Proteins binding to the leader of the 6.0 kb mRNA of human insulin-like growth factor 2 influence translation

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The leader of the 6.0 kb human insulin-like growth factor 2 (IGF-2) mRNA, leader 3, has been reported to partially repress translation. In the regulation of this phenomenon, RNA-binding proteins may play a role. Using UV-irradiation crosslinking, we found specific binding of four proteins (57, 43, 37 and 36 kDa) to this leader. Binding of these proteins to RNA proved to be highly sensitive to the potassium chloride concentration in the buffer solution, each protein having its own optimum. The 57 kDa protein was indistinguishable by size, binding properties and immunoprecipitation from the polypyrimidine tract binding protein (PTB), first described as a nuclear protein binding to the polypyrimidine tracts (PPTs) in introns. Cross-competition experiments showed that leader 3 has a much higher affinity for this

57 kDa protein than the PPT on which PTB was originally characterized. By competition with different fragments of leader 3, we were able to localize the binding of the 57 kDa protein to a 162 nt RNA fragment (*AsnI-PvuII*) in the 3'-part of the leader. When placed before a chloramphenicol acetyltransferase (CAT) open reading frame, this RNA fragment stimulated translation in reticulocyte lysate 3-fold, while other fragments of leader 3 repressed translation. The efficient translation directed by the 162 nt *AsnI-PvuII* fragment fused to CAT could be repressed by adding free *AsnI-PvuII* RNA fragment, indicating that the high translation efficiency of the *AsnI-PvuII*-CAT synthetic mRNA was due to the binding of protein and not to the structure of the RNA itself.

INTRODUCTION

The human insulin-like growth factor 2 (IGF-2) gene is a complex transcription unit containing four promoters, as depicted in Figure 1 (Sussenbach et al., 1992). Each promoter drives the transcription of a distinct 5'-untranslated region (leader) that is spliced to the coding exons. The mRNA transcribed from promoter 3 is found as a major 6.0 kb and a minor 2.1 kb species, due to alternative polyadenylation in the last exon (exon 9). All five mRNAs that can thus be transcribed from the gene encode the same pre-pro-IGF-2 molecule. The four promoters exhibit development-dependent and tissue-specific expression. Promoter 1 activity has been demonstrated only in adult liver, whereas promoters 2, 3 and 4 are co-expressed in a variety of fetal tissues, notably fetal liver, and, at a lower level, in many adult tissues with the exception of adult liver. In normal fetal tissues and in several cell lines, promoter 3 is by far the most active, generating mRNA levels 4- to 10-fold higher than promoters 2 and 4. Curiously, very little of the 6.0 kb mRNA derived from promoter 3 appears to be translated, judging by its poor association with the polyribosomes in cultured cells (e.g. Hep3B hepatoma cells) and in fetal liver (Nielsen et al., 1990; De Moor et al., 1994). In contrast, the IGF-2 mRNAs transcribed from promoters 2 and 4 are found completely in the polyribosomal fractions, suggesting efficient translation. The only difference between these IGF-2 mRNA species is the leader. Apparently, leader 3 inhibits entry into the polyribosomes, but a fraction of leader 3-containing IGF-2 mRNA molecules somehow manages to escape from this inhibition.

Translation of mRNAs is influenced by the structure of the leader. Leader 3 is exceptionally long (1190 nucleotides), con-

sidering that the majority of leaders do not exceed 100 nucleotides (Kozak, 1987). It is extremely rich in cytosine residues (48%) and relatively rich in guanosine residues (25%), giving ample

Figure 1 Schematic representation of the human IGF-2 gene and its mRNAs

The nine exons of the human IGF-2 gene are shown as boxes, numbered sequentially from 1 to 9. Four promoters with their corresponding transcription start sites are indicated with arrows and they are numbered P1 to P4. P3 is the major promoter in most tissues (thick arrow). The hatched area represents the coding region. The coding regions are identical for all IGF-2 mRNAs, since exons 7, 8 and 9 are present in all transcripts. The leaders (5'-UTRs) are numbered after the promoters that drive their transcription. There is a minor polyadenylation site in exon 9, the use of which has only been demonstrated in leader 3-containing transcripts. The insulin gene is located 1.5 kb upstream of promoter 1, as indicated. The sizes of the exons are not exactly to scale. For further details, see the Introduction.

Abbreviations used: IGF-2, insulin-like growth factor 2; PPT, polypyrimidine tract (from adenovirus intron); PTB, polypyrimidine tract binding protein; CAT, chloramphenicol acetyltransferase; GST, glutathione-S-transferase; RSB, ribosomal suspension buffer; DTT, dithiothreitol.

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possibilities for the formation of secondary structure while also generating single-stranded cytosine stretches. Several mRNAs encoding growth-related proteins have long GC-rich leaders that can suppress translation (Kozak, 1987; Ratner et al., 1987; Rao et al., 1988). Extensive secondary structure in the leader has been shown to be deleterious for normal translation initiation (Kozak, 1992). This can be overcome in part by improved unwinding activity of the initiation complex, for instance by activating initiation factors through phosphorylation or dephosphorylation (Rhoads, 1993). Such a change in the general translation-initiation machinery is likely to affect translation of a large number of mRNAs. A more specific regulation of translation, however, could be mediated by regulatory proteins that bind to a leader and modify the translation-initiation efficiency of its mRNA (Kozak, 1992; Hershey, 1991; Melefors and Hentze, 1993). We therefore investigated the possible existence of proteins that bind to IGF-2 leader 3 and the effect they have on translation.

MATERIAL AND METHODS

Cell culture and extracts

The human hepatoma cell line Hep3B (Knowles et al., 1980) was cultured in α minimal essential medium with 10% fetal calf serum. For the preparation of cytoplasmic extracts, subconfluent cells were harvested by treatment with trypsin, suspended in cold medium with serum, washed once with cold PBS (8 g/l NaCl/0.2 g/l KCl/2.31 g/l $\text{NaH}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$ /0.2 g/l KH_2PO_4) and counted. Cells (3×10^7) were suspended gently in 1 ml of cold ribosomal suspension buffer (RSB-10: 10 mM KCl/1.5 mM MgCl_2 /10 mM Tris-acetic acid, pH 7.4) and spun for 1 min at 70 g in a microcentrifuge at 4 °C. The cell pellet was suspended in 450 μ l of cold RSB-10 to which 1 mM dithiothreitol (DTT) and 50 μ l of a solution of 5% sodium desoxycholate and 5% Nonidet P40 was added. After mixing, this suspension was incubated on ice for 10 min with repeated mixing. The extract was obtained by spinning at 70 g for 1 min in a microcentrifuge at 4 °C. Supernatant (450 μ l) was collected and heparin was added to 1 mg/ml. The extract was aliquotted and stored at -20 °C. HeLa nuclear extracts were prepared as described (Garcia-Blanco et al., 1989).

Plasmids and *in vitro* RNA synthesis

The different leaders of IGF-2 were synthesized employing the PCR on DNA templates. A *Bam*HI site was added to the 5'-terminus of the PCR product and a *Hind*III site to the 3'-terminus by inclusion in the PCR primers. The 5'-termini were approximately 10 nt upstream of the presumed cap-site, e.g. for leader 3 at the *Nar*I site preceding exon 5 (Sussenbach et al., 1992). The leaders thus obtained were cloned into pBlueScript KS⁺ (Stratagene) in the *Bam*HI and *Hind*III sites. Linearization for sense transcription with T7 RNA polymerase was by digestion with *Hind*III.

For several of the leader 3 fragments depicted in Figure 3(a) we used a recently designed vector that includes a minimum of polylinker sequence in the leaders. This vector, termed pLeaderScript, was constructed from two vectors, pT₀ and pNco-CAT (CAT is chloramphenicol acetyltransferase) (Berben-Bloemhevel et al., 1992). pT₀ was constructed by cloning the 220 nt *Sph*I-*Bam*HI fragment from pET7 into the corresponding polylinker sites of pEMBL19. This fragment contains an RNA polymerase T7 promoter fused to an *Stu*I site, with the following sequence: GACTCACTATAGGCCTGGATCC (in which the *Stu*I site is underlined and the transcription start of the T7 promoter is in bold) (Rosenberg et al., 1987). pNco-CAT is a

plasmid derived from pSuperCAT, from which, by *Bal*I digestion and religation, the CAT leader and part of the polylinker have been removed. The remaining nucleotides of the *Pst*I site have been fused to the initiator ATG in such a way that an *Nco*I site is created. This deletes the CAT leader, which is present in most CAT constructs. A *Bam*HI-*Sau*III fragment containing the CAT coding region was cloned into the *Bam*HI site of pT₀, creating *Bam*HI sites on both sides of the insert. This results in the following structure of the polylinker region: T7 promoter/*Stu*I-*Bam*HI-*Xba*I-*Sal*I/*Hind*II-*Nco*I/initiator ATG of CAT. In order to be able to cut the *Stu*I site in this plasmid it must be produced in *dam*⁻ methylation-defective *Escherichia coli*, such as JM110. The fragments *Acyl*147, *Nae*270, *Nae*I-*Pvu*II, *Nru*I-*Asn*I, *Acyl*-*Eco*RI, *Asn*I-*Pvu*II, *Pvu*II-*Hind*III and *Rsa*I-*Hind*III [indicated in Figures 5(a) and 6] were cloned into the *Stu*I and *Hind*II sites of pLeaderScript after filling in protruding ends with Klenow DNA polymerase. This way, only one additional G is added to the RNA transcribed by the T7 polymerase. Linearization for transcription was with *Nco*I. *Bam*HI-*Sal*I and *Bam*HI-*Nru*I were transcribed from leader 3 in pLeaderScript linearized with *Sal*I and *Nru*I respectively. *Nae*I-*Nru*I was transcribed from *Nae*I-*Pvu*II in pLeaderScript linearized with *Nru*I. For translation *in vitro* the constructs in pLeaderScript were digested with *Sca*I. The polypyrimidine tract (PPT) of adenovirus pre-mRNA (Garcia-Blanco et al., 1989) was constructed by annealing the oligonucleotides 5'-CTAGATATCCCTTTTTTTCACAGG-3' (sense) and 5'-TCGACCTGTGGAAAAAAGGGATAT-3' (antisense) and cloning them in the *Xba*I and *Sal*I sites of pLeaderScript. Linearization for transcription was by digestion with *Nco*I.

Transcription was performed with T7 polymerase in transcription buffer (10 mM NaCl/10 mM MgCl_2 /4 mM spermidine/40 mM Tris-HCl, pH 8.0). Ribonucleotides were present at 1 mM. For radioactive transcription the labelled nucleotide was not supplemented with the non-radioactive nucleotide. The specific activity of the radioactive nucleotides was 400–800 Ci/mmol. For capped transcripts, GTP was present at 50 μ M, cap-analogue (³mGpppG) at 0.5 mM. The reactions were incubated for 1 h (radioactive, 10–20 μ l) or 2 h (bulk synthesis, 25–50 μ l) at 37 °C in siliconized microtubes. The transcripts were separated from the free nucleotides by a spun column of Sephadex G50 in water, the flow-through was extracted with phenol, and 0.1 vol. of 3 M sodium acetate, pH 5.4, was added. For radioactive probe synthesis 0.5 μ g of yeast tRNA was used as a carrier. All synthetic RNAs were precipitated with 3 vols. of ethanol, washed with 70% ethanol and dried. The pellet was dissolved in an appropriate volume of water or RSB-10. The RNAs for competitions were quantified by gel electrophoresis with a dilution series of a standard RNA, the mRNAs for translation *in vitro* were quantified similarly and this was checked by Northern blotting and hybridization with the CAT coding region.

UV-crosslinking

RNAs (both probes and competitors) used in UV-crosslinking assays were renatured in RSB-10 by cooling from 90 to 30 °C in approximately 3 h. The renaturation was carried out in siliconized microtubes.

Unless indicated otherwise, crosslinking reactions were executed in RSB-50 (50 mM KCl/1.5 mM MgCl_2 /10 mM Tris-HAc, pH 7.4). Reaction volumes were 4–6 μ l, with 1 μ l of cytoplasmic or nuclear extract diluted 10-fold in RSB-10 with 1 mM DTT. For leader 3, 10⁶ c.p.m. was used per assay, for the PPT (1–2) $\times$ 10⁵ c.p.m.. UV-crosslinking was achieved in a

Stratalinker 2400 device (Stratagene) at a dose of 10 J/cm² in 96-well tissue culture dishes (Nunc) floating on ice-water. After crosslinking, 25 μ l of RNase cocktail (100 μ g/ml RNase A/100 i.u./ml RNase T1/1 mM EDTA/10 mM Tris-HCl, pH 7.5) was added and the dish was incubated on water at 37 °C for 1 h. Subsequently, SDS/PAGE loading buffer was added and the samples were collected from the dish into microtubes. After heating for 5 min at 90 °C, the samples were subjected to SDS/PAGE electrophoresis (Laemmli, 1970). The gels were stained with Coomassie Brilliant Blue R250, dried, and exposed to X-ray film.

The PPT-binding protein (PTB) antibody was a gift from Drs. A. Gil and P. A. Sharp (M.I.T., Cambridge, MA, U.S.A.). Immunoprecipitation was performed as described (Gil et al., 1991). Dr. P. A. Sharp also donated the construct for the preparation of the glutathione-S-transferase (GST)-PTB fusion protein.

Translation in reticulocyte lysate

Translation *in vitro* was performed as described (Pelham and Jackson, 1976), in 5 μ l reaction mixtures containing 2 μ l of reticulocyte lysate, 1 μ Ci of [³⁵S]methionine (1000 Ci/mmol), 0.2 μ g of rat liver tRNA, 50 μ M amino acids minus methionine, 120 mM potassium acetate, 1 mM magnesium acetate, 5 mM 2-aminopurine and 32 fmol of mRNA. Reaction mixtures were incubated at 30 °C for 30 min. The mixtures were put on ice and a 1 μ l sample was spotted on Whatman 3MM filter paper to determine the incorporated radioactivity by hot trichloroacetic acid precipitation. The remaining reaction mixture was electrophoresed as described above for the crosslinking assays. After staining, the gels were washed for 15 min in 10% methanol and incubated for another 15 min in 1 M sodium salicylate/Tris (pH 9), dried, autoradiographed and quantified by phosphoimaging (Molecular Dynamics). The stability and the quantity of the mRNAs were checked by RNA isolation and Northern blotting, followed by hybridization with a CAT coding region probe. Quantification was performed by phosphoimaging. CAT and leader 3-CAT mRNAs were equally stable within the 30 min of our assays.

RESULTS AND DISCUSSION

Salt-dependence of RNA binding proteins

Using Hep3B cytoplasmic extracts as a source of RNA-binding proteins, we employed a UV-crosslinking method to detect proteins binding to IGF-2 leader 3 RNA. After UV-crosslinking of radioactively labelled RNA to proteins in the extract, unprotected RNA was degraded by RNase digestion. Subsequently, the radiolabelled RNA-tagged proteins were separated by SDS/PAGE and visualized by autoradiography. Labelling the RNAs either with [α^{32} P]CTP or [α^{32} P]UTP changed the relative intensity of the crosslinked bands but did not cause any bands to appear or disappear completely. Some of the proteins binding to leader 3, however, appeared to bind only in a fairly narrow range of KCl concentrations: a 43 kDa protein bound optimally at 10–25 mM KCl, whereas a 57 kDa protein had an optimum at 50–75 mM and a protein of 26 kDa bound only at 75–100 mM (Figure 2). Because of this sensitivity of the method to variations in salt concentration, all subsequent experiments were carried out in 50 mM KCl unless indicated otherwise, and inadvertent introduction of salts was carefully avoided.

The remarkably low KCl optimum for binding of some of the proteins, e.g. the 43 kDa protein(s), seems non-physiological. In

Figure 2 Crosslinking of RNA binding proteins is salt dependent

Hep3B cytoplasmic proteins were crosslinked to [³²P]CTP-labelled leader 3 at different potassium chloride concentrations, treated with RNase and electrophoresed on SDS/PAGE. Bands of interest and their apparent molecular masses (kDa) are indicated.

recent experiments, however, it could be shown that the 43 kDa protein(s) can tolerate much higher concentrations of potassium acetate than of potassium chloride. Our results therefore do not preclude binding of these proteins under physiological conditions.

Four proteins bind specifically to leader 3

In order to determine which of the proteins seen in Figure 2 bound specifically to IGF-2 leader 3 RNA, unlabelled competitor RNA was added to the crosslinking reactions. Four proteins could be competed out with unlabelled leader 3 but not with IGF-2 leader 1 (Figure 3); their apparent sizes were 57, 43, 37 and 36 kDa. At 50 mM KCl the 57 kDa band was by far the most prominent, the 43 kDa band could be detected in most experiments, but the intensity of the 37 and 36 kDa bands approached the limit of detection and their appearance was more variable (Figures 3a and 3b). Because of their appearance as a double band they may represent degradation products of the

Figure 3 Competition of proteins crosslinking to leader 3

Hep3B cytoplasmic proteins were crosslinked to leader 3 (labelled with CTP) in the presence or absence of competitor RNA. L3, leader 3; L1, leader 1. Numbers above the lanes indicate the molar excess of competitor over the probe: (a) shows the proteins crosslinked to leader 3 in 50 mM KCl; (b) shows a longer exposure of three lanes of (a); and (c) shows the proteins crosslinked to leader 3 in 10 mM KCl, without (—) and in the presence of 25-fold molar excess of unlabelled leader 3 (L3 25x). As a second specificity control, the proteins that are crosslinked to antisense leader 3 probe are shown (α -L3 probe). Migration of proteins is indicated as in Figure 2.

Figure 4 Proteins binding to leader 3 are ubiquitous

Cytoplasmic extracts of different cells were crosslinked to leader 3. Specificity of binding was ascertained by competition with a 25-fold molar excess of unlabelled leader 3 (20 ng). Cell lysates of Hep3B, IN157, SHSY5Y and HeLa cells were prepared as stated and diluted 10-fold, as described in the Materials and methods section. Reticulocyte lysate was prepared as for translation *in vitro* and diluted 2-fold.

57 kDa protein(s), which sometimes also resolve as a double band (Figure 4). One-third to one-half of the signal in the 43 kDa band could not be competed out with leader 3 at 50 mM KCl. At 10 mM KCl, however, the 43 kDa band was by far the most prominent and competition was more efficient (Figure 3c). Most likely, therefore, this band consists of a leader 3-specific 43 kDa RNA-

binding protein with optimal binding at a low KCl optimum, and another, non-specific RNA-binding protein that binds at higher KCl concentrations. In addition to the competition experiments, the specificity of binding of the four proteins was confirmed by the lack of signal at their positions if another probe was used, for instance antisense leader 3 (Figure 3c, α -L3 probe). We also examined the cross-linking of proteins from extracts of several other cell lines to leader 3, such as HeLa, the neuroblastoma cell line SHSY5Y (Schneid et al., 1992), the rhabdomyosarcoma line IN157 (Nielsen et al., 1990) and rabbit reticulocyte lysate, and found the patterns of the proteins detected to be identical (Figure 4).

The specific binding of proteins to leader 3 is confined to a 162 nucleotide region

To localize the binding sites of these proteins, particularly the 57 kDa protein, we did competitions with labelled leader 3 and various unlabelled fragments of leader 3 (Figure 5a). As can be seen in Figure 5(b), only two RNA fragments did compete: a larger fragment, *NaeI-PvuII*, spanning nucleotides 328–906, and a smaller one, *AcyI-EcoRI*, spanning nucleotides 614–1003 and overlapping with the 3'-part of the former. Since *NaeI-NruI* (328–464) and *NruI-AsnI* (465–746) did not compete, the high-affinity binding of the 57 kDa protein appeared to be confined to the RNA fragment between *AsnI* and *PvuII*. To prove this, we competed labelled leader 3 with this fragment and, conversely, labelled *AsnI-PvuII* with unlabelled leader 3 (Figure 5c). The 57 kDa protein was competed from the leader 3 probe by the *AsnI-PvuII* fragment and crosslinked with high affinity to a labelled *AsnI-PvuII* fragment.

Figure 5 Localization of the binding site of the 57 kDa protein

(a) Depicts the RNA fragments used in this Figure and in Figure 6. (b) Shows the competition obtained with various fragments of leader 3 in 40-fold molar excess for crosslinking of proteins from Hep3B cytoplasmic extract to leader 3 probe. (c) Shows the crosslinking of Hep3B cytoplasmic proteins to leader 3 (L3) and *AsnI-PvuII* (AP) and cross-competition. BN is a 5' RNA-fragment of leader 3 (*Bam*HI-*Nru*I); it is a negative control. The other proteins (43, 36 and 37 kDa) are not visible on this exposure. In (d) the sequence of *AsnI-PvuII* is depicted. The three closely spaced PPTs are underlined.

Figure 6 The 57 kDa protein is similar to or identical with PTB

(a) Shows the crosslinking of leader 3 (labelled with CTP) and the PPT (labelled with CTP) with Hep3B cytoplasmic and HeLa nuclear proteins. In (b) the competition of crosslinking of cytoplasmic proteins to PPT-U (PPT labelled with UTP) with various RNAs is depicted. L4, leader 4; L2, leader 2. Numbers above the lanes indicate the molar excess of the competitor over the probe. (c) Depicts the competition of leader 3 (labelled with CTP) with various RNAs and (d) shows the immunoprecipitation of PTB. After crosslinking of cytoplasmic extract to leader 3 (CTP labelled) or PPT (UTP labelled) and RNase digestion, some material was applied directly to gel (c), other material was immunoprecipitated, either with anti-PTB antibodies (i) or with pre-immune serum (p). (e) Shows crosslinking of GST-PTB fusion protein (83 kDa) to PPT and *AsnI-PvuII*. Numbers with arrows indicate the position of the molecular mass markers.

The sequence of the *AsnI-PvuII* fragment is depicted in Figure 5(d). It contains three closely spaced PPTs (underlined). This situation is reminiscent of the leaders of picorna virus RNAs, where a PPT is present in the 3'-part of a region necessary for internal initiation of translation (Morris et al., 1993). Several authors could demonstrate binding of a 57 kDa protein to this region (Pestova et al., 1991; Jang and Wimmer, 1990; Luz and Beck, 1991; Borovjagin et al., 1991). This protein was similar or identical with the PTB, a predominantly nuclear protein that binds to the PPTs found in the 3'-part of introns (Garcia-Blanco et al., 1989; Gil et al., 1991; Patton et al., 1991). A number of variants of this protein exist because of alternative splicing, causing a characteristic double or triple band around 57 kDa. Since the 57 kDa protein in our experiments sometimes resolves as a double band and binds to a highly pyrimidine-rich fragment between the *AsnI* and *PvuII* sites of leader 3, it was suggested that the 57 kDa protein in our experiments might be PTB.

The 57 kDa protein binding to leader 3 is identical with or closely related to the PTB

Crosslinking of Hep3B cytoplasmic proteins to the PPT of adenovirus pre-mRNA (Ad10 RNA), a known binding site for PTB (Garcia-Blanco et al., 1989), revealed a protein migrating at the same velocity in SDS/PAGE as the 57 kDa protein crosslinked to leader 3 (Figure 6a). Conversely, crosslinking of either leader 3 or PPT to HeLa nuclear proteins resulted in comigrating 57 kDa bands of the same size as the 57 kDa protein from cytoplasmic extract that was crosslinked to leader 3 (Figure 6a).

In competition experiments, leader 3 proved to be an extremely efficient competitor for binding of the 57 kDa cytoplasmic protein to a PPT probe, as shown in Figure 6(b). A mere 2.5-fold molar excess of leader 3 prevented the crosslinking of the 57 kDa protein to the PPT probe completely, without affecting the signal for other proteins crosslinking to PPT. Unlabelled PPT competed much less efficiently than leader 3 for binding of the 57 kDa

protein to the PPT probe, competition starting only at a 50-fold molar excess of unlabelled over labelled PPT. Of the other IGF-2 leaders, leader 4 competed efficiently at a 20-fold molar excess, leader 2 only at 50-fold molar excess, and leader 1 not at all (Figure 6b). As expected, an unrelated mRNA of similar size, transcribed from the polylinker of a plasmid, did not compete at all (results not shown). The relatively efficient competition of leader 4 for a PTB is not surprising, since this leader contains a 10-fold CCU repeat.

Another protein, of 50 kDa, was somewhat more sensitive to competition by PPT. This protein was much more prominent relative to the 57 kDa protein if the probe was labelled with UTP instead of CTP (compare Figure 6a with 6b) and was virtually absent in nuclear extracts. It may therefore be a cytoplasmic U-tract-binding protein.

Figure 6(c) shows a competition of the IGF-2 leader 3 probe with leader 3 itself and with PPT. Leader 3 abolished the binding of the 57 kDa protein to the leader 3 probe at only a 10-fold molar excess. PPT did not compete for the 57 kDa protein binding to leader 3, even at molar excesses as high as 400-fold, indicating a much lower affinity of the 57 kDa protein for PPT than for leader 3. Leaders 1, 2 and 4 did not compete within the range of RNA concentrations tested (50–100-fold excess, results not shown).

In order to investigate whether the 57 kDa protein is immunologically related to PTB we performed immunoprecipitations of cytoplasmic extract crosslinked to labelled leader 3 and PPT. In both cases the immune serum specifically precipitated a 57 kDa band, indicating that the 57 kDa protein crosslinking to leader 3 is closely related to or identical with PTB (Figure 6d). In view of the apparent uniformity of this band with respect to its KCl optimum for binding and its behaviour in the competition experiments with PPT and leader 3 fragments, we would expect it to be homogeneous. As shown in Figure 6(d), however, only part of the 57 kDa band was precipitated. Unfortunately, the available amount of α -PTB antibody did not allow us to perform

It appeared that a CAT messenger starting with the complete leader 3 was hardly translated at all, while a messenger starting with the polylinker region was translated with high efficiency (set at 100 %, Figure 7a). All 5'-fragments of leader 3 tested, up to the *AcyI* site at nucleotide 614, repressed translation as severely as the full leader 3. The *AcyI*-*EcoRI* fragment resulted in a moderately efficient translation, as did the *PvuII*-*HindIII* and *RsaI*-*HindIII* fragments. Surprisingly, the *AsnI*-*PvuII* fragment in the sense orientation stimulated translation approximately 3-fold as compared with the polylinker leader. In the antisense orientation the translation was comparable to that directed by the other leader 3 fragments downstream of the 3' *AcyI* site. To check for a direct role of the RNA-binding proteins in this enhanced translation, we added excess *AsnI*-*PvuII* and control RNAs to different CAT-fusion messengers. As illustrated in Figure 7(b), *AsnI*-*PvuII* inhibited the translation of *AsnI*-*PvuII*-CAT and, to a lesser extent, polylinker-CAT mRNA. In another batch of reticulocyte lysate, *AsnI*-*PvuII*-CAT and polylinker-

CAT were equally repressed (Figure 7c). No RNA fragment other than *AsnI-PvuII* could cause repression of translation, except *AcyI-EcoRI*, a fragment that encompasses *AsnI-PvuII* (other RNAs tested were PPT, leader 4, leader 3 fragments *Acy147*, *Nae270*, *NruI-AsnI* and *PvuII-HindIII*, see Figures 7b and 7c). This inhibition of translation by competition with the *AsnI-PvuII* RNA fragment is indicative of a phenomenon occurring *in trans* by one or more RNA-binding proteins. Whether this is the 57 kDa PTB-like protein, or other proteins binding to this fragment, cannot be determined from our experiments. The fact that *AsnI-PvuII* also inhibited the translation of other messengers, such as CAT, suggests that the *AsnI-PvuII* RNA fragment binds a general translation-initiation factor in addition to one or more specific factors. Curiously enough, this phenomenon has also been described for two picorna virus leaders that direct internal initiation. These RNAs have been reported to bind the translation-initiation factor eIF-2 (Scheper et al., 1991; Del Angel et al., 1989).

In reticulocyte lysates, all fragments of leader 3 upstream of nt 614 severely repressed translation, even relatively small ones, such as *Acy147*, a 147 nt fragment located in the far 5'-end of the leader. Since most of the fragments were non-overlapping, this implicates a widespread repressive activity in this part of leader 3. It seems likely that this is caused by extensive stable secondary structure in this region, since besides being C-rich it also contains a large number of G residues. Computer predictions of the secondary structure of leader 3, however, diverge widely with different programs, probably because the C-richness allows the formation of several alternative foldings of similar free energy. Determination of the structure will therefore have to be addressed experimentally, for instance by using primer extension and nuclease mappings.

Leader 3 of IGF-2 represses translation both in cells, as indicated by its polysomal distribution (Nielsen et al., 1990; De Moor et al., 1994), and in reticulocyte lysate, as demonstrated here with leader 3-CAT fusion mRNAs. The positive effect on translation mediated by the *AsnI-PvuII* fragment seems insufficient, at least *in vitro*, to overcome the repressive influences in the 5' part of the leader. Possibly, the *AsnI-PvuII* region and the proteins binding to it are involved in the generation of the small population of leader 3-containing IGF-2 mRNAs found in the polysomes in IGF-2-expressing cell lines (De Moor et al., 1994). It seems odd, however, that an mRNA made in such abundance would be only marginally translated, especially since other, less abundant but translationally more efficient IGF-2 mRNAs are also present. Currently, only one report in the literature indicates that leader 3-containing IGF-2 mRNA may be translationally activated: in polysomal profiles of early human placenta all of the IGF-2 mRNA is polysomal, as assayed in a dot-blot hybridization with IGF-2 coding sequence. Since the major IGF-2 mRNA in this tissue is the 6.0 kb mRNA headed by leader 3, it should be polysomal too (Ohlsson et al., 1989). We have been unable to reproduce this result. In our hands the IGF-2 mRNAs with leader 3 predominantly sedimented with the free messenger ribonucleoprotein particle in first trimester human placenta, quite similar to the situation in fetal liver (De Moor et al., 1994). It can be hypothesized that these mRNAs are only expressed in a very narrow time window, so that the exact developmental

stage is essential in this respect. Since the characterization of the polysomal distribution of an mRNA is an elaborate procedure requiring fairly large amounts of fresh tissue, extensive screening of fetal and embryonic tissues with this procedure is not readily feasible. Now that we have characterized the proteins binding to leader 3 from cells that do not translate the leader 3-containing IGF-2 mRNAs efficiently, another approach might be to screen for changes in the proteins associated with leader 3 during development.

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