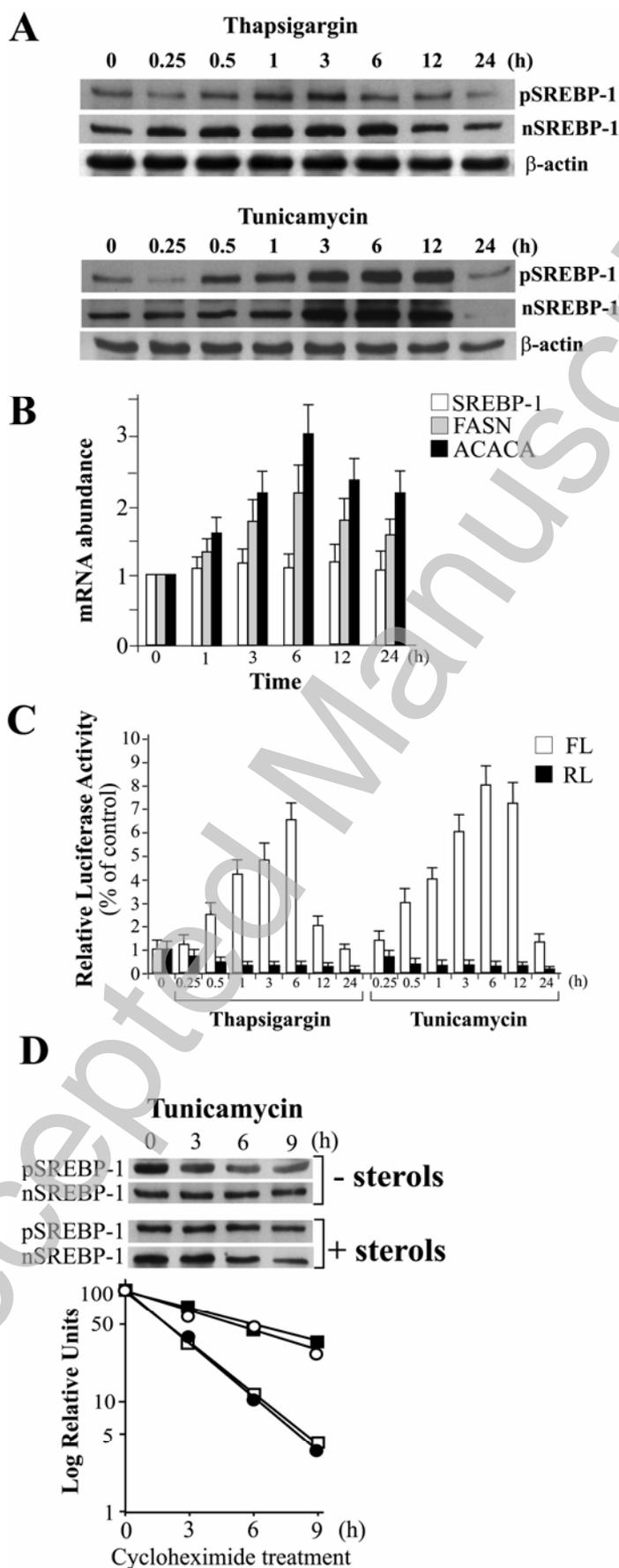


Figure 6



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