Growth-condition-dependent regulation of insulin-like growth factor II mRNA stability

Wiep SCHEPER, P. Elly HOLTHUIZEN* and John S. SUSSENBACH

Laboratory for Physiological Chemistry, Utrecht University, Stratenum, P.O. Box 80042, 3508 TA Utrecht, The Netherlands

Insulin-like growth factor II (IGF-II) is synthesized in many tissues, but the main site of production is the liver. In this paper we show that IGF-II mRNA levels are dependent on the growth conditions of the cells. In Hep3B cells, serum deprivation leads to a marked increase in IGF-II mRNA levels. Serum stimulation of starved Hep3B cells induces a decrease in the amount of IGF-II mRNA, which is not caused by a change in promoter activity. IGF-II mRNAs are subject to endonucleolytic cleavage, a process that requires two widely separated elements in the 3' untranslated region of the mRNA. Specific regions of these elements can form a stable stem structure which is involved in the formation of RNA–protein complexes. By employing electrophoretic mobility

shift assays, two complexes have been identified in cytoplasmic extracts of Hep3B cells. The formation of these complexes is related to the growth conditions of the cells and is correlated with the regulation of IGF-II mRNA levels. Our data suggest that, depending on whether serum is present or absent, a transition from one complex to the other occurs. A decrease in the IGF-II mRNA level is also observed when IGF-I or IGF-II is added to serum-deprived Hep3B cells, possibly providing a feedback mechanism for IGF-II production. The serum-induced degradation of IGF-II mRNAs does not require *de novo* protein synthesis, and is abolished by rapamycin, an inhibitor of p70 S6 kinase.

INTRODUCTION

Human insulin-like growth factor II (IGF-II) is encoded by a single gene containing four promoters, P1–P4, which give rise to a family of mRNAs. These promoters are differentially active in a tissue- and development-specific manner and render mRNAs of 5.3 (P1), 5.0 (P2), 6.0 (P3) and 4.8 (P4) kb respectively [1]. The transcripts share the IGF-II coding region, but have different 5' untranslated regions (UTRs), and show differential translatability [2,3]. In fetal tissues and tumour cells the 6.0 kb mRNA is the major transcript. In a recent study, it was shown that this transcript is efficiently translated only in rapidly growing cells, possibly regulated by the p70/p80 S6 kinase signalling pathway [4].

All IGF-II mRNAs are targets for site-specific endonucleolytic cleavage in the > 4 kb long 3' UTR [5]. This results in an unstable capped 5' cleavage product containing the coding region and a stable uncapped polyadenylated 3' cleavage product of 1.8 kb consisting of the 3' terminal region of the 3' UTR [6]. Two putative functions for the cleavage are possible that are not mutually exclusive: endonucleolytic cleavage may be a first step in the degradation of IGF-II mRNAs, and/or the 1.8 kb cleavage product itself may exert a specific function.

We have identified two *cis*-acting elements in the 3' UTR that are required for cleavage of IGF-II mRNAs [7,8]. These elements are separated by approx. 2 kb of RNA that can be deleted without affecting the cleavage efficiency. Element I is located at positions –2116 to –2013 and contains no remarkable features in its primary or secondary structure. Element II at positions –173 to +150 encompasses the cleavage site and contains two regions that are highly conserved among human, rat and mouse IGF-II mRNAs [7]: two stem loops upstream of the cleavage site

(–139 to –3) and a G-rich region at positions –14 to +60 (positions are relative to the cleavage site, which is set at +1; Figure 1A). Cleavage *in vivo* requires the presence of an intact cleavage unit comprising elements I and II. We have shown that a region of element I (–2108 to –2029) and a region of element II downstream of the cleavage site (+18 to +101) together form a very stable stem structure (Figure 1B) that is conserved among human, rat and mouse IGF-II mRNAs. Both the secondary structure and the sequence of the double-stranded stem structure are important for cleavage [8].

An RNA–protein complex has been identified by electrophoretic mobility shift assays (EMSAs) which is formed specifically with the stem structure and is called the IGF-II cleavage unit RNA–protein complex (ICU-RPC). In this complex, a 50 kDa protein called the ICU binding protein (ICU-BP) is the main protein component that is in direct contact with the RNA [9]. In the human hepatoma cell line Hep3B, which endogenously expresses IGF-II mRNAs, the formation of this protein complex decreases with increasing cell density. This correlates inversely with IGF-II mRNA levels, which are approx. 10-fold higher in confluent cells than in cells grown at 20% density. In parallel with the decreased formation of ICU-RPC, the formation of an as yet unidentified lower mobility complex increases. This complex is not present in cell lines that do not express IGF-II.

Regulation of mRNA stability is a common mechanism of controlling gene expression in eukaryotic cells [10–12]. *Cis*-acting sequences involved in stability regulation are often found in the 3' UTR, although there are also reports of stability determinants present in the coding region or 5' UTR [12,13]. It is clear that, in these processes, *cis*-acting elements in the mRNA interact with specific *trans*-acting factors. Often, the stability of mRNAs is

Abbreviations used: IGF-II, insulin-like growth factor II; UTR, untranslated region; ICU-RPC, IGF-II cleavage unit RNA–protein complex; ICU-BP, IGF-II cleavage unit binding protein; FCS, fetal calf serum; EMSA, electrophoretic mobility shift assay.

* To whom correspondence should be addressed.

Figure 1 Location of the *cis*-acting elements involved in the cleavage of IGF-II mRNAs

(A) Schematic representation of the positions of the *cis*-acting elements involved in endonucleolytic cleavage in the 3' UTR of IGF-II mRNAs. Positions in exon 9 of the human IGF-II gene are indicated relative to the cleavage site, which is set at +1. The stop codon of the IGF-II open reading frame is located at position -2185. Elements I and II are separated by approx. 2 kb (drawing not to scale). Element II encompasses two stem loops (positions -139 to -3), the cleavage site (+1) and a G-rich region at positions -14 to +60 which is highly conserved among human, rat, and mouse [7]. (B) Schematic representation of the structure of the minimal cleavage unit. As was shown previously, the region between elements I and II (positions -2012 to -174) can be deleted without disrupting the cleavage reaction and efficiency [7]. Indicated are the stem structure that is formed by an interaction of element I (positions -2108 to -2029) and element II (+18 to +101), the two stem loops (-139 to -3) in element II and the cleavage site (+1).

regulated by external stimuli such as hormones, growth factors and ions, although the elucidation of pathways transducing the signal from the stimulus to the mRNA is only just evolving [12]. In the present study we have investigated the effect of growth conditions on the stability of the 6.0 kb IGF-II mRNA as well as on the formation of complexes between the stem structure in the 3' UTR of IGF-II mRNAs and specific proteins.

MATERIALS AND METHODS

Materials

Plasmid pBluescript II (KS⁻) was obtained from Stratagene (La Jolla, CA, U.S.A.). Enzymes were purchased from Boehringer Mannheim, with the exception of RNase-free DNase (Promega, Madison, WI, U.S.A.) and RNase T1 (CalBiochem, La Jolla, CA, U.S.A.). Enzymes were used according to the instructions of the manufacturers. NTPs and dNTPs were obtained from Kabi-Pharmacia (Uppsala, Sweden). A random primer DNA labelling kit was purchased from Boehringer Mannheim. Guanidinium thiocyanate was obtained from Fluka (Buchs, Switzerland), and GeneScreen membranes were from du Pont de Nemours (Dreieich, Germany). [α -³²P]dCTP (3000 Ci/mmol), [γ -³²P]ATP (3000 Ci/mmol) and [α -³²P]CTP (760 Ci/mmol) were purchased from Amersham International (Amersham, Bucks., U.K.). IGF-I and IGF-II were obtained from GroPep (Adelaide, Australia), rapamycin was a gift from Dr. D. Roelen (AZL, Leiden, The Netherlands) and cycloheximide was obtained from Sigma (St. Louis, MO, U.S.A.).

Cell culture and extract preparation

Hep3B cells were grown to a density of approx. 50% confluence in α -minimal essential medium supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin and 300 μ g/ml glutamine in

the presence or absence of 10% (v/v) fetal calf serum (FCS). Cytoplasmic extracts were prepared employing a method adapted from that of Vakalopoulou et al. [14] with slight modifications. Briefly, the cells were lysed in 10 mM Tris, pH 8.0, 10 mM NaCl, 3 mM MgCl₂, 1 mM DTT, 1 mM PMSF, 0.5% Nonidet P40 and 0.5% sodium deoxycholate on ice for 10 min. Subsequently, the lysate was spun in a Microfuge at 14000 *g* for 5 min at 4°C and the supernatant was collected and used for cytoplasmic RNA isolation or stored at -80°C in 10% glycerol (cytoplasmic extract). Protein concentrations were determined by the Bradford protein assay (Bio-Rad, München, Germany).

Cellular RNA isolation and analysis

Total RNA was isolated from Hep3B cells by the single-step guanidinium thiocyanate method [15]. A sample of 10 μ g of RNA was glyoxalated and size-separated on a 1% agarose/10 mM sodium phosphate gel and transferred to a GeneScreen membrane. The RNA was fixed on the membrane by irradiation with long-wavelength UV light for 2.5 min and baking at 80°C for 2 h. Northern blots were hybridized in the presence of 50% formamide according to GeneScreen protocols in glass cylinders with continuous rotation at 42°C. A 1.0 kb *Sma*I fragment from human IGF-II exon 9 (positions +81 to +1094) was used as a probe to detect IGF-II mRNAs and the 3' specific cleavage product. The fragment was labelled by random priming with [α -³²P]dCTP and added after 3 h of prehybridization at a final concentration of 10⁶ c.p.m./ml. After overnight hybridization, blots were washed to a final stringency of 0.1 $\times$ SSC/1% SDS at 65°C (1 $\times$ SSC is 0.15 M NaCl, 0.015 M sodium citrate) and exposed on Fuji RX X-ray film. A 26 nt long oligonucleotide, 5'-AACGATCAGAGTAGTGGTATTTCACC-3', was used as a 28 S rRNA probe, and labelled with T4 polynucleotide kinase and [γ -³²P]ATP. Hybridization conditions for this probe were

identical to those for the IGF-II probe except for the formamide concentration (25 %). Blots were washed for 30 min in $2 \times \text{SSC}/1\%$ SDS and 30 min in $1 \times \text{SSC}/1\%$ SDS at 30°C . The autoradiographs were analysed by densitometric scanning.

Transfections

Hep3B cells were transfected at a density of approx. 50 % confluence in 25 cm^2 flasks using the calcium phosphate coprecipitation method [16]. Each transfection mixture contained $3\text{ }\mu\text{g}$ of a (thymidine kinase promoter)–luciferase fusion construct or a luciferase reporter construct containing the *Bss*HII/*Nar*I promoter 3 fragment (positions -1183 to -7 , relative to the transcription start site) of IGF-II. This construct was obtained by cloning the *Bss*HII fragment into the *Pst*I/*Bss*HII sites of pFLAG $-540/-7$ [17]. To correct for differences in transfection efficiency, 500 ng of pRSV–LacZ was included. The cells were shocked 4 h after addition of the precipitate in 10 % (v/v) DMSO for 1.5 min and allowed to recover in medium containing 10 % (v/v) FCS. After 4 h, the medium was replaced by either serum-free medium or medium containing 10 % (v/v) FCS and the cells were cultured for 16–17 h. Subsequently the cells were harvested or stimulated with 10 % (v/v) FCS and grown for an additional 6 h before harvesting. Preparation of enzyme extracts and luciferase and β -galactosidase assays were performed as described [18,19]. Luciferase values were determined using a Lumac/3M biocounter M2010A.

Synthesis of RNA probes

Radiolabelled RNA probes were synthesized using T7 RNA polymerase on linearized DNA templates, according to the instructions of the manufacturer, in the presence of 1 mM of each of ATP, GTP and UTP and $20\text{ }\mu\text{Ci}$ of $[\alpha\text{-}^{32}\text{P}]\text{CTP}/0.1\text{ mM}$ CTP. As templates for the synthesis of RNAs, BS constructs containing IGF-II exon 9 inserts were used. The starting construct BS-S/S contains the region -2116 to -2013 (element I) linked to the region -173 to $+169$ (element II). In BS-AS/S element I is inverted, and in BS-AS/AS both element I and the region $+12$ to $+82$ from element II are inverted. BS-AS/AS Δ was prepared by deletion of nucleotides -104 to -15 from BS-AS/AS. BS-II contains the region -173 to $+169$. After linearization of these constructs with *Eco*RI (at position $+170$), these templates were used for the synthesis of S/S, AS/S, AS/AS, AS/AS Δ and II RNAs respectively. Linearization of BS-S/S with *Bgl*II (at position -110) results in a template for the synthesis of RNA I. The pBluescript constructs used as templates for the synthesis of RNA probes are described in detail in [8,9]. After synthesis (1 h), the template was removed by DNaseI treatment (1 unit in $20\text{ }\mu\text{l}$ of reaction mixture for 15 min at 37°C) and the RNA was separated from unincorporated nucleotides by Sephadex G25 spin dialysis. Subsequently, the samples were phenol/chloroform-extracted, ethanol-precipitated, washed in 70 % (v/v) ethanol and renatured in renaturation buffer (20 mM Tris/HCl, pH 7.5, 100 mM KCl, 2 mM MgCl_2) for 5 min at 90°C and then 30 min on ice. The integrity of the RNAs was checked by gel electrophoresis.

Analysis of RNA–protein interactions

For EMSAs, radiolabelled RNA probes (10^5 c.p.m.) were incubated with cytoplasmic extracts from Hep3B cells grown under different conditions, with a protein content of $10\text{ }\mu\text{g}$ in a $10\text{ }\mu\text{l}$ reaction mixture containing 20 mM Hepes/KOH, pH 7.5, 100 mM KCl, 1 mM MgCl_2 , 1 mM dithiothreitol and 0.01 %

Nonidet P40, for 45 min on ice. After the binding reaction, the samples were incubated at room temperature for 20 min with a mixture of $12.5\text{ }\mu\text{g/ml}$ RNase A and 2 units/ml RNase T1. Samples were analysed on a non-denaturing 6 % polyacrylamide gel containing 0.01 % Nonidet P40.

The molecular mass of the protein present in ICU-RPC2 was determined as described in [20]. First, the complex was UV cross-linked (254 nm ; 5 J/cm^2) in an EMSA gel and a gel slice containing this complex (localized by wet gel autoradiography) was excised. Subsequently the gel slice was inserted into a 10 % (w/v) polyacrylamide gel in the presence of SDS and subjected to electrophoresis with $[^{14}\text{C}]$ methylated protein markers (Amersham) loaded in parallel.

RESULTS

IGF-II mRNA levels are dependent on the growth conditions of the cells

It was observed previously that the level of the major 6.0 kb IGF-II mRNA is 10-fold lower in Hep3B cells grown at low density than in fully confluent cells [9]. This indicates that the IGF-II mRNA levels are dependent on the growth state of the cells. To investigate this phenomenon in more detail, we studied the IGF-II mRNA levels in cells grown in the presence or in the absence of 10 % (v/v) FCS for 16–17 h. Subsequently, starved cells were refed with 10 % FCS and RNA was isolated at different time points thereafter up to 6 h. The samples were analysed by Northern blotting of total RNA, followed by hybridization with a probe specific for the 3' part of the IGF-II mRNA. This shows that the levels of the 6.0 kb mRNA and the 1.8 kb cleavage product were markedly increased in the starved cells compared with cells grown in 10 % FCS (Figure 2).

Upon refeeding of the serum-deprived cells, the level of the

Figure 2 The IGF-II mRNA level is dependent on the growth conditions of the cells

Northern blot analysis of total RNA isolated from Hep3B cells grown under different conditions. Cells were cultured in the presence (S+) or absence (S–) of 10 % (v/v) FCS for 16–17 h (ON). Starved cells were stimulated with 10 % FCS and RNA was isolated at different time points thereafter (30, 60, 180 and 360 min S+). Samples of $10\text{ }\mu\text{g}$ of RNA were loaded in each lane and the blot was hybridized with the 3'–specific probe ($+81$ to $+1094$; positions are relative to the cleavage site, which is set at $+1$) that detects full-length IGF-II mRNAs and the 3' cleavage product of 1.8 kb. Sizes of the RNAs are indicated in kb. In the lower panel the 28 S rRNA hybridization of the same blot is shown.

Table 1 Serum down-regulates IGF-II mRNA levels

Quantitative representation of the IGF-II 6.0 kb mRNA level 3 and 6 h after administration of 10% (v/v) FCS to serum-starved Hep3B cells. Data were obtained from three separate experiments by densitometric scanning of the autoradiographs, and are means $\pm$ S.E.M. RNA loading differences were normalized after hybridization with a 28 S rRNA probe. The amount of 6.0 kb mRNA in starved cells (0 h) was set at 100%.

Time after refeeding (h)	6.0 kb mRNA/28 S rRNA (%)
0	100
3	56 $\pm$ 7
6	31 $\pm$ 11

6.0 kb mRNA decreased (Figure 2; Table 1). There was a decrease to 56% of the level in serum-deprived cells within 3 h, and a further decrease to 31% at 6 h after serum administration (Table 1; the level of 6.0 kb mRNA in serum-deprived cells at 0 min was set at 100%). Because the level of the 1.8 kb RNA decreased more slowly than that of the 6.0 kb mRNA, the ratio of the 1.8 kb cleavage product to the 6.0 kb mRNA increased upon serum administration, up to 1.6-fold at 6 h.

The serum-induced down-regulation of IGF-II mRNA levels is not caused by a decrease in transcriptional activity

The lower levels of IGF-II mRNA observed in cells grown in 10% FCS compared with starved cells could be due either to lower promoter activity or to decreased stability of the transcripts. We have attempted to study the stability of the mRNA directly in starved and refed cells by employing actinomycin D. Unfortunately, this agent is not suitable for half-life determinations of IGF-II mRNA, because it affects mRNA degradation in general (results not shown). This effect of transcriptional inhibitors on the RNA degradation machinery has also been reported for other systems [13].

To study IGF-II promoter activity, we employed transient transfection assays of Hep3B cells using a luciferase reporter construct containing a region of the IGF-II promoter 3 (P3; positions -1183 to -7) [17]. The promoter activity determined by this assay can be interpreted as the transcriptional activity of P3 under various conditions. Hep3B cells were transfected with the P3-luciferase construct and with an RSV-LacZ fusion construct to control for transfection efficiency, and the cells were cultured for 16–17 h in either serum-free medium or medium containing 10% FCS. The luciferase values were lower in starved cells than in cells grown overnight in 10% FCS (Table 2). At 6 h after refeeding of the starved cells, the P3 activity had reached a level similar to that in cells grown overnight in 10% FCS (Table 2). This indicates that, in the absence of serum, IGF-II P3 promoter activity is down-regulated. Similar behaviour was found for a (thymidine kinase promoter)-luciferase fusion construct, suggesting that transcription in general is inhibited in Hep3B cells deprived of serum (Table 2).

The activity of P3 was higher in cells grown in 10% FCS than in serum-deprived cells (Table 2). In contrast, the level of the 6.0 kb mRNA was lower in the presence of serum (Figure 2; Table 1). This shows that the effects of serum on the activity of P3 and on the level of the 6.0 kb mRNA are opposite to each other. Obviously, changes in transcriptional activity are not responsible for the observed serum-dependent differences in IGF-II mRNA abundance, and consequently these effects must

Table 2 Serum administration stimulates transcriptional activity

Results of transient transfections with an (IGF-II P3)-luciferase fusion construct (P3) and a (thymidine kinase promoter)-luciferase fusion construct (TK) in Hep3B cells grown under different conditions: 16–17 h in the presence (S+) or absence (S-) of 10% (v/v) FCS, and 6 h after refeeding (Refed). The luciferase values were set at 100% for cells grown in 10% FCS (S+) for both P3 and TK. Variations in transfection efficiency were corrected for by using RSV-LacZ as an internal control. Data were obtained from three (P3) and one (TK) experiments in triplicate, and are means $\pm$ S.E.M.

Conditions	Transcription (%)	
	P3	TK
S+	100 $\pm$ 12	100 $\pm$ 16
S-	64 $\pm$ 12	59 $\pm$ 2
Refed	102 $\pm$ 36	88 $\pm$ 7

be due to changes in mRNA stability. In conclusion, these data suggest that serum stimulation induces degradation of IGF-II mRNAs.

ICU-RPC formation is dependent on the growth conditions of the cells

We have identified two RNA-protein complexes in Hep3B cells that form specifically with the stem structure in the 3' UTR of IGF-II mRNAs and are dependent on the cell density. The higher-mobility complex can be detected in several cell lines, irrespective of the presence of endogenous IGF-II mRNA expression. The lower-mobility complex is not found in cells that do not express IGF-II, but can be detected under certain conditions in the Hep3B cell line [9]. We will denote the complex with the higher mobility as ICU-RPC1 (ICU-RPC in [9]) and the other complex as ICU-RPC2 (not previously named). The formation of ICU-RPC1 in cytoplasmic Hep3B extracts gradually decreased with increasing cell density. In parallel with the decreased formation of ICU-RPC1, the formation of ICU-RPC2 was increased [9]. These changes in the formation of RNA-protein complexes are correlated inversely with IGF-II mRNA levels, which are approx. 10-fold higher in confluent cells than in cells grown at 20% density [9].

In the present study we have shown that IGF-II mRNA levels in Hep3B cells are also regulated by serum (Figure 2; Table 1). Therefore we have investigated whether serum also affects the formation of RNA-protein complexes containing the cleavage unit. To study this, Hep3B cells were grown to a density of approx. 50% confluence. Cells from parallel dishes were cultured for 16–17 h in medium either containing or lacking 10% FCS, and subsequently cytoplasmic extracts were prepared. Furthermore, starved cells were refed with 10% FCS and harvested at different time points after serum administration. In order to study the formation of ICU-RPC1 and ICU-RPC2 under these different growth conditions, the various extracts were analysed in an EMSA using a radiolabelled RNA probe consisting of both elements required for cleavage of IGF-II mRNAs (probe I/II). With extracts from cells grown under normal conditions, both ICU-RPC1 and ICU-RPC2 were formed (Figure 3). In contrast, with extracts from starved cells, practically no ICU-RPC1 was formed, whereas the amount of ICU-RPC2 was increased. At only 30 min after serum stimulation the binding of ICU-RPC1 was greatly increased, whereas ICU-RPC2 formation had resumed the same level as in cells grown in 10% FCS overnight. At later time points, up to 6 h, the ICU-RPC1 level approached

Figure 3 ICU-RPC formation is dependent on the growth conditions of the cells

EMSA of cytoplasmic extracts from Hep3B cells grown under different conditions and probe I/II. Cells were cultured in the presence (S+) or absence (S-) of 10% (v/v) FCS for 16–17 h (ON). Starved cells were stimulated with 10% FCS and extracts were prepared at different time points thereafter (30, 60, 180 and 360 min S+). ICU-RPC1 and ICU-RPC2 are indicated by arrows.

Figure 4 Characteristics of ICU-RPC2

(A) EMSA of cytoplasmic extract from Hep3B cells grown in 10% (v/v) FCS to a density of 50% confluence, and different cleavage unit mutants as probe. The probes used are indicated above the lanes: S/S (wild type), I (element I only), II (element II only), AS/S (element I in the antisense configuration), AS/AS (both parts of the stem structure in the antisense configuration) and AS/ASΔ (AS/AS with the stem loops deleted). ICU-RPC1 and ICU-RPC2 are indicated by arrows. (B) Size determination of the protein component of ICU-RPC2. [α - 32 P]CTP-labelled RNA was incubated with Hep3B cytoplasmic extract, treated with RNase and run on a non-denaturing gel. Subsequently the gel was UV-irradiated. ICU-RPC2 was then removed from the gel and analysed by electrophoresis on a SDS/10%-PAGE gel. The apparent size of the protein, designated ICU-BP2, was determined to be approx. 50 kDa. The sizes of 14 C-labelled molecular mass markers are indicated on the left in kDa.

that in cells grown in 10% FCS. No additional changes occurred in ICU-RPC2 formation (Figure 3). A minor band was sometimes observed between ICU-RPC1 and ICU-RPC2; this was probably due to incomplete digestion by RNase. The greatest change in ICU-RPC formation was observed after only 30 min, indicating that changes in the binding of ICU-RPC1 and ICU-RPC2 occur rapidly.

ICU-RPC1 and ICU-RPC2 share RNA binding properties

In Hep3B cells grown at different densities [9] or under different serum conditions (Figure 3), the formation of ICU-RPC1 is inversely correlated with that of ICU-RPC2. To investigate the

relationship between ICU-RPC1 and ICU-RPC2 in more detail, we first compared the binding specificities of the two complexes. For this purpose cytoplasmic extracts were prepared from Hep3B cells grown to a confluence of approx. 50%. We performed an EMSA with several cleavage unit mutants as probes (described in detail in [8,9]): S/S [contains element I (positions -2116 to -2013) and element II (-173 to +169) in the wild-type configurations; see Figure 1B], I (element I only), II (element II only), AS/S (element I in the antisense configuration; element II wild type) and AS/AS (both element I and region +13 to +83 in element II in the antisense configuration, resulting in an inverted stem structure). ICU-RPC1 and ICU-RPC2 both exhibited a similar binding specificity: since they are formed efficiently with the combination of the elements (S/S) but not with the separate elements (I or II), they obviously require both elements I and II (Figure 4A). The complexes are also not formed with the AS/S mutant, which cannot form the stem structure. In the AS/AS mutant the interaction between the elements is restored, and with this RNA complex-formation was as efficient as with S/S RNA. These results indicate that the RNA-protein complexes are formed with the stem structure but, since the stem loops upstream of the cleavage site are stabilized by the presence of the stem [8], the possibility cannot be excluded that the complexes require these stem loops. Therefore the AS/ASΔ mutant (AS/AS with the region -104 to -15 containing the stem loops deleted) was used in an EMSA. This RNA also gave rise to the formation of both ICU-RPC1 and ICU-RPC2, albeit with lower efficiency (Figure 4A). We conclude that both ICU-RPC1 and ICU-RPC2 form with the stem structure between elements I and II. Formation of both ICU-RPC1 and ICU-RPC2 in itself does not require the stem loops upstream of the cleavage site. However, in view of the lower efficiency of complex-formation with the AS/ASΔ RNA, these structures may facilitate the formation of ICU-RPC1 and ICU-RPC2 on the stem structure.

We have shown previously that the main protein component of ICU-RPC1 that directly contacts the stem structure is a protein with an apparent molecular mass of approx. 50 kDa, which we named ICU-BP [9]. This molecular mass determination was performed by UV cross-linking a gel slice containing ICU-RPC1, followed by separation of cross-linked proteins on a denaturing protein gel. The same method was applied to analyse ICU-RPC2 (Figure 4B). The protein that directly contacts the RNA in this complex (ICU-BP2) also has an apparent molecular mass of approx. 50 kDa. Comparing ICU-RPC1 and ICU-RPC2, we can conclude that they are both formed with the stem structure present between elements I and II, and that in both complexes a protein of approx. 50 kDa is in direct contact with the RNA.

Administration of IGFs mimics the effect of serum on IGF-II mRNA levels

Administration of serum to starved cells induces a decrease in the level of the 6.0 kb mRNA, but it is not clear which component in serum exerts this effect. An interesting possibility is that it is mediated by growth factor(s) in serum, maybe even the IGFs themselves. This could then provide a negative-feedback mechanism for the production of IGF-II. To study this, Hep3B cells were deprived of serum for 16–17 h and subsequently stimulated for 6 h with 10% FCS or 10 ng/ml IGF-I or IGF-II. The IGFs down-regulated the level of the 6.0 kb mRNA in starved cells to 29% (IGF-I) and 34% (IGF-II) of the control (Table 3). This implies that physiological levels of both IGFs can induce a 3-fold decrease in IGF-II mRNA levels.

Table 3 IGFs can mimic the effect of serum on the level of IGF-II mRNA

Quantitative representation of Northern blot analysis of total RNA isolated from Hep3B cells grown under different conditions. Cells were cultured in the absence of 10% (v/v) FCS for 16–17 h (S–). Subsequently, the starved cells were stimulated with 10% FCS (S+), 10 ng/ml IGF-I or 10 ng/ml IGF-II, and RNA was isolated after 6 h. RNA samples were analysed by Northern blotting and hybridized with the 3'-specific probe (positions +81 to +1094). The amount of 6.0 kb mRNA was quantified by densitometric scanning of the autoradiograph and normalized for RNA loading differences by hybridization with a 28 S rRNA probe. The amount of 6.0 kb mRNA in starved cells (S–) was set at 100%. Data are means $\pm$ S.E.M. of at least four different observations in three separate experiments.

Addition	6.0 kb mRNA/28 S rRNA (%)
S–	100
S+	31 $\pm$ 11
IGF-I	29 $\pm$ 21
IGF-II	34 $\pm$ 25

Figure 5 The serum-induced degradation of IGF-II mRNAs does not require *de novo* protein synthesis, and is abolished by rapamycin

Northern blot analysis of total RNA isolated from Hep3B cells grown under different conditions. Cells were cultured in the absence (S–) of 10% (v/v) FCS for 16–17 h. Subsequently the starved cells were stimulated with 10% FCS for 6 h (6 h S+) without other additions (–) or in the presence of either 20 μ g/ml cycloheximide (CHX) or 20 ng/ml rapamycin (RM), and RNA was isolated after 6 h. Samples of 10 μ g of RNA were loaded in each lane and the blot was hybridized with the 3'-specific probe (+81 to +1094). In the lower panel the 28 S rRNA hybridization of the same blot is shown.

Growth-factor-regulated stability of IGF-II mRNAs is mediated by a pathway involving p70 S6 kinase

Within 6 h after the administration of serum or IGFs to Hep3B cells, the IGF-II mRNA level had decreased 3-fold. The mechanism by which growth factor administration triggers the degradation of IGF-II mRNAs is not known. In order to learn more about the mechanism, we first investigated whether *de novo* protein synthesis is required for the serum-induced decrease in IGF-II mRNA levels. Starved Hep3B cells were serum-stimulated in the presence of the translational inhibitor cycloheximide. This treatment did not affect the serum-induced mRNA degradation, indicating that *de novo* protein synthesis is not required (Figure 5).

Translation of the 6.0 kb mRNA has also been shown to be

regulated in a growth-dependent manner. The transcript is translated efficiently only in actively growing cells. This effect was shown to be mediated by a signal transduction pathway involving p70 S6 kinase (p70^{S6k}) [4], since it could be inhibited by rapamycin, a specific inhibitor of this kinase [21,22]. For many mRNAs a coupling between translation and degradation of the mRNA is observed [12]. Therefore we investigated whether the degradation of the 6.0 kb mRNA upon growth factor stimulation also involves the p70^{S6k} pathway. To study this, we stimulated serum-deprived Hep3B cells for 6 h with 10% FCS in the presence of rapamycin, which completely abolished the serum-induced decrease in the level of the 6.0 kb mRNA (Figure 5). This suggests that serum/growth-factor-induced activation of p70^{S6k} can lead to degradation of IGF-II mRNAs.

DISCUSSION

Previously we have shown that IGF-II mRNA levels in Hep3B cells increase with increasing cell density [9]. In the present paper we extend the study of the growth-dependent regulation of IGF-II mRNA levels to serum dependence. In Hep3B cells deprived of serum the IGF-II mRNA level is greatly increased compared with that in cells grown in 10% (v/v) FCS. If the starved cells are refed by administration of 10% FCS, the amount of mRNA decreases, by approx. 3-fold in 6 h. Transfections with an (IGF-II promoter P3)–luciferase reporter construct indicate that a change in transcription is not responsible for the differences observed in the mRNA level, but that most probably IGF-II mRNAs are less stable in the presence of serum.

Human IGF-II mRNAs are a target for site-specific endonucleolytic cleavage in the 3' UTR. The increase in the 1.8 kb cleavage product relative to the 6.0 kb mRNA seen after serum induction suggests that endonucleolytic cleavage is the mechanism by which IGF-II mRNAs are degraded in the presence of serum, although alternative explanations are possible. The involvement of cleavage is further substantiated by the observation that the formation of RNA–protein complexes with *cis*-acting elements that are involved in cleavage (ICU-RPC1 and ICU-RPC2) changes in response to serum in a manner that correlates with the IGF-II mRNA levels. After serum administration, a transition from ICU-RPC2 to ICU-RPC1 takes place. This change takes place rapidly; therefore it is likely to occur post-translationally. Interestingly, the serum-induced degradation of IGF-II mRNA takes place in the absence of *de novo* protein synthesis, in good accordance with this rapid change in ICU-RPC formation. High mRNA levels are correlated with low ICU-RPC1 levels and high ICU-RPC2 levels, suggesting that ICU-RPC1 is an RNA–protein complex that facilitates cleavage.

ICU-RPC2 is not found in cell lines that do not endogenously express IGF-II [9]. To further investigate the properties of ICU-RPC2, we studied the requirements for the formation of this complex. We have shown previously that the stem structure formed between the regions of elements I and II located at positions –2108 to –2029 and +18 to +101 respectively appear to be involved in ICU-RPC1 formation [9]. By employing cleavage unit mutants we have shown that the formation of ICU-RPC2 also requires this stem structure. Since deletion of the stem loops upstream of the cleavage site reduces the efficiency of formation of both RNA–protein complexes, these structures may facilitate ICU-RPC formation, although their presence is not a prerequisite for complex-formation. The protein that interacts directly with the RNA in ICU-RPC2 (ICU-BP2) has a similar size on an SDS/polyacrylamide gel as ICU-BP, i.e. 50 kDa. Taken together, these results suggest that ICU-BP is present in both ICU-RPC1 and ICU-RPC2. The two complexes

Figure 6 Negative-feedback model for growth-condition-dependent degradation of IGF-II mRNAs

IGF-II activates a signalling pathway that involves $p70^{S6k}$. This leads to a transition from ICU-RPC2 to ICU-RPC1, which results in endonucleolytic cleavage of the mRNA, thereby reducing the amount of IGF-II mRNA and eventually the amount of IGF-II.

may represent different glycosylation or phosphorylation states of the same protein(s). Alternatively, ICU-RPC2 may be derived from ICU-RPC1 by binding of an additional protein. Further studies are required to address these possibilities.

IGF-II is known to act in an autocrine/paracrine manner. We hypothesize that the down-regulation of IGF-II mRNA in response to serum is a negative-feedback mechanism for the production of IGF-II. This is strengthened by the observation that addition of IGF-I or IGF-II to the culture medium also induces a decrease in IGF-II mRNA levels. Some other proteins such as β -tubulin [23] and histones [24,25] have been reported to regulate their expression via negative feedback on the stability of their own mRNA. The present study indicates that IGF-II expression may also be regulated in this way.

Nielsen et al. [4] recently showed that the 6.0 kb IGF-II mRNA is translated efficiently only in exponentially growing cells. This growth-dependent regulation of translation is sensitive to rapamycin, an inhibitor of $p70^{S6k}$ [4]. In many systems there is coupling between translation and the stability of an mRNA, e.g. *c-myc* mRNA [26], *c-fos* mRNA [27], β -tubulin mRNA [28] and several others (reviewed in [12,29]). Therefore we investigated the role of the $p70^{S6k}$ signalling pathway in the growth-condition-dependent degradation of the 6.0 kb IGF-II mRNA. We found that rapamycin prevents the serum-induced degradation of IGF-II mRNA, suggesting that $p70^{S6k}$ is involved in transduction of the signal for degradation. The data in the present paper, combined with the data obtained by Nielsen et al. [4], suggest a highly regulated post-transcriptional system for the production of IGF-II. Rapidly growing cells require IGF-II, and therefore the mRNA is translated efficiently. This causes an increased protein level, which in turn induces down-regulation of the mRNA level by mRNA degradation. In quiescent cells the level of IGF-II mRNA is high, so if a mitogenic signal is received a large amount of mRNA is directed to the polysomes. The amount of mRNA will then be down-regulated to prevent overproduction of IGF-II. This may occur by a growth-factor-

induced transition from ICU-RPC2 to ICU-RPC1, mediated by a pathway involving $p70^{S6k}$, thereby facilitating cleavage of the IGF-II mRNAs (Figure 6). Interestingly, it has been reported that the type I IGF receptor can signal to $p70^{S6k}$ [30]. The present study indicates that mRNA stability provides an additional level of control in the regulation of IGF-II expression.

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