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Original Article

Controlled translation initiation on Insulin-like growth factor 2-leader 1 during *Xenopus laevis* embryogenesis

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ABSTRACT A number of growth factors, whose spatio-temporal expression is essential for embryonic development, are encoded by mRNAs with a complex 5'UTR. Human insulin-like growth factor 2 mRNA contains a long (592 nucleotides) 5'UTR (IGFl1) with one upstream open reading frame and stable stem-loop structures, elements which might be important for controlled translation. To investigate whether these unusual features of IGFl1 can control translation initiation during embryogenesis, we examined the initiation efficiency on this 5'UTR during development of *Xenopus laevis*. The results demonstrate that IGFl1 strongly represses translation of a reporter in early embryos, compared with the *Xenopus* β-globin 5'UTR. The inhibition is alleviated soon after the midblastula transition, suggesting a stimulatory effect of the transcription start. However, a similar stimulation of IGFl1-driven translation is seen in embryos in which *de novo* transcription was inhibited by actinomycin D. Furthermore, it is shown that up-regulation of IGFl1 activity is independent of eIF4E levels, and activity of IGFl1 is observed in all tissues of transgenic *Xenopus* embryos. These results indicate that post-translational modulation of a trans-acting factor enables efficient initiation on this complex 5'UTR after the midblastula transition.

KEY WORDS: *Insulin-like growth factor 2, 5'UTR, translation regulation, Xenopus laevis, development.*

Introduction

Translational control mechanisms are of vital importance throughout embryonic development (Curtis *et al.*, 1995; Wickens *et al.*, 1996). Early embryogenesis is characterized by pronounced changes in the pattern of gene expression with little or no transcription in the zygotic nucleus. Therefore, decisions concerning cell division, specification of cell fates, and the establishment of body axes rely on control of translation. The mechanisms involved in translational control during early development are diverse and complex. The 3' and 5' untranslated regions (UTR) of the mRNA are important by controlling mRNA stability, localization, and translational activation or repression. Elements within the 3'UTR are known to control translation by poly- or deadenylation, localization, masking, and protein binding. The regulatory potential of the 5'UTR is becoming increasingly apparent during developmental processes (Spirin, 1994; Curtis *et al.*, 1995; Van der Velden and Thomas, 1999). The major determinants influencing the efficiency of translation initiation, which is the rate limiting step in protein synthesis, are the length, structure, and AUG codon content of the 5'UTR.

Translation initiation starts with the binding of eukaryotic initiation factor (eIF) 4E to the 7-methyl guanosine cap-structure at the 5' end of the mRNA, and formation of the eIF4F complex, consisting of eIF4E, 4A, and 4G. eIF4A, an ATP-dependent RNA helicase unwinds the secondary structure of the 5'UTR, thereby creating a landing platform for the 43S ribosomal subunit. After having bound at or near the cap, the 43S complex scans the 5'UTR for the AUG initiation codon with a favourable context (Kozak, 1989a; 1999). Here the 60S ribosomal subunit joins and protein synthesis begins.

This cap-dependent scanning model explains initiation on the majority of eukaryotic mRNAs. However, problems arise with long 5'UTRs, containing secondary structure formed by C-G, A-U and G-U base pairing, and upstream open reading frames (uORF). These characteristics are known to inhibit ribosomal scanning, and thereby reduce the translational efficiency of the mRNA (Pelletier and Sonenberg, 1985; Kozak, 1989b). Strikingly, many growth

Abbreviations used in this paper: ActD, actinomycin D; CAT, chloramphenicol acetyl transferase; eIF, eukaryotic initiation factor; FGF, fibroblast growth factor; GFP, green fluorescent protein; IGF, insulin-like growth factor; IGFl1, insulin-like growth factor 2-leader 1; IRES, internal ribosomal entry site; MBT, midblastula transition; uORF, upstream open reading frame; UTR, untranslated region.

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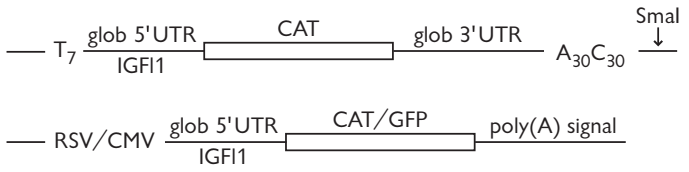

Fig. 1. Schematic representation of the reporter plasmids. *Plasmids for in vitro transcription contain either the Xenopus β-globin 5'UTR or IGFI1, followed by the CAT coding region, the Xenopus β-globin 3'UTR, and a track of 30 A and C residues. The SmaI site was used to linearize the plasmids for in vitro transcription. Plasmids for injection and transgenesis contain respectively the RSV or CMV promoter and the CAT or GFP reporter gene, followed by the SV40 polyadenylation signal.*

factors, as well as other proteins functioning in growth and differentiation are encoded by mRNAs with complex 5'UTRs, for example, fibroblast growth factor-2 (FGF-2), insulin-like growth factor (IGF) 2, transforming growth factor-β1, platelet-derived growth factor 2, epidermal growth factor, the c-mos, c-myc, c-syn, c-bcl-2 proto-oncogene products, retinoic acid receptor-β2, and poly(A)-binding protein (Kozak, 1987). Despite the complex 5'UTRs, most of these mRNAs are translated efficiently, at least in some tissues or at some time points (Zelus *et al.*, 1989; Bernstein *et al.*, 1995; Reynolds *et al.*, 1996).

For a number of these 5'UTRs, alternative mechanisms for translation initiation have been described, circumventing secondary structures and uORFs. The 5'UTRs of Antennapedia, FGF-2, and vascular endothelial growth factor mRNA contain an internal ribosomal entry site (IRES) in their 3' parts, able to bind ribosomes independently of the cap (OH *et al.*, 1992; Vagner *et al.*, 1995; Stein *et al.*, 1998). The 5'UTR of c-myc uses a shunt mechanism; ribosomes bind the cap, scan only part of the 5'UTR, and are discontinuously transferred to a ribosome acceptor site at the 3' end of the 5'UTR (Carter *et al.*, 1999). There are strong indications that these alternative initiation mechanisms require regulatory co-factors besides canonical eIFs (Van der Velden and Thomas, 1999). Therefore, these mechanisms provide a very specific way of controlling protein expression, independent from the bulk of cellular mRNAs.

Indeed, interesting examples exist of 5'UTRs specifically regulating protein expression during embryonic development. The IRES in Antennapedia mRNA directs a spatial and temporal expression pattern in transgenic flies (Ye *et al.*, 1997). The 5'UTRs of retinoic acid receptor-β2 and c-mos mRNA inhibit translation in a tissue-specific way by the precise organization of uORFs. Translation of the short uORF4 inhibits receptor expression in the heart and brain (Zimmer *et al.*, 1994; Reynolds *et al.*, 1996), and the uAUGs in the c-mos 5'UTR restrict protein expression to post-meiotic spermatids (Steel *et al.*, 1996). The ability of *Xenopus* embryos to overcome a structural barrier (a synthetic stable hairpin) in the 5'UTR depends on the developmental stage; the hairpin strongly inhibits translation in oocytes as well as in midblastula embryos, but is neglected during early cleavages (Fu *et al.*, 1991).

Besides intrinsic 5'UTR features, also canonical eIFs can control expression of specific mRNAs. Overexpression of eIF4E specifically enhances initiation on extensive secondary structure-containing 5'UTRs (Koromilas *et al.*, 1992). Proto-oncogene and other growth-related mRNAs contain complex 5'UTRs, explaining how eIF4E overexpression results in oncogenic transformation (Lazaris-Karatzas *et al.*, 1990). In *Xenopus* embryos, injection of eIF4E mRNA causes mesoderm induction in ectodermal explants. This is caused by preferential translation of the mesoderm inducer activin (Klein and Melton, 1994).

IGF2 mRNA can be transcribed from four different promoters resulting in mRNAs with the same coding region but with different 5'UTRs (Sussenbach *et al.*, 1992). The existence of four 5'UTRs is a strong indication for post-transcriptional regulation, which indeed has been shown (Nielsen *et al.*, 1990, 1995; De Moor *et al.*, 1994). Recently, we described the translation initiation properties of IGF2-leader 1 (IGFl1) (Van der Velden *et al.*, submitted). This 5'UTR of 592 nucleotides with one uORF and stable structures does not fit in one of the described mechanisms for translation initiation; a ribosomal shuffling mechanism was proposed guided by a cellular co-factor. In this report, we investigated whether IGFl1 activity is controlled during embryonic development. IGFl1-containing mRNAs and DNA constructs were injected into fertilized eggs of *Xenopus laevis*. During the initial stages of development, IGFl1-driven expression is severely suppressed, whereas its efficiency is stimulated after the midblastula transition. Possible mechanisms and implications of this up-regulated 5'UTR activity are investigated and discussed.

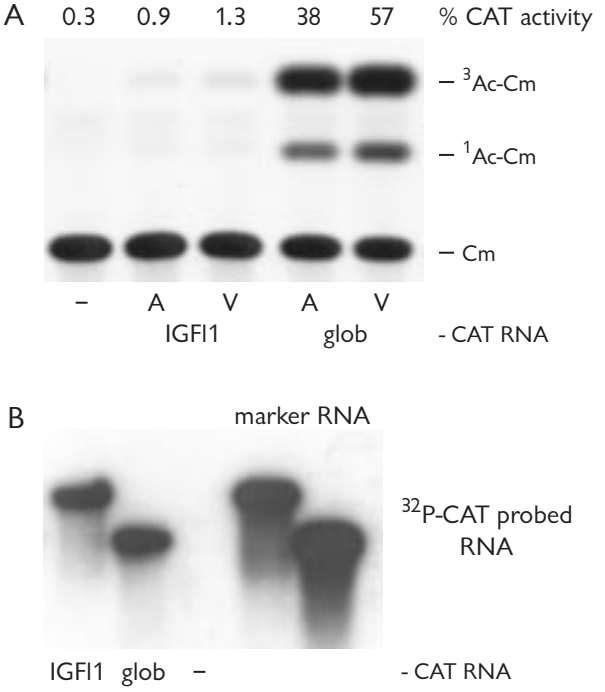

Fig. 2. Efficiency of IGF2-leader 1 in *Xenopus* embryos prior to MBT. *Fertilized Xenopus eggs were injected with in vitro synthesized capped IGFI1-CAT or glob-CAT mRNA (as indicated under each panel) into the animal (A) or vegetal (V) pole of the egg.* **(A)** *Embryo extracts were prepared at stage 6.5 and the equivalent of 0.01 embryo was assayed for CAT activity. Extract derived from uninjected embryos was used as a negative control (-). The percentage of chloramphenicol (Cm) converted to the acetylated forms (1/3Ac-Cm) is indicated.* **(B)** *Total RNA was isolated at stage 6 from injected and uninjected (-) embryos, and assayed by Northern blotting using a randomly primed 32P-labelled CAT probe; 5 ng of injected transcripts were used as marker RNA.*

Results

Translation initiation on IGF2-leader 1 is repressed in early Xenopus *embryos, and is potentiated in embryos after the midblastula transition (MBT)*

Control of translation initiation on the human IGF2-leader 1 (IGFl1) during embryonic development was studied by injecting synthetic CAT mRNAs and DNA plasmids into fertilized eggs of *Xenopus laevis*. RNA was synthesized *in vitro* from the T_7TS-IGFl1-CAT and T_7TS-glob-CAT constructs (Fig. 1) resulting in mRNAs containing the CAT reporter, preceded by either IGFl1 or the control *Xenopus* β-globin 5'UTR, and followed by the *Xenopus* β-globin 3'UTR and 30 A and C residues. Both 3' elements stabilize the synthetic RNA within the *Xenopus* embryo (results not shown). The transcripts were injected into the cytoplasm at the animal or vegetal pole of the fertilized egg. Embryo extracts were prepared at developmental stage 6.5 and assayed for CAT activity (Fig. 2A). Translation of IGFl1-CAT mRNA was 60-fold lower than glob-CAT translation (after correction for the CAT activity by extracts from uninjected embryos), which was independent of the site of injection. This differential CAT expression did not result from a difference in stability of the injected transcripts, as was determined by Northern blot analysis (Fig. 2B). The amount of CAT transcripts present within the embryos was comparable, indicating that the difference in CAT activity was due to the inability of early *Xenopus* embryos to efficiently initiate on IGFl1.

The translational potential of IGFl1 was further investigated by injecting DNA plasmids encoding the IGFl1-CAT and glob-CAT mRNAs. CAT expression was studied at various developmental stages after stage 8.5, the MBT, at which the embryo initiates transcription (Newport and Kirschner, 1982a, b). As is shown in Fig. 3A, post-MBT embryos were capable to translate IGFl1-CAT with an only 4-fold lower efficiency than glob-CAT. The Northern blot analysis of RNA isolated at stage 20 shows that the embryos produced comparable levels of both transcripts, and that the transcripts were of the expected sizes (Fig. 3B). Apparently, pre-MBT *Xenopus* embryos could hardly initiate on IGF2-leader 1, whereas embryos after MBT were very well capable of initiating on this 5'UTR sequence.

After transfection of the same plasmids to Cos-1 cells, the efficiencies of IGFl1 and the β-globin 5'UTR were similar (Van der Velden *et al*., submitted). The 4-fold lower potential of IGFl1 in *Xenopus* embryos could indicate that only specific cells or tissues support initiation on IGFl1 with an efficiency comparable to the globin 5'UTR. To investigate this possibility, embryos transgenic for the CMV-glob-GFP and CMV-IGFl1-GFP encoding sequences were generated. Embryos were visualized throughout development for GFP-dependent green light emission, and embryos between stage 17 and 42 are shown in Fig. 4. No major differences in the GFP expression patterns driven by IGFl1 and the globin 5'UTR could be detected. To be able to detect differences prior to stage 17, expression within the embryo was visualized after manual dissection of the transgenics. At these early stages, attention was paid to the ecto-, endo-, and mesodermal layers and to neural tissue; no differences were found either (results not shown). Northern blot analysis showed that transgenic embryos transcribed similar levels of the GFP RNAs, of the expected sizes (not shown).

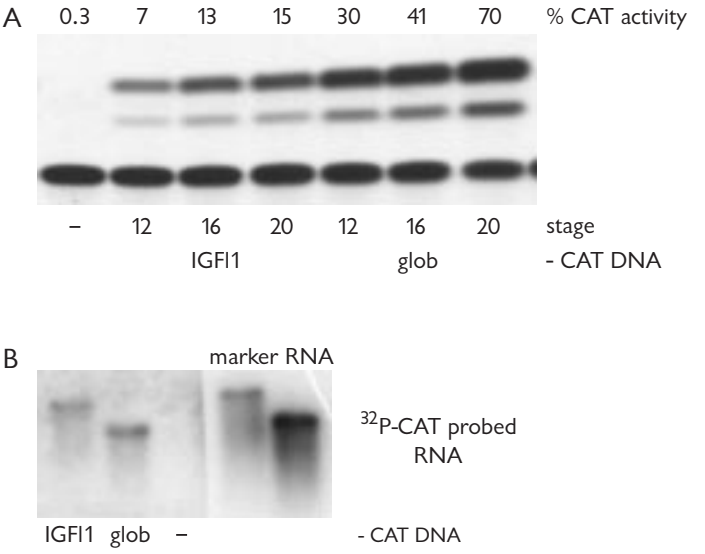

Fig. 3. Efficiency of IGF2-leader 1 in *Xenopus* embryos after MBT. *Fertilized Xenopus eggs were injected with the RSV-IGFl1-CAT or RSV-glob-CAT DNA constructs.* **(A)** *Embryo extracts were prepared at stages 12, 16, and 20, and the equivalent of one embryo was tested in the CAT assay. Extract from uninjected embryos was used as a negative control (-). The percentage of converted chloramphenicol is indicated.* **(B)** *Total RNA was isolated at stage 20, and assayed by Northern blotting using a ^{32}P-labelled CAT probe. RNA from uninjected embryos was used as a negative control (-), and in vitro synthesized transcripts as marker RNA. The signal from embryonic RNA was enhanced using an intensifying screen.*

Translation initiation on IGF2-leader 1 is upregulated around developmental stage 9

Combination of the RNA and DNA injection experiments suggests that translation initiation on IGFl1 is enhanced during embryonic development. This was investigated by injecting the CAT mRNAs, and determining CAT expression at various stages before and after MBT. This approach also gave the opportunity to determine the exact developmental stage of enhanced IGFl1 activity. Figure 5A shows a typical time-course experiment with embryo extracts from developmental stages 5, 8, 9, 12, 13, and 15. The results from several independent experiments were combined in the graph in Fig. 5B. From both figures can be concluded that initiation on IGFl1 was stimulated around stage 9 in *Xenopus* development. Prior to MBT the ratio between IGFl1 and the globin 5'UTR activities was about 1:50, which rapidly changed after stage 9 to 1:8. The moment of IGFl1 activation was developmental stage- and not time-dependent. We also cultured injected embryos at higher or lower temperatures, resulting in accelerated or delayed development. In these experiments, stimulation of IGFl1 activity was also seen around stage 9 (results not shown).

The Northern blot in the inset shows that the majority of IGFl1-CAT mRNA was still present and full-length in the stage 12 embryo. Therefore, we conclude that the enhanced initiation occurred on the full-length IGFl1, and not on a truncated, less complex form. The amount of glob-CAT mRNA decreased more rapidly in the embryo, coinciding with the decrease in the rate of glob-CAT RNA translation. A significant decrease in the stability of an efficiently translated mRNA was also observed in another *Xenopus* injection

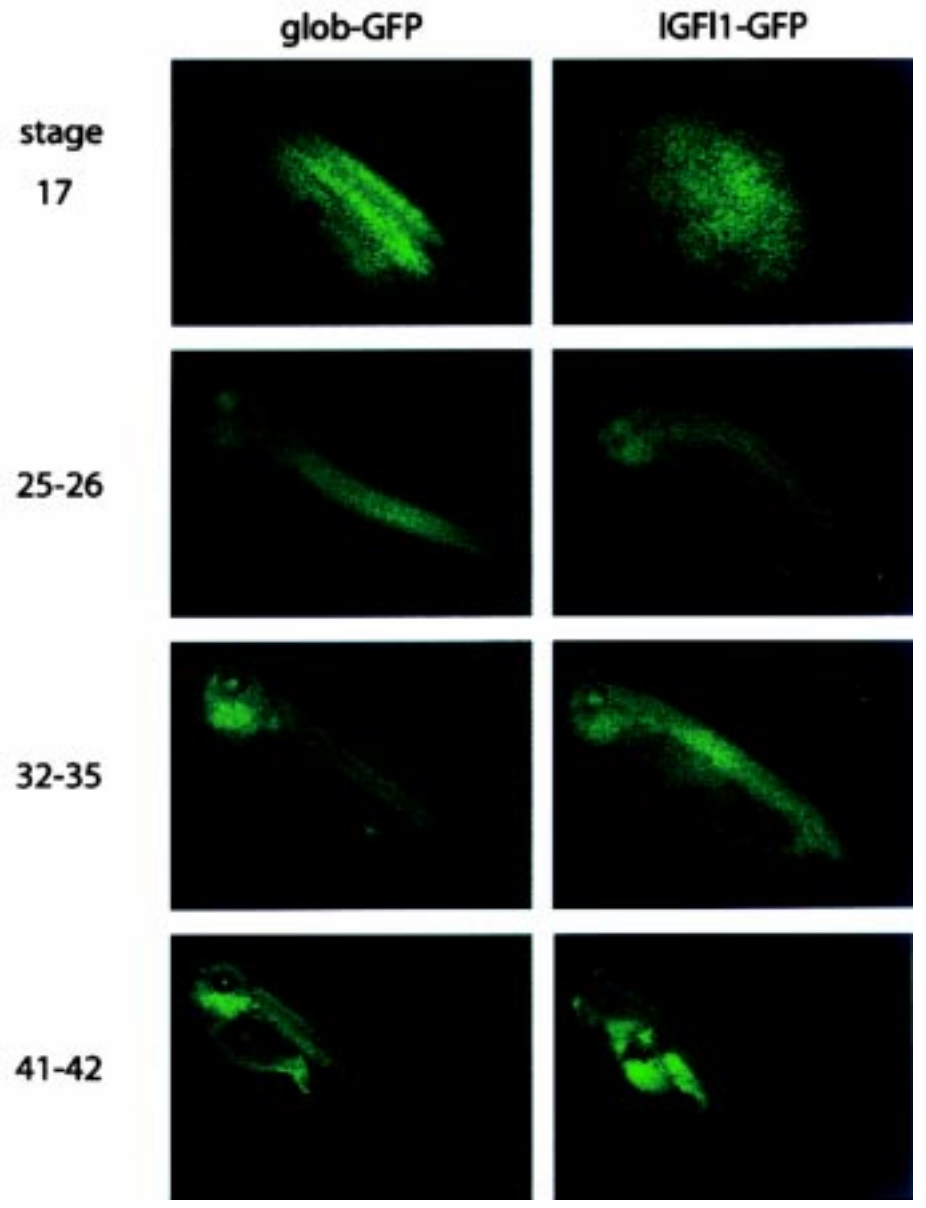

Fig. 4. GFP expression patterns directed by IGF2-leader 1 and by the β-globin 5′UTR in transgenic *Xenopus* embryos. *Mature Xenopus eggs were fertilized by injecting sperm DNA containing either the linearized CMV-glob-GFP or CMV-IGFl1-GFP DNA construct. Fluorescence of stage 17, 25-26, 32-35, and 41-42 embryos was visualized using a mercury lamp. The signal from transgenics was about equalized by adjusting the gain and/or the number of added frames. At these settings, control embryos only showed autofluorescence in the yolk (not shown).*

study (Lazarus, 1992). A control for linearity of globin 5'UTR directed translation was performed by injecting *in vitro* synthesized glob-GFP mRNA. Glob-GFP RNA appeared to be more stable in the embryo than glob-CAT RNA, and a straight line from stage 1 to 14 for accumulated GFP was found (results not shown).

Effect of eIF4E overexpression, and general transcription inhibition on the up-regulation of IGF2-leader 1 activity

As mentioned in the Introduction, elevated levels of eIF4E specifically enhance initiation on structured 5'UTRs. To test whether the suppressed IGFl1 activity in pre-MBT embryos was due to low eIF4E levels, we co-injected fertilized eggs with IGFl1-CAT and an mRNA encoding human eIF4E. Embryo extracts were made at developmental stage 8 to see whether eIF4E overexpression stimulated IGFl1-CAT translation in pre-MBT embryos. As shown in Fig. 6A, CAT translation driven by either IGFl1 or by the globin 5'UTR was not stimulated by eIF4E, although eIF4E protein was synthesized as was determined by immunoblotting (Fig. 6B).

Stimulation of IGFl1 activity occurred a few hours after transcription initiation in the embryo, around developmental stage 9. Therefore, we determined whether a co-factor resulting from de novo transcription enabled efficient initiation on IGFl1. For this purpose, transcription in the embryo was inhibited by injecting Actinomycin D (ActD) into the blastocoel of stage 7 embryos, reducing transcription of glob-CAT DNA by 84% (Fig. 7). It was technically impossible to further repress transcription, as higher amounts of ActD, or injection at earlier stages resulted in embryonic death. Although the amount of ActD used in our experiments resulted in a gastrulation block or exogastrulation, embryonic cells were still dividing. At the time of ActD injection at stage 7, IGFl1 activity was 50-fold lower than the activity of the globin 5'UTR. At stage 14, IGFl1-CAT translation was stimulated in normal embryos, as well as in ActD injected embryos. We therefore conclude that the stimulation of translation initiation on IGFl1 is independent of eIF4E levels and of de novo transcription.

Discussion

The length, uAUG content, and structure of a 5'UTR are important in the control of translation initiation. The expression of growth-related proteins, like growth factors, proto-oncogenes, and cyclins, needs to be tightly regulated during embryonic development. The transcripts encoding these factors often contain architecturally complex 5'UTRs. IGF2-leader 1 is complex and displays all characteristics inhibitory for ribosomal scanning. However, we have shown that neither internal initiation nor ribosomal shunting occurs on IGFl1 (Van der Velden *et al.*, submitted); a ribosomal shuffling mechanism was proposed, guided by a cellular co-factor. In this study, we present evidence for temporal translational control by IGFl1 during embryonic development of *Xenopus laevis*.

IGFl1 strongly inhibited reporter translation in fertilized eggs of *Xenopus* until stage 9 (Fig. 2). The complex 5'UTRs of c-myc, poliovirus RNA, the TAR-region of HIV-1, and synthetic stem-structures strongly impeded translation throughout *Xenopus* development (Lazarus *et al.*, 1988; Fu *et al.*, 1991; Lazarus, 1992; Fraser *et al.*, 1996). It was concluded that the degree of secondary structure is too excessive for efficient unwinding, thereby impairing the scan-

ning of ribosomes, or that the viral, human, murine, or synthetic structures are incompatible with the *Xenopus* translation system.

In our study, we have demonstrated that IGFl1 is compatible with the *Xenopus* translational machinery. Around developmental stage 9, a strong potentiation was observed resulting in an efficiency approaching the very efficient *Xenopus* β-globin 5'UTR (Figs. 3 and 5). IGFl1 activity was stimulated just after MBT (stage 8.5), a very important moment in *Xenopus* development. Zygotic transcription is initiated, resulting in new mRNAs needed for early patterning and differentiation events, like gastrulation where the three primordial germ layers are established. The localized expression of members of the FGF and transforming growth factor-β families is required for mesoderm induction and early differentiation (Kimelman and Kirschner, 1987; Amaya *et al.*, 1991). During these important patterning and differentiation events, the translational inhibition by IGFl1 is relieved. We investigated whether IGFl1 is active in all embryonic tissues, or whether for example mesoderm inducing tissue or the mesoderm itself is better equipped to initiate on IGFl1. We detected no differences between IGFl1 or globin 5'UTR-driven GFP expression in transgenic embryos (Fig. 4). However, enhanced expression in some cells within a tissue, or a transient stimulation added to the normal expression level are very hard to detect using this method. Therefore, we conclude that the vast majority of cells gain the potential to initiate on IGFl1 after MBT.

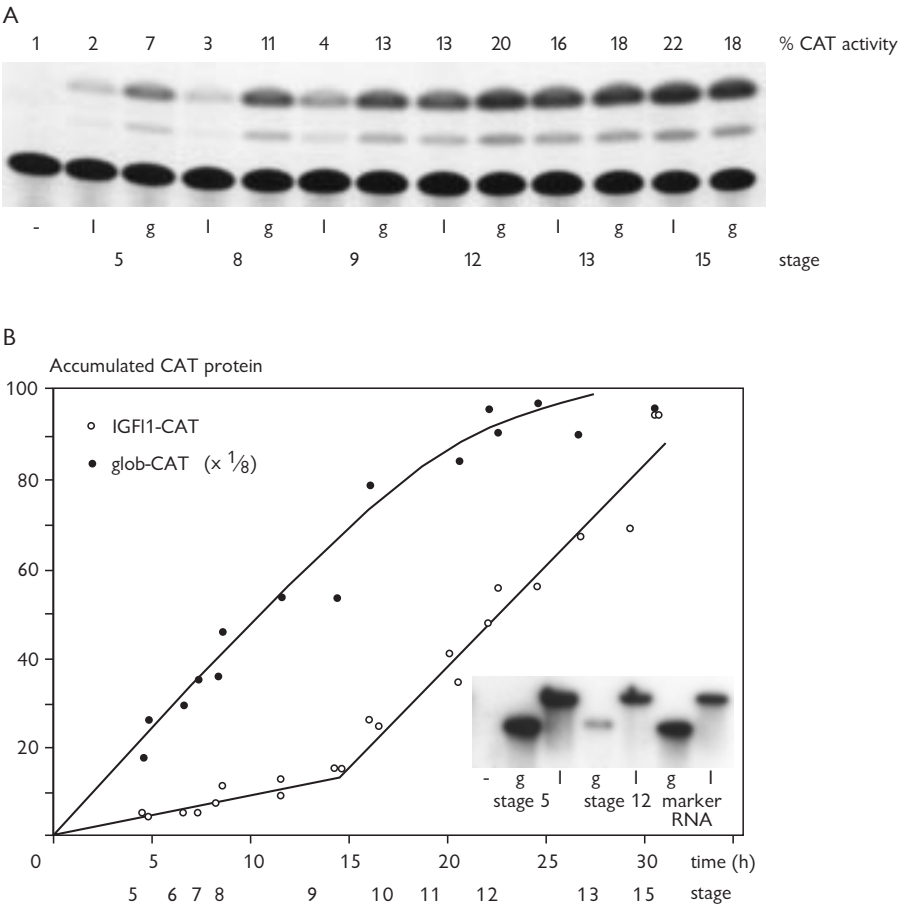

Fig. 5. Time-course experiments of IGFl1-CAT and glob-CAT translation during *Xenopus* development. *Fertilized Xenopus eggs were injected with in vitro synthesized capped IGFl1-CAT (l) or glob-CAT (g) mRNA.* **(A)** *Embryo extracts were prepared at stages 5, 8, 9, 12, 13, and 15; the equivalents of 0.02 IGFl1-CAT injected embryo and 0.0025 glob-CAT injected embryo were tested for CAT activity.* **(B)** *Several independent time-course experiments were combined. Accumulated CAT (normalized to 100) was plotted against time and developmental stage. In each experiment 8-fold less extract derived from glob-CAT injected embryos was tested. The inset shows a Northern analysis with RNA isolated at stages 5 and 12 from injected and uninjected (-) embryos, probed with a ^{32}P -labelled CAT probe.*

What can be responsible for the up-regulation of IGFl1 activity? A well-known mechanism for translational control during development is poly- or deadenylation of mRNAs (Curtis *et al.*, 1995; Richter, 1996). The 3' region of the injected RNAs lacks signals for polyadenylation. Furthermore, we have compared the sizes of the injected transcripts before and after MBT and did not find an alteration in length (Fig. 5B), indicating that modulation of the poly(A) tail length is not responsible for IGFl1 activation. Enhanced RNA helicase activity was suggested during early cleavages in *Xenopus* embryogenesis (Fu *et al.*, 1991), whereas IGFl1 becomes stimulated at later stages. As IGFl1 activity is enhanced just after transcription initiation, the most straightforward explanation would be a newly transcribed trans-acting factor, enabling efficient initiation on IGFl1. This hypothesis was tested by inhibiting zygotic transcription by ActD (Fig. 7). Although transcription was severely reduced, IGFl1 activity was normally stimulated. Therefore, activation by a newly synthesized trans-acting factor seems unlikely. We cannot however exclude the possibility that the very low level of transcription occurring before stage 7, or the remaining activity after ActD injection is sufficient for de novo synthesis of a trans-acting factor.

Given our results we propose the stage-specific activation of a trans-acting factor, already present before stage 9. This factor could be activated by post-translational modification, induced via signal transduction pathways. An example of the involvement of a signal transduction route in specific translational control is the p70 S6 kinase route regulating the 5' terminal oligopyrimidine tract, present in mRNAs encoding ribosomal proteins, and translation elongation factors, and in IGF2-leader 3 (Brown and Schreiber, 1996; Nielsen *et al.*, 1995). IGF2-leader 1 has no 5' terminal oligopyrimidine tract. The only known example of a signaling pathway regulated during *Xenopus* embryogenesis is MAP-kinase. MAP-kinase activity is low during cleavage stages and increases during gastrulation, the moment of mesoderm induction and IGFl1 up-regulation (LaBonne and Whitman, 1997). An interesting possibility is that activation of the MAP-kinase route results in an enhanced co-factor phosphorylation, thereby activating it.

Which factor could be phosphorylated by the MAP-kinase or another signaling pathway, and then stimulate IGFl1-driven translation? First, it can be an unidentified trans-acting factor binding the 5'UTR, thereby influencing initiation. Trans-acting protein factors

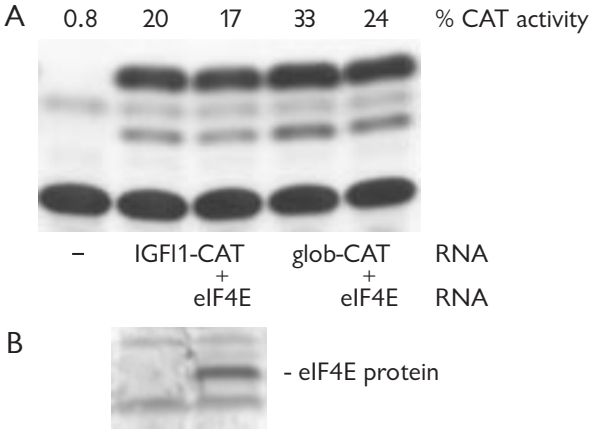

Fig. 6. Effect of eIF4E overexpression on the up-regulation of IGF2-leader 1 activity. *Fertilized eggs were injected with IGFI1-CAT or glob-CAT RNA alone, or in combination with human eIF4E encoding RNA.* **(A)** *Extracts were prepared at stage 8, and assayed for CAT activity: 2 embryo equivalents of IGFI1-CAT injected embryos, and 0.04 of glob-CAT injected embryos.* **(B)** *Extracts from IGFI1-CAT and IGFI1-CAT+eIF4E injected embryos were analyzed by immunoblotting using a polyclonal eIF4E antibody.*

have been implicated in the regulation of several complex 5'UTRs, for example FGF-2 and platelet-derived growth factor 2/c-*sis*, although their identities are unknown (Van der Velden and Thomas, 1999). Translation lysates were prepared from cell lines and stage 20 *Xenopus* embryos, both efficiently translating IGFI1-CAT *in vivo*. *In vitro*, these extracts failed to support IGFI1-CAT translation, whereas glob-CAT mRNA was translated (results not shown). This suggests that the co-factor, its activity, or essential interactions are easily lost during extract preparation.

A second possible candidate to stimulate IGFI1-driven translation is eIF4E. We have tested whether the amount of eIF4E is limiting in pre-MBT embryos, by co-injecting an mRNA encoding human eIF4E, as IGFI1 is a human 5'UTR. As eIF4E overexpression did not stimulate IGFI1-driven translation (Fig. 6), we conclude that the absolute amount of eIF4E is not limiting. However, not only the absolute amount of eIF4E, but most importantly, its phosphorylation state and binding to proteins determine eIF4E activity. The amount of free eIF4E is regulated via sequestering by eIF4E-binding proteins. This inhibitory interaction is relieved following phosphorylation of the binding proteins by a PI3K-dependent pathway, involving FRAP/mTOR (Raught and Gingras, 1999). No data are available on the activities of these proteins during *Xenopus* embryogenesis. Free eIF4E is phosphorylated by MNK1, a substrate of the MAP-kinase and ERK signaling pathways (Raught and Gingras, 1999). Developmentally regulated activation of the MAP-kinase pathway might result in more phosphorylated eIF4E. This can increase initiation on complex 5'UTRs, present in growth factor mRNAs needed for mesoderm induction. An interesting observation with respect to this hypothesis, is that MAP-kinase activity is absolutely required for mesoderm induction (Gotoh *et al.*, 1995). If regulation via eIF4E would be the case, there are two explanations why eIF4E overexpression in pre-MBT embryos did not enhance IGFI1 activity. First, the overexpressed eIF4E can be sequestered by the binding proteins. Second, due to low pre-MBT *Xenopus* eIF4E kinase activity, human eIF4E is not phosphorylated and activated. The idea of a transient eIF4E activation has not been tested yet in *Xenopus* embryos. *Xenopus* eIF4E cDNAs have been obtained but mRNA, protein, and phosphorylation levels have not been determined between blastula and tailbud stages (Wakiyama *et al.*, 1995).

Concluding, we hypothesize that developmentally regulated activity of protein kinases is responsible for the activation of a specific trans-acting factor or translation initiation factor eIF4E. This subsequently results in enhanced translation of mRNAs with architecturally complex 5'UTRs. We are planning to investigate efficiencies of *Xenopus* growth factor 5'UTRs during embryogenesis to determine whether the described up-regulation is a general phenomenon.

Materials and Methods

Construction of the plasmids

The RSV-glob-CAT, RSV-IGFI1-CAT, T_7TS-glob-CAT, T_7TS-IGFI1-CAT, and CMV-glob-GFP constructs were described elsewhere (Van der Velden *et al.*, submitted), and are schematically shown in Fig. 1. CMV-IGFI1-GFP was constructed by inserting IGF2-leader 1 (StuI-NcoI fragment), and the GFP coding sequence (NcoI-XbaI fragment from pEGFP-N1, Clontech) via a 3-point ligation into the EcoRV/XbaI-digested modified pCS2 (Van der Velden *et al.*, submitted). The T_7TS-4E plasmid was generated by replacing the CAT coding sequence in T_7TS-glob-CAT by the eIF4E coding sequence (NcoI-BamHI fragment, Stern *et al.*, 1993).

In vitro *transcription*

Plasmids were linearized with SmaI, and transcribed using the T_7MEGAscript kit (Ambion) with 7.5 mM of m^7GpppG, ATP, CTP, UTP, and 1.5 mM of GTP. After template digestion with DNaseI, transcripts were purified by gel-filtration. The amount and integrity of RNA was examined by gel electrophoresis in an ethidium bromide containing agarose gel.

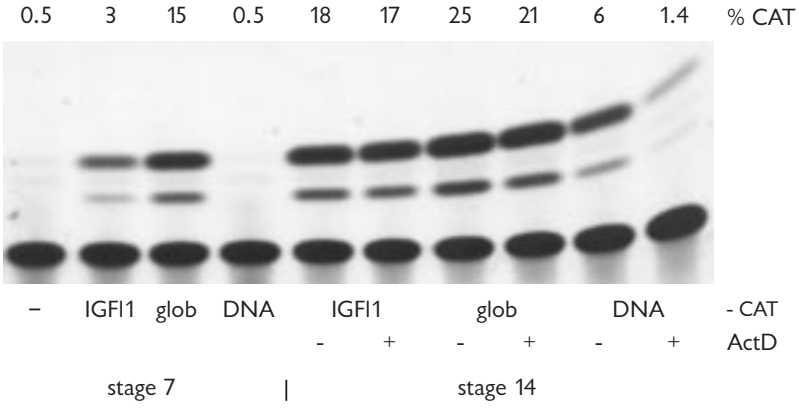

Fig. 7. Effect of transcription inhibition on the up-regulation of IGF2-leader 1 activity. *IGFI1-CAT, glob-CAT mRNA, or RSV-glob-CAT DNA (DNA) were injected into fertilized eggs. At stage 7 extracts were prepared, and half of the remaining embryos received 0.5 ng of ActD into the blastocoel. These embryos were homogenized at stage 14. CAT activity was determined in one DNA injected embryo, in 0.02 IGFI1-CAT, and 0.0025 glob-CAT injected embryo equivalent.*

Manipulation of embryos, micro-injection and transgenesis

In vitro fertilized *Xenopus laevis* eggs were dejellied using 2% cysteine (pH 7.8), and kept in 0.25xMMR (modified amphibian Ringers' solution: 25 mM NaCl, 0.5 mM KCl, 0.25 mM $MgSO_4$, 0.5 mM $CaCl_2$, 1.25 mM HEPES-NaOH (pH 7.8), 0.025 mM EDTA). Fertilized eggs were injected in 0.25xMMR containing 3% Ficoll with 50 pg of supercoiled circular DNA or with 1 ng of *in vitro* synthesized capped RNA. In the co-injection experiment, 1.4 ng of eIF4E RNA was injected together with 0.1 ng of CAT RNA. After 4-6 hours the medium was changed to 0.25xMMR, and the embryos were allowed to develop until the desired stages (Nieuwkoop and Faber, 1967) at 18-20°C. Transcription in the embryo was inhibited by injecting 0.5 ng of ActD into the blastocoel of stage 7 embryos.

Transgenic embryos were generated by injecting detergent- and restriction enzyme- treated sperm nuclei into the mature oocyte (Kroll and Amaya, 1996; Amaya, 1999). We used the procedure provided by E. Amaya on Internet (www.welc.cam.uk/~ea3/), with some minor modifications. *Xenopus* eggs were partially dejellied using 2.2% cysteine (pH 7.9). The restriction enzyme mediated integration reaction contained 4 μl of sperm nuclei (from a frozen stock) with 1 μl of the PvuII-linearized plasmids, CMV-glob-GFP or CMV-IGFl1-GFP (500 ng/μl). The extract mixture contained 0.5 Unit of PvuII. Embryos were cultured overnight in 0.1xMMR, 3% Ficoll, with 50 μg of gentamycin/ml, and from then on in the same solution with 1% Ficoll, always at 16°C.

CAT assay

At least 8 healthy embryos per time point were homogenized in 0.25 M Tris-HCl (pH 7.5), frozen in liquid nitrogen, thawed at 37°C, mixed, and centrifuged at 12,000 rpm for 5 min at 4°C. Supernatants were transferred to a new tube, and cleared by a second centrifugation step. These embryo extracts were tested for CAT enzyme activity (Gorman *et al.*, 1982); to ensure conversion in the linear range, different amounts of extract (corresponding to 0.0025 to 2 embryo equivalents) were tested. The 150 μl reaction mixture (0.25 M Tris-HCl, pH 7.5), containing 70 μg of acetyl coenzyme A and 0.25 μCi of [^{14}C]chloramphenicol, was incubated for 45 min at 37°C. Chloramphenicol and the acetylated forms were extracted with ethylacetate and separated by TLC-chromatography with 95% chloroform-5% methanol. After autoradiography, conversion was quantified using a Phosphor-imager (Molecular Dynamics).

Isolation of RNA from** Xenopus **embryos and Northern blotting

At least 50 embryos were homogenized in 6 M urea, 3 M LiCl. RNA was allowed to precipitate overnight at 4°C, and was subsequently pelleted by centrifugation for 1 hour at 16,000 g at 4°C. The pellet was dissolved in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% SDS, extracted with phenol, twice with phenol/chloroform, and with chloroform, and RNA was precipitated in ethanol. Total RNA (5 or 20 μg) was separated by electrophoresis in an 1.5% agarose gel, containing 6.5% formaldehyde, 20 mM MOPS (pH 7), 5 mM NaAc, and 1 mM EDTA, and was transferred to a nylon membrane (Qiabrane). After UV-cross linking and prehybridization for 4 hours at 65°C, filters were hybridized overnight at 65°C with a randomly primed ^{32}P-labelled CAT or GFP probe in 3xSSC, 5xDenhardt's, 0.1% SDS, 10% dextran sulphate, and salmon sperm DNA (0.05 mg/ml). After washing, RNA was visualized by autoradiography.

GFP visualization

GFP in transgenic embryos was visualized with a microscope (Leica, MZ FLIII), equipped with a mercury lamp to detect fluorescence. The image was electronically recorded with a 3CCD camera (Sony, XC-003P), and the analogue output was sent to a video digitizer. To enhance the signal two options were available: first, the signal from the 3CCD camera could be amplified (gain), and second, several frames could be superimposed using a home-made computer program.

Western immunoblotting

Protein extracts from *Xenopus* embryos were separated in a SDS-12.5% polyacrylamide gel, and transferred onto an Immobilon filter. Blocking was done with 5% milk powder in 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.075% Tween-20. Human eIF4E protein was detected using a rabbit polyclonal antibody against a human eIF4E peptide (1:2000, kindly provided by N. Sonenberg), and a goat-anti-rabbit antibody coupled to alkaline phosphatase (1:5000).

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References

AMAYA, E. (1999). A method for generating transgenic frog embryos. In *Methods in Molecular Biology* (Eds. P. Sharpe and I. Mason). Molecular Embryology: Methods and Protocols, Vol. 97. Humana Press Inc., Totowa, pp. 394-414.

AMAYA, E., MUSCI, T.J. and KIRSCHNER, M.W. (1991). Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in *Xenopus* embryos. *Cell* 66: 257-270.

BERNSTEIN, J., SHEFLER, I. and ELROY-STEIN, O. (1995). The translational repression mediated by the platelet-derived growth factor 2/c-*sis* mRNA leader is relieved during megakaryocytic differentiation. *J. Biol. Chem.* 270: 10559-10565.

BROWN, E.J. and SCHREIBER, S.L. (1996). A signaling pathway to translational control. *Cell* 86: 517-520.

CARTER, P.S., JARQUIN-PARDO, M. and DE BENEDETTI, A. (1999). Differential expression of Myc1 and Myc2 isoforms in cells transformed by eIF4E: evidence for internal ribosome repositioning in the human c-*myc* 5'UTR. *Oncogene* 18: 4326-4335.

CURTIS, D., LEHMANN, R. and ZAMORE, P.D. (1995). Translational regulation in development. *Cell* 81: 171-178.

DE MOOR, C.H., JANSEN, M., SUSSENBACH, J.S. and VAN DEN BRANDE, J.L. (1994). Differential polysomal localization of human insulin-like growth factor 2 mRNAs both in cell lines and foetal liver. *Eur. J. Biochem.* 222: 1017-1024.

FRASER, S.D., WILKES-JOHNSTON, J. and BROWDER, L.W. (1996). Effects of c-*myc* first exons and 5' synthetic hairpins on RNA translation in oocytes and early embryos of *Xenopus laevis*. *Oncogene* 12: 1223-1230.

FU, L., YE, R., BROWDER, L.W. and JOHNSTON, R.N. (1991). Translational potentiation of messenger RNA with secondary structure in *Xenopus*. *Science* 251: 807-810.

GORMAN, C.M., MOFFAT, L.F. and HOWARD, B.H. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2: 1044-1051.

GOTOH, Y., MASUYAMA, N., SUZUKI, A., UENO, N. and NISHIDA, E. (1995). Involvement of the MAP kinase cascade in *Xenopus* mesoderm induction. *EMBO J.* 14: 2491-2498.

KIMELMAN, D. and KIRSCHNER, M. (1987). Synergistic induction of mesoderm by FGF and TGF-β and the identification of an mRNA coding for FGF in the early *Xenopus* embryo. *Cell* 51: 869-877.

KLEIN, P.S. and MELTON, D.A. (1994). Induction of mesoderm in *Xenopus laevis* embryos by translation initiation factor 4E. *Science* 265: 803-806.

KOROMILAS, A.E., LAZARIS-KARATZAS, A. and SONENBERG, N. (1992). mRNAs containing extensive secondary structure in their 5' non-coding region translate efficiently in cells overexpressing initiation factor eIF-4E. *EMBO J.* 11: 4153-4158.

KOZAK, M. (1987). An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucl. Acids Res.* 15: 8125-8148.

KOZAK, M. (1989a). The scanning model for translation: An update. *J. Cell Biol.* 108: 229-241.

KOZAK, M. (1989b). Circumstances and mechanisms of inhibition of translation by secondary structure in eukaryotic mRNAs. *Mol. Cell. Biol.* 9: 5134-5142.

KOZAK, M. (1999). Initiation of translation in prokaryotes and eukaryotes. *Gene* 234: 187-208.

KROLL, K.L. and AMAYA, E. (1996). Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation.

Development 122: 3171-3183.

LABONNE, C. and WHITMAN, M. (1997). Localization of MAP kinase activity in early *Xenopus* embryos: Implications for endogenous FGF signaling. *Dev. Biol.* 183: 9-20.

LAZARIS-KARATZAS, A., MONTINE, K.S. and SONENBERG, N. (1990). Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap. *Nature* 345: 544-547.

LAZARUS, P. (1992). The regulation of translation by the 5' untranslated region of *Xenopus* c-*myc* I mRNA during early development. *Oncogene* 7: 1037-1041.

LAZARUS, P., PARKIN, N. and SONENBERG, N. (1988). Developmental regulation of translation by the 5' non-coding region of murine c-*myc* mRNA in *Xenopus laevis*. *Oncogene* 3: 517-521.

NEWPORT, J. and KIRSCHNER, M. (1982a). A major developmental transition in early *Xenopus* embryos: I. Characterization and timing of cellular changes at the midblastula stage. *Cell* 30: 675-686.

NEWPORT, J. and KIRSCHNER, M. (1982b). A major developmental transition in early *Xenopus* embryos: II. Control of the onset of transcription. *Cell* 30: 687-696.

NIELSEN, F.C., GAMMELTOFT, S. and CHRISTIANSEN, J. (1990). Translational discrimination of mRNAs coding for human insulin-like growth factor II. *J. Biol. Chem.* 265: 13431-13434.

NIELSEN, F.C., ØSTERGAARD, L., NIELSEN, J. and CHRISTIANSEN, J. (1995). Growth-dependent translation of IGF-II mRNA by a rapamycin-sensitive pathway. *Nature* 377: 358-362.

NIEUWKOOP, P.D. and FABER, J. (Eds.) (1967). *Normal table of Xenopus laevis (Daudin): a systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis.* North Holland Publishing company, Amsterdam.

OH, S.-K., SCOTT, M.P. and SARNOW, P. (1992). Homeotic gene *antennapedia* mRNA contains 5'-noncoding sequences that confer translational initiation by internal ribosome binding. *Genes Dev.* 6: 1643-1653.

PELLETIER, J. and SONENBERG, N. (1985). Insertion mutagenesis to increase secondary structure within the 5' non-coding region of a eukaryotic mRNA reduces translational efficiency. *Cell* 40: 515-526.

RAUGHT, B. and GINGRAS, A.-C. (1999). eIF4E activity is regulated at multiple levels. *Int. J. Biochem. Cell Biol.* 31: 43-57.

REYNOLDS, K., ZIMMER, A.M. and ZIMMER, A. (1996). Regulation of RARβ2 mRNA expression: Evidence for an inhibitory peptide encoded in the 5'-untranslated region. *J. Cell Biol.* 134: 827-835.

RICHTER, J.D. (1996). Dynamics of poly(A) addition and removal during development. In *Translational Control* (Eds. J.W.B. Hershey, M.B. Mathews and N. Sonenberg). Cold Spring Harbor Laboratory Press, New York, pp. 481-503.

SPIRIN, A.S. (1994). Storage of messenger RNA in eukaryotes: envelopment with protein, translational barrier at 5' side, or conformational masking by 3' side? *Mol. Rep. Dev.* 38: 107-117.

STEEL, L.F., TELLY, D.L., LEONARD, J., RICE, B.A., MONKS, B. and SAWICKI, J.A. (1996). Elements in the murine c-*mos* messenger RNA 5'-untranslated region repress translation of downstream coding sequences. *Cell Growth Diff.* 7: 1415-1424.

STEIN, I., ITIN, A., EINAT, P., SKALITER, R., GROSSMAN, Z. and KESHET, E. (1998). Translation of vascular endothelial growth factor mRNA by internal ribosome entry: implications for translation under hypoxia. *Mol. Cell. Biol.* 18: 3112-3119.

STERN, B.D., WILSON, M. and JAGUS, R. (1993). Use of non-reducing SDS-PAGE for monitoring renaturation of recombinant protein synthesis initiation factor, eIF-4α. *Prot. Expr. Pur.* 4: 320-327.

SUSSENBACH, J.S., STEENBERGH, P.H. and HOLTHUIZEN, P. (1992). Structure and expression of the human insulin-like growth factor genes. *Growth Reg.* 2: 1-9.

VAGNER, S., GENSAC, M.-C., MARET, A., BAYARD, F., AMALRIC, F., PRATS, H. and PRATS, A.-C. (1995). Alternative translation of human fibroblast growth factor 2 mRNA occurs by internal entry of ribosomes. *Mol. Cell. Biol.* 15: 35-44.

VAN DER VELDEN, A.W. and THOMAS, A.A.M. (1999). The role of the 5' untranslated region of an mRNA in translation regulation during development. *Int. J. Biochem. Cell Biol.* 31: 87-106.

VAN DER VELDEN, A.W., VAN NIEROP, K., VOORMA, H.O. and THOMAS, A.A.M. (2000). Ribosomal scanning on the highly structured IGF2-leader 1. *Submitted for publication.*

WAKIYAMA, M., SAIGOH, M., SHIOKAWA, K. and MIURA, K. (1995). mRNA encoding the translation initiation factor eIF-4E is expressed early in *Xenopus* embryogenesis. *FEBS Lett.* 360: 191-193.

WICKENS, M., KIMBLE, J. and STRICKLAND, S. (1996). Translational control of developmental decisions. In *Translational Control* (Eds. J.W.B. Hershey, M.B. Mathews and N. Sonenberg). Cold Spring Harbor Laboratory Press, New York, pp. 411-450.

YE, X., FONG, P., IIZUKA, N., CHOATE, D. and CAVENER, D.R. (1997). *Ultrabithorax* and *Antennapedia* 5' untranslated regions promote developmentally regulated internal translation initiation. *Mol. Cell. Biol.* 17: 1714-1721.

ZELUS, B.D., GIEBELHAUS, D.H., EIB, D.W., KENNER, K.A. and MOON, R.T. (1989). Expression of the poly(A)-binding protein during development of *Xenopus laevis*. *Mol. Cell. Biol.* 9: 2756-2760.

ZIMMER, A., ZIMMER, A.M. and REYNOLDS, K. (1994). Tissue specific expression of the retinoic acid receptor-β2: Regulation by short open reading frames in the 5'-noncoding region. *J. Cell Biol.* 127: 1111-1119.

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