Translation initiation on the insulin-like growth factor II leader 1 is developmentally regulated

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The majority of cellular mRNAs have relatively short and unstructured 5' untranslated regions (UTRs) that allow efficient translation, such as the β -globin mRNA. An exception to this rule is the group of growth factor mRNAs which, in general, have long 5' UTRs with a high G+C content. An example is insulin-like growth factor II (IGF-II), which is encoded by four mRNAs, arising from four different promoters. Transcripts having the human IGF-II leader 1 are only expressed in adult liver where IGF-II protein synthesis is solely under direction of this 5' UTR. We investigated the translational efficiency *in vitro* of this 5' UTR, linked to the chloramphenicol acetyltransferase (CAT) encoding region. As expected from the primary structure of IGF-II leader 1, translational efficiency was very low compared

with β -globin 5' UTR–CAT mRNA. Addition of cell extract from undifferentiated P19 embryonal carcinoma (EC) cells preferentially stimulated translation of an IGF-II 5' UTR RNA construct. No translational stimulation was found when cell extract from differentiated P19 EC cells was added. In contrast with the β -globin 5' UTR, translation initiation on the IGF-II 5' UTR was not dependent on the presence of a cap structure. The results imply that only in undifferentiated P19 EC cells and not in their differentiated derivatives is a factor present that specifically stimulates IGF-II RNA translation, thereby suggesting translational regulation of IGF-II production during early embryonic development. A mechanism for translation initiation on the 5' UTR of IGF-II is discussed.

INTRODUCTION

Insulin-like growth factors (IGFs) play an important role in fetal and post-natal development. The genes of two major human IGFs, IGF-I and IGF-II, have been fully characterized (Rinderknecht and Humbel, 1978a,b; Sussenbach et al., 1992). The human IGF-II gene consists of nine exons. The 5' located part of the gene consists of non-coding leader exons 1–6. Exons 1, 4, 5 and 6 are each preceded by a separate promoter region, leading to the formation of four IGF-II mRNAs with different untranslated regions (van Dijk et al., 1991). mRNAs derived from the four different promoters (P1–P4) are expressed in a developmentally regulated manner (de Pagter-Holthuis et al., 1987, 1988). mRNAs derived from promoters P2–P4 are found in many embryonic tissues. In the liver, which is thought to be the major source for circulating IGF-II, promoters P2–P4 are shut off after birth, at which time promoter P1 is activated. The existence of a number of IGF-II mRNAs with different 5' untranslated regions (UTRs) raises the possibility that these mRNAs are differentially translated. It has been demonstrated, that the four human IGF-II mRNAs provide an example of translational discrimination in which the transcript derived from promoter P4 (formerly P3) is actively engaged in protein synthesis while the predominant P3 (formerly P2) transcript is present in ribonucleoprotein particles (Nielsen et al., 1990). The human IGF-II mRNA derived from promoter P1 has a 5' UTR (IGF-II leader 1) of 590 nucleotides and is the subject of this study.

The length of the 5' UTR of eukaryotic mRNA molecules varies from only a few to several thousands of nucleotides (Kozak, 1987). In general, highly structured 5' UTRs inhibit translation, because they hamper the necessary unwinding during protein synthesis initiation (Pelletier and Sonenberg, 1985;

Lawson et al., 1989; Meerovitch et al., 1989; Jang and Wimmer, 1990). Therefore, a long 5' UTR (more than about 200 nucleotides) is a hallmark for inefficient initiation, unless an alternative mode of initiation is possible. Currently two different models exist for translation initiation: the scanning-model, as first described to account for initiation of most cellular mRNAs (Kozak, 1992), and the internal initiation-model, as first described for picornaviruses (Jang et al., 1988; Pelletier and Sonenberg, 1988). Sarnow and co-workers described two cellular mRNAs that also seemed to initiate internally (Macejak and Sarnow, 1991; Oh et al., 1992). The two models mainly contradict at the moment where the 40S ribosome subunit binds to the mRNA molecule: in the Kozak model the entry site for the 40S subunit is at the 5' cap structure and the 40S subunit is supposed to stay on the mRNA until termination, whereas in internal initiation, the 40S entry site is at internal sequences.

It has been shown that the efficiency of growth factor synthesis is inhibited by their 5' UTRs. The 5' UTRs of platelet-derived growth factor 2 (PDGF-2) and of transforming growth factor (TGF)- β 1 and - β 3 are all potent translational inhibitors (Rao et al., 1988; Arrick et al., 1991; Kim et al., 1992). For TGF- β 1 the inhibitory effect on translation by its 5' UTR was specific for certain cell types. This means that growth factor mRNA molecules with 5' UTRs, that are extremely long and rich in G+C content, are being translated efficiently under certain conditions. Recently, it was reported that translation of mRNA encoding PDGF-AA is decreased upon differentiation of F9 teratocarcinoma cells induced by retinoic acid (RA) (Wang and Styles, 1993). Also, it was postulated, that an increased phosphorylation grade of the eukaryotic initiation factor eIF-4E is required for more efficient translation of growth factor mRNAs (Koromilas et al., 1992). All these aspects seem to indicate that growth factor

Abbreviations used: BiP, immunoglobulin heavy chain-binding protein; CAT, chloramphenicol acetyltransferase; EC, embryonal carcinoma; eIF, eukaryotic initiation factor; EMC, encephalomyocarditis virus; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; RA, retinoic acid; TGF, transforming growth factor; TPT, tripartite; UTR, untranslated region.

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mRNAs are poorly translated and that translation may be developmentally regulated. This behaviour of growth factor mRNAs does not simply follow the rules of translation initiation, as explained above. The 5' UTRs of several growth factor mRNAs share two properties with the picornaviral 5' UTR: they contain extensive secondary structure due to their extreme length and have upstream AUGs, that hinder scanning to the initiation codon (Kozak, 1983). In spite of the similarity to picornaviral 5' UTRs, internal initiation of translation has not yet been found for growth factor mRNAs.

P19 mouse embryonal carcinoma (EC) cells, the undifferentiated stem cells of teratocarcinomas, are frequently used as a model system to study cell differentiation and early events in embryonic development because of the many properties they have in common with the pluripotent cells of the embryo itself (Martin, 1975). Undifferentiated P19 EC cells synthesize IGF-II, that might act as a mitogen (Jenkins Eicher et al., 1993). A comparison of the genomic organization of the human IGF-II gene with the IGF-II genes from rat and mouse shows extensive structural sequence similarity (Gray et al., 1987; Matsuguchi et al., 1990; Ikejiri et al., 1991).

To explore the potential molecular mechanisms involved in translational control throughout development, directed by the human IGF-II leader 1, we measured the effect of the addition of extracts from undifferentiated and RA-induced differentiated P19 EC cells during *in vitro* translation.

MATERIALS AND METHODS

P19 EC cells (Berg and McBurney, 1990) were cultured on gelatin-coated flasks in a bicarbonate-buffered 1:1 (v/v) mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium, supplemented with 7.5% (v/v) fetal-calf serum (Mummery et al., 1984). Cells were induced to differentiate in the presence of 1×10^{-6} M all-*trans* RA (Sigma) to cultures in monolayer or in aggregates (Mummery et al., 1985) resulting in endoderm-like or neuron-like cells. Cells started to differentiate after 2 to 3 days, which was visible by microscopy.

Preparation of a ribosomal salt wash, the source of initiation factors, and subfractionation of the ribosomal salt wash to a 0–40% $(\text{NH}_4)_2\text{SO}_4$ fraction (A wash) and to a 40–70% $(\text{NH}_4)_2\text{SO}_4$ fraction (BC wash) were as described (Voorma et al., 1979). Initiation factor eIF-4F was purified from rabbit reticulocytes according to procedures described earlier (Buckley and Ehrenfeld, 1987).

Plasmid L Σ IGFCAT contains the human IGF-II leader 1 (van

Dijk et al., 1991) fused to the chloramphenicol acetyltransferase (CAT) reporter gene under control of the T7 promoter. The IGF-II 5' UTR exons (de Pagter-Holthuizen et al., 1986, 1987) were used as a template for PCR. The blunted IGF-II leader 1 PCR product was ligated in the *StuI/HincII*-digested expression vector LeaderScript (pLSCAT) (C. H. de Moor, M. Jansen, E. J. Bonte, A. A. M. Thomas, J. S. Sussenbach and J. L. van den Brande, unpublished work). This resulted in the following structure for the plasmid: T7 promoter/IGF-II 5' UTR/GACC/CAT sequence. The bicistronic construct pTZ4AIGFCAT, containing the IGF-II-5' UTR as an intercistronic region, was created by cloning the *BamHI* IGF-CAT fragment from pL Σ IGFCAT directly behind the eIF-4A1 gene in pTZ4AI (Scheper et al., 1992), digested by *BamHI*. Plasmid pGEMBiPCAT was created by cloning the CAT fragment, isolated by (partial) *NcoI/SphI* digestion of pTZEMCCAT (Scheper et al., 1992), into *NcoI/SphI*-digested pGEM5ZBiP5' (provided by Dr. P. Sarnow). In the latter plasmid the immunoglobulin heavy chain-binding protein (BiP) 5' UTR (Ting and Lee, 1988), consisting of nucleotides 9 to 216, was flanked by the *SacI* and *NcoI* sites of pGEM5Z (Promega). The bicistronic construct pTZ4ABiPCAT, containing the BiP 5' UTR as an intercistronic region, was created by cloning the *SacI/BamHI* BiP-CAT fragment from pGEM5ZBiPCAT directly behind the eIF-4A1 gene in pTZ4AI (Scheper et al., 1992), digested by *SacI* and *BamHI*. Plasmid GEM β -globCAT containing the CAT-encoding sequence directly downstream of the 5' UTR of *Xenopus* β -globin, was made by replacing the chimp α -globin-coding region in pGEMSVgl (obtained from Dr. D. Andrews) for the *NcoI/BamHI* CAT fragment from pTZEMCCAT. All manipulations were checked by DNA sequencing. Plasmids TZ4AEMCCAT, TPTCAT and 26SCAT (Scheper et al., 1992; Thomas et al., 1992; Berben-Bloemhevel et al., 1992) were described elsewhere.

Transcription of linearized DNA was performed as described (Pelletier and Sonenberg, 1985) with SP6 or T7 RNA polymerase either in the presence of 0.5 mM $^3\text{mGpppG}$ and 0.1 mM GTP to obtain capped transcripts, or with 1 mM GTP for uncapped transcripts. Transcripts were purified by phenol/chloroform extraction and chromatography on a Sephadex G-50 column. Yield and integrity of the transcripts were checked by agarose electrophoresis. A schematic representation of the transcripts used is given in Figure 1.

Translation of transcripts in a rabbit reticulocyte lysate was carried out as described (Pelham and Jackson, 1976). The 5 μl assays contained 37 kBq of [^{35}S]methionine (3.7×10^7 MBq/mmol) and 30 ng of transcript. Lysates were homemade. Cytoplasmic extracts used to supplement the reticulocyte lysate for *in vitro* translation were made from cells collected by scraping in a hypotonic buffer. Briefly, cells were harvested in 10 mM Hepes/KOH, pH 7.6, 140 mM NaCl, 1.5 mM magnesium acetate and 0.1 mM EDTA and lysed by douncing in the same buffer supplemented with 10 mM KCl and 10 mM 2-mercaptoethanol. CAT assays and t.l.c. (Gorman et al., 1982) were performed by using aliquots from *in vitro* translation assays, containing equal amounts of ^{35}S -labelled proteins.

Preparation of extracts from P19 EC cells used for *in vitro* translation was based on a procedure described elsewhere (de Benedetti et al., 1991). Briefly, cells were harvested in 20 mM Tris/HCl, pH 7.6, 140 mM potassium acetate and 1.5 mM magnesium acetate and pelleted cells were homogenized in 2.5 vol. of 20 mM Hepes/KOH, pH 7.6, 50 mM potassium acetate, 1.5 mM magnesium acetate and 0.3% Brij 58. Leupeptin (2 $\mu\text{g}/\text{ml}$) and RNasin (300 units/ml) were added before removal of cell debris and nuclei. *In vitro* translation assays were

Figure 1 Schematic representation of the *in vitro*-synthesized transcripts used

T7 RNA-polymerase-derived transcripts consist of a 5' UTR upstream of the CAT reporter-encoding sequence (monocistronic) or as an intercistronic region (ICR) between CAT and the sequence encoding eukaryotic initiation factor 4A (bicistronic). Abbreviations: Semliki Forest virus (SFV), tripartite leader (TPT).

performed under conditions similar to translation in reticulocyte lysates. Following translation, 1 μ l aliquots were taken to determine trichloroacetic acid-precipitable radioactivity and 2 μ l samples were analysed by SDS/PAGE. The gels were prepared for fluorography by treatment with 0.5 M sodium salicylate/Tris, pH 9. Densitometry was performed by using an automatic Interactive Image Analysing System from Kontron Bildanalyse (Germany).

RESULTS

The similarity of the IGF-II leader 1 to other growth factor 5' UTRs and the inefficiency of the PDGF- and TGF-5' UTRs in translation initiation (Rao et al., 1988; Arrick et al., 1991; Kim et al., 1992) indicate that the IGF-II leader 1 may also be inefficient in translation. To investigate this, the efficiency of translation of IGF-II-CAT RNA was compared with other 5' UTR-CAT transcripts during translation in reticulocyte lysate assays and analysis of ^{35} S-labelled CAT by SDS/PAGE (Figure 2). CAT synthesis, supported by the IGF-II 5' UTR, could only be demonstrated after long exposure of the gel (about 70-fold longer for lanes 1–3, compared with lanes 4–11). Thus replacement of the β -globin 5' UTR for the IGF-II 5' UTR decreased initiation of translation about 100-fold. The 5' UTRs from tripartite (TPT) and β -globin were included in this experiment because they belong to the most efficient 5' UTRs in our hands (lanes 8 and 9). The presence of a cap structure at the 5'-end of the IGF-II 5' UTR partially inhibited translation (lane 2), suggesting cap-independent initiation, as has been described for the 5' UTR of picornaviruses (Jang et al., 1988; Pelletier and Sonenberg, 1988). For comparison, the subgenomic SFV 26S-CAT RNA was translated. This 5' UTR was dependent on the cap structure for full activity (lanes 6 and 7), as described earlier (Berben-Bloemhevel et al., 1992). The uncapped BiP 5' UTR (lane 5) was 50% less active, an unexpected result as the BiP 5' UTR can mediate initiation from a downstream cistron in a bicistronic construct (Macejak and Sarnow, 1991). As expected from the data given in the introduction, translation of a CAT RNA directed by the IGF-II 5' UTR was indeed very inefficient, about 100-fold less active than the β -globin 5' UTR.

Figure 2 Translation of IGF-II-CAT RNA in reticulocyte lysate

In vitro synthesis of mRNA was either in the presence or absence of ^{75} mGpppG to obtain capped (^{75} mG) or uncapped transcripts. mRNA (30 ng) was translated, as described in the Materials and methods section. Aliquots of 2 μ l were analysed by SDS/PAGE and fluorography. Exposure time was 9 days for lanes 1–3 and 3 h for lanes 4–11. Lane 1, no RNA added; lane 2, ^{75} mG-IGF-II-CAT RNA; lane 3, IGF-II-CAT RNA; lane 4, ^{75} mG-BiP-CAT RNA; lane 5, BiP-CAT RNA; lane 6, ^{75} mG-26S-CAT RNA; lane 7, 26S-CAT RNA; lane 8, ^{75} mG-TPT-CAT RNA; lane 9, ^{75} mG- β -globin-CAT RNA; lane 10, ^{75} mG-4A-BiP-CAT RNA; lane 11, ^{75} mG-4A-EMC-CAT RNA. Migration of 4A (46 kDa) and CAT (25 kDa) is indicated on the right.

The partial independence of the cap structure of IGF-II-CAT RNA translation seems to indicate that IGF-II leader 1 may mediate internal initiation. This was tested by translation of three bicistronic constructs, in which the encephalomyocarditis virus (EMC) 5' UTR, the BiP 5' UTR, or the IGF-II 5' UTR were used as intercistronic spacers (Figure 1). The reticulocyte lysate efficiently initiated translation on the EMC-5' UTR (Table 1; Figure 2, lane 11). However, both the BiP 5' UTR (Figure 2, lane 10) and the IGF-II 5' UTR (result identical with Figure 2, lane 10) did not lead to synthesis of CAT product. It has been shown that, after transfection of HeLa cells with a bicistronic construct, containing the BiP 5' UTR as an intercistronic region, the activity of the downstream cistron was strongly increased compared with a construct lacking the BiP 5' UTR as an intercistronic region (Macejak and Sarnow, 1991). The result shows that the reticulocyte lysate may lack proteins necessary for internal initiation on the BiP 5' UTR, as also can be concluded for the IGF-II 5' UTR. However, autoradiography may not be sensitive enough to detect synthesis of ^{35} S-labelled CAT. Therefore, translation mixtures were also analysed for CAT enzyme activity (Table 2). The control template RNA 4A- Δ EMC-CAT, lacking an internal ribosomal entry site (indicated with Δ EMC) resulted in background levels of CAT enzyme activity. Similar results were obtained with the BiP 5' UTR, or the IGF-II 5' UTR as an intercistronic region. As expected, synthesis of CAT protein

Table 1 Cap-dependence and efficiency of translation in reticulocyte lysate and P19 cell extract

RNA	Translational efficiency* (%)		Stimulation (fold)
	Reticulocytes	P19 EC cells	
^{75} mG-IGF-CAT	$\leq 0.5^{\dagger}$	0	—
IGF-CAT	$\leq 0.5^{\dagger}$	3	6
^{75} mG-BiP-CAT	31	13	0.5
BiP-CAT	16	16	1
^{75} mG-26S-CAT	45	51	1
26S-CAT	9	ND	—
^{75} mG-TPT-CAT	23	9	0.4
^{75} mG- β -globin-CAT	100	100	1
^{75} mG-4A-EMC-CAT	16	15	1

*Efficiency was calculated by densitometry of the autoradiograms (Figures 2 and 6) relative to the translation of ^{75} mG- β -globin-CAT RNA, which was set at 100%.

† Due to inefficient translation, a comparison with ^{75} mG- β -globin-CAT was impossible from one single autoradiogram; the value given is therefore an estimate of the maximal relative efficiency.

Table 2 Efficiency of internal initiation from bicistronic transcripts in reticulocyte lysate

CAT enzyme activity was determined after *in vitro* translation. The relative conversion of [^{14} C]chloramphenicol into the acetylated forms was measured by t.l.c. (Gorman et al., 1982).

Intercistronic region*	Chloramphenicol converted (%)
BiP	1
EMC	70
IGF	0
Δ EMC	1

*See also Figure 1.

Figure 3 Effect of P19 cell extract during *in vitro* translation

In vitro-synthesized capped transcripts (30 ng) were translated in a 5 μ l reticulocyte lysate assay with or without addition of increasing amounts of extract from undifferentiated P19 cells, prepared as described in the Materials and methods section. Aliquots of 2 μ l were analysed by SDS/PAGE and fluorography. Exposure time was 7 days for lanes 1–8 and 3 h for lanes 9–24. Lanes 1–4, no RNA added; lanes 5–8, ^{7} mG-IGF-II-CAT RNA; lanes 9–12, ^{7} mG- β -globin-CAT RNA; lanes 13–16, ^{7} mG-BiP-CAT RNA; lanes 17–20, ^{7} mG-4A-BiP-CAT RNA; lanes 21–24, ^{7} mG-4A-EMC-CAT RNA. No P19 cell extract was added in lanes 1, 5, 9, 13, 17 and 21. In lanes 2, 6, 10, 14, 18 and 22 0.55 μ g of P19 cell extract was added; 1.1 μ g of extract in lanes 3, 7, 11, 15, 19 and 23, and 1.65 μ g of extract in lanes 4, 8, 12, 16, 20 and 24. Migration of 4A (46 kDa) and CAT (25 kDa) is indicated.

during translation of the 4A-EMC-CAT RNA was efficient in this experiment. The results indicate that either the IGF-II 5' UTR does not contain an entry site for the ribosome, or that the reticulocyte lysate lacks proteins necessary for internal initiation on the IGF-II 5' UTR.

In an attempt to improve the translational efficiency of IGF-II leader 1 in a reticulocyte lysate, increasing amounts of cell extract from undifferentiated P19 EC cells were added during translation in reticulocyte lysates of *in vitro*-synthesized capped transcripts (Figure 3). The P19 cell extract contained mRNAs that led to a background translation, only visible after prolonged exposure of the gel (lanes 1–8). However, translation of IGF-II-CAT RNA (lanes 5–8) was stimulated by P19 cell extract at least 7-fold, as determined by scanning of the autoradiogram, whereas translation of monocistronic transcripts containing the 5' UTR of β -globin (lanes 9–12) or BiP (lanes 13–16) was inhibited by P19 cell extract. The inability of the BiP 5' UTR to initiate in a bicistronic construct could not be relieved by adding P19 extract (lanes 17–20), whereas internal initiation from the EMC 5' UTR was unaffected or even slightly decreased (lanes 21–24). Apparently, stimulation of translation by P19 cell extract is specific for IGF-II leader 1-CAT RNA and the P19 cell extract does not contain proteins that allow internal initiation from the IGF-II and BiP 5' UTR. This result suggests that P19 cell extract contains factors that support translation initiation on the IGF-II 5' UTR and that these factors are lacking in reticulocyte cell extracts. Also, the specific effect on IGF-II leader 1 suggests that stimulation is not due to a *bona fide* translation initiation factor. Nevertheless, the effect could be caused by the addition of translation initiation factors that are limiting in reticulocytes. The least abundant initiation factor is eIF-4E, that functions together with eIF-4A and eIF-4 γ (formerly p220) as the cap-binding complex eIF-4F (Rhoads, 1988). To exclude the possibility that the IGF-II leader 1-specific stimulation of translation by P19 cell extract (Figure 3) was due to the addition of extra eIF-4E or eIF-4F, increasing amounts of exogenous eIF-4F were added to the *in vitro* translation of *in vitro*-synthesized transcripts (Figure 4).

Figure 4 Effect of eIF-4F on *in vitro* translation

In vitro-synthesized capped (^{7} m) or uncapped transcripts (60 ng) were translated in a 5 μ l reticulocyte lysate assay with or without the addition of increasing amounts of eIF-4F, purified as described in the Materials and methods section. Aliquots of 2 μ l, except for ^{7} mG- β -globin-CAT RNA translation (0.5 μ l), were analysed by SDS/PAGE and fluorography. Exposure time was 3 days for lanes 1–8 and 16 h for lanes 9–24. Lanes 1–4, no RNA added; lanes 5–8, ^{7} mG-IGF-II-CAT RNA; lanes 9–12, BiP-CAT RNA; lanes 13–16, ^{7} mG-BiP-CAT RNA; lanes 17–20, ^{7} mG-4A-EMC-CAT RNA; lanes 21–24, ^{7} mG- β -globin-CAT RNA. No eIF-4F was added in lanes 1, 5, 9, 13, 17 and 21. In lanes 2, 6, 10, 14, 18 and 22 0.05 μ g of eIF-4F was added, in lanes 3, 7, 11, 15, 19 and 23 0.1 μ g of eIF-4F and in lanes 4, 8, 12, 16, 20 and 24 0.2 μ g of eIF-4F. Migration of 4A (46 kDa) and CAT (25 kDa) is indicated on the right.

Translation of both the uncapped BiP-CAT RNA and the bicistronic 4A-EMC-CAT transcript was stimulated 4- and 1.5-fold. The stimulation of uncapped mRNA translation by eIF-4F is not without precedent (Fletcher et al., 1990; Scheper et al., 1992). For the capped transcripts of IGF-II-CAT and BiP-CAT, a slight increase in translation was observed when eIF-4F was added. However, no specific effect on IGF-II-CAT RNA translation was observed. Thus, the specific effect on IGF-II-CAT RNA translation as shown in Figure 3 cannot be attributed to addition of eIF-4E or eIF-4F present in P19 cell extract. Hemin and 2-aminopurine were present during translation, to prevent phosphorylation of eIF-2 α (Proud, 1992). Nevertheless, the effect of adding exogenous eIF-2 and eIF-2B (in the form of BC-wash; see the Materials and methods section) to translation assays was measured and, as expected, no change in translation was found.

In the mouse embryo IGF-II functions as an autocrine and paracrine growth factor and is necessary for normal growth (DeChiara et al., 1990; Rappolee et al., 1992). In undifferentiated P19 EC cells endogenous IGF-II acts as a mitogen (Jenkins Eicher et al., 1993). If the stimulation of translation of IGF-II leader 1 RNA constructs is specific for this 5' UTR, one might expect a change of stimulation after differentiation of P19 cells by RA. Indeed this was found as shown in Figure 5. For this purpose extracts were made both from undifferentiated P19 cells and from cells differentiated into epithelial or neuronal cells. Increasing amounts of these extracts were added during *in vitro* translation of *in vitro*-synthesized capped transcripts containing the 5' UTR from IGF-II or BiP (Figure 5). As already shown in Figure 3, extract of undifferentiated P19 cells (extract C) stimulated translation supported by IGF-II leader 1 (lanes 7 and 8), whereas translation efficiency of uncapped (lanes 14 and 15) and capped (lanes 21 and 22) BiP-CAT RNA was more or less unaffected. However, translational stimulation by P19 cell extract was abolished by adding extracts from cells differentiated with RA. Addition of extracts from neuron-like differentiated P19 cells (lanes 3, 4, 10, 11, 17 and 18) and especially from epithelial-like differentiated P19 cells (lanes 5, 6, 12, 13, 19 and 20) inhibited translation of transcripts containing the 5' UTR from IGF-II or BiP. Translational activity from endogenous mRNA

Figure 5 Influence of cell differentiation on the stimulation of translation *in vitro* by P19 cell extract

In vitro-synthesized capped (^{7} m) or uncapped transcripts (30 ng) were translated in a 5 μ l reticulocyte lysate assay with or without (—) addition of increasing amounts of extract from undifferentiated (designated below the figure as P19 extract C) or RA-induced differentiated P19 cells grown as a monolayer (extract B) or in aggregates (extract A), as described in the Materials and methods section. Aliquots of 2 μ l were analysed by SDS/PAGE and fluorography. Exposure time was 7 days for lanes 1–8 and 16 h for lanes 9–22. Lane 1, no RNA added; lanes 2–8, ^{7} mIGF-II-CAT RNA; lanes 9–15, BiP-CAT RNA; lanes 16–22, ^{7} mBiP-CAT RNA. No P19 cell extract was added in lanes 2, 9 or 16, 1.6 μ g of P19 cell extract was added in lanes 3, 5, 7, 10, 12, 14, 17, 19 and 21 and 3.2 μ g of P19 cell extract was added in lanes 1, 4, 6, 8, 11, 13, 15, 18, 20 and 22. Migration of CAT (25 kDa) is indicated on the right.

Figure 6 Translation of IGF-II-CAT RNA in extract of undifferentiated P19 EC cells

In vitro synthesis of mRNA was either in the presence or absence of ^{7} mGpppG to obtain capped (^{7} mG) or uncapped transcripts. mRNA (30 ng) was translated in a 5 μ l P19 translation assay, prepared as described in the Materials and methods section. Aliquots (2 μ l) were analysed by SDS/PAGE and fluorography. Exposure time was 3 days. Lane 1, no RNA added; lane 2, ^{7} mG-IGF-II-CAT RNA; lane 3, IGF-II-CAT RNA; lane 4, ^{7} mG-BiP-CAT RNA; lane 5, BiP-CAT RNA; lane 6, ^{7} mG-TPT-CAT RNA; lane 7, ^{7} mG-26S-CAT RNA; lane 8, ^{7} mG- β -globin-CAT RNA; lane 9, ^{7} mG-4A-IGF-II-CAT RNA; lane 10, ^{7} mG-4A-EMC-CAT RNA. Migration of 4A (46 kDa) and CAT (25 kDa) is indicated on the right.

molecules was significantly lower in the latter cell extract (lanes 5 and 6). Both features indicate a lower rate of protein synthesis in the differentiated cells used for the preparation of this extract. Figure 5 shows that IGF-II-CAT RNA translation is stimulated by addition of cell extracts from undifferentiated P19 cells only. All transcripts mentioned in Figure 1 were tested as in Figure 5 (results not shown), but stimulation was only found for the IGF-II-CAT RNA.

In order to examine the translational efficiency of IGF-II leader 1-CAT RNA, we set up an *in vitro* translation system derived from undifferentiated P19 cells. To determine the translational efficiency, *in vitro*-synthesized monocistronic and bicistronic CAT transcripts were translated in this P19 translation assay (Figure 6). In Table 1 the relative amounts of CAT product formed during the *in vitro* translation of the different transcripts

Table 3 Translation efficiency of IGF-II-CAT RNA relative to β -globin-CAT RNA in extracts containing different ratios of P19 translation assay and reticulocyte lysate

In vitro-synthesized IGF-II-CAT RNA and ^{7} mG- β -globin-CAT RNA (30 ng of each) was translated in 5 μ l assays in extracts containing 0, 20, 40, 60, 80 or 100% (v/v) of P19 translation assay and respectively 100, 80, 60, 40, 20 and 0% (v/v) reticulocyte lysate.

For measurement of translational efficiency aliquots of 4 μ l were analysed by SDS/PAGE and fluorography. Efficiency of IGF-II-CAT RNA translation relative to the translation of ^{7} mG- β -globin-CAT RNA, which was set at 100%, was calculated by densitometry of the autoradiogram.

Extracts (%)		Translational efficiency (%)
Reticulocyte	P19	
100	0	2
80	20	3
60	40	4
40	60	3
20	80	5
0	100	18

are shown and a comparison is made to translation in a reticulocyte lysate, as shown before in Figure 2.

In the P19 translation assay, IGF-II leader 1 (Figure 6, lane 3) supports translation much more efficiently than in a reticulocyte lysate, confirming that P19 cell extracts contained an activity that specifically stimulated initiation on the IGF-II 5' UTR. Relative to the β -globin 5' UTR (lane 8), inhibition of translation was measured for CAT transcripts containing the UTRs of ^{7} mG-BiP (lane 4) and ^{7} mG-TPT (lane 6), whereas translation of uncapped CAT transcript containing the 5' UTR of BiP (lane 5), or the capped transcripts ^{7} mG-26S (lane 7) and ^{7} mG-4A-EMC (lane 10) was more or less unaffected in P19 cell extract (Table 1). The presence of a cap structure on the IGF-II-CAT transcript (lane 2) completely abolished translation in this system, whereas translational efficiency of uncapped (lane 5) and capped BiP-CAT RNA (lane 4) was similar. These results suggest that IGF-II-CAT RNA initiates in a cap-independent manner, stimulated by factors only present in undifferentiated P19 cells. However, similar to translation in reticulocyte lysate (Table 2), initiation on the 5' UTR from IGF-II as intercistronic region (Figure 6, lane 9) remained below background levels.

The efficiency of IGF-II-CAT RNA translation compared with β -globin-CAT RNA was increased in Figure 3. The specific stimulation of translation on IGF-II-CAT RNA was further examined in an experiment in which the translation assay from P19 cells (as in Figure 6) was mixed with the reticulocyte assay (as in Figure 2). Therefore, translation mixtures were made, composed of different ratios of reticulocyte lysate and P19 translation assay. In these composed extracts, uncapped IGF-II-CAT RNA and capped β -globin-CAT RNA were translated and translation efficiency of the IGF-II 5' UTR relative to the β -globin 5' UTR was determined (Table 3). Relative to the β -globin 5' UTR, translation directed by the 5' UTR of IGF-II was stimulated up to 9-fold. This stimulation is similar to that found when translation in reticulocyte lysate was compared with the P19 translation assay (Table 1). Apparently, a factor present in P19 cell extract is able to stimulate IGF-II-CAT RNA initiation preferentially over β -globin-CAT RNA initiation.

The stimulatory effect of P19 cell extract on IGF-II-CAT RNA translation in reticulocyte lysate assays, as in Figures 3 and 5, is apparently not similar to the effect of the P19 translation assay in composed extracts containing different ratios of P19 translation assay and reticulocyte lysate (Table 3). These

differences are not entirely understood. An obvious explanation is the difference in preparation of P19 translation assay compared with the P19 cell extract, as described in the Materials and methods section.

DISCUSSION

The human IGF-II mRNA derived from promoter P1 has a 590-nucleotide-long 5' UTR (IGF-II leader 1). We determined its translational efficiency in a reticulocyte lysate and in a translation assay derived from P19 cells by translating an IGF-II-CAT construct with this leader upstream of a CAT-encoding cistron. Compared with the 5' UTR of β -globin mRNA, IGF-II leader 1 was translated very inefficiently. Moreover, the presence of a cap slightly decreased efficiency of translation initiation mediated by the IGF-II 5' UTR. Addition of cell extract from undifferentiated, but not from differentiated P19 EC cells, to the *in vitro* translation assay specifically stimulated translation of IGF-II-CAT RNA. This stimulatory effect could not be attributed to eIF-4F or eIF-2, which are the most important regulatory factors involved in translation initiation (Rhoads, 1993). Relative to the 5' UTR of β -globin, translational efficiency of IGF-II-CAT RNA was significantly higher in a translation assay derived from P19 cells than in reticulocyte lysates. In both the reticulocyte lysate and in the P19 translation assay IGF-II leader 1 did not support internal initiation, as determined by translation of a bicistronic transcript containing the IGF-II 5' UTR between the cistrons eIF-4A and CAT.

Undifferentiated P19 cells produce IGF-II that induces DNA synthesis, thus acting as a mitogen (Jenkins Eicher et al., 1993). The preferential stimulatory effect on IGF-II leader 1 translation by extract from undifferentiated, and not from differentiated cells, might imply a role for IGF-II in vertebrate embryo development. It has been described previously that IGF-II indeed plays an important role in growth and development (Froesch et al., 1986). The IGF-II promoter 1 was found to be active only in human adult liver and not in embryonic tissue (de Pagter-Holthuizen et al., 1987). Although transcriptional regulation of IGF-II is similar in human, rat and mouse cells (Gray et al., 1987; Matsuguchi et al., 1990; Ikejiri et al., 1991), transcripts with a 5' UTR similar to human IGF-II leader 1 have not been observed in mouse cells. Therefore, the effect of extracts from undifferentiated (and lack of such from differentiated) mouse P19 cells was rather surprising. The effect might not only be IGF-II-specific, but more general for 5' UTRs with similar characteristics, such as PDGF and TGF. PDGF-AA RNA translation was indeed abolished in F9 teratocarcinoma cells after RA-induced differentiation (Wang and Styles, 1993), similar to our results *in vitro*. The failure of extracts from differentiated P19 cells to specifically stimulate IGF-II leader 1 translation could be attributed to the absence of factors specifically involved in translation initiation of the IGF-II 5' UTRs and perhaps other growth factor 5' UTRs. During preparation of P19 cell extract the stimulatory effect was easily lost (results not shown), indicating a low stability of the stimulatory factor. The nature of this factor is unknown; its inactivation upon freeze-thawing suggests a protein or RNA nature.

The presence of IGF-II mRNAs containing different 5' UTRs provides a basis for post-transcriptional regulation of gene expression. IGF-II leader 1 exhibits an unusual 5' UTR of 590 nucleotides (van Dijk et al., 1991) containing one upstream AUG codon with an overall G+C content of 67%, indicating a strong potential for higher-order structuring. It is striking that long G+C-rich leaders are found among transcripts from growth

control genes, where they could work as *cis*-acting translational inhibitors (Rao et al., 1988; Arrick et al., 1991; Kim et al., 1992). Therefore, it is conceivable that the higher-order structure of the 5' UTR prevents efficient binding or migration of the 40S ribosomal subunit along the 5' UTR (Kozak, 1992). Indeed, decreasing the higher-order structure of IGF-II leader 1 by lowering the potassium concentration increased its translation efficiency, whereas no difference was seen for the β -globin 5' UTR (results not shown).

In vivo IGF-II mRNAs will undoubtedly be capped. The preferential stimulation of translation, as shown in Figures 3 and 4, was observed with capped IGF-II mRNA constructs. The increased activity of uncapped IGF-II-CAT RNA is a phenomenon observed *in vitro*, and is most likely irrelevant to the *in vivo* situation. Although the absence of a cap structure stimulated *in vitro* translation of IGF-II leader 1, suggesting cap-independent initiation of translation, no evidence was found for internal initiation, as has been shown for the EMC 5' UTR (Jang et al., 1988; Pelletier and Sonenberg, 1988) and the BiP 5' UTR (Macejak and Sarnow, 1991). Moreover, the failure of P19 cell extract to stimulate translation initiation on the EMC- or BiP 5' UTR suggests a cap-independent initiation on IGF-II leader 1 different from that described for EMC or BiP. The ability of IGF-II leader 1 to support cap-independent translation with a free 5' end, but not as an intercistronic 5' UTR, fits in neither one of the current models of translation initiation. A possible explanation for this discrepancy could be the requirement for a free 5'-end for translation initiation whether the transcript is capped or not. A cistron fused to the 5'-end of IGF-II leader 1 might influence the secondary structure resulting in a translational block, as was determined in this study by translating a bicistronic transcript. Another possibility for the inability of IGF-II leader 1 to support internal initiation of translation might be the obvious explanation that these experiments were performed *in vitro*. Characterization of the factor(s) needed for translation initiation on the IGF-II 5' UTR will be necessary. IGF-II leader 1 mRNA translation initiation seems to deviate from the majority of cellular mRNAs, which were shown to be translated in a cap-dependent way. We are currently trying to elucidate how translation of IGF-II leader 1 is initiated *in vivo* and how its translational efficiency is regulated upon differentiation of P19 EC cells.

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