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Original Article

Sequence and translation initiation properties of the *Xenopus* TGFβ5, PDGF-A, and PDGF-α receptor 5' untranslated regions

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ABSTRACT The properties of the architecturally complex *Xenopus laevis* TGFβ5, PDGF-A and PDGF-α receptor 5′UTRs were investigated. 5' extended cDNAs were obtained by 5′RACE, resulting in long 5′UTRs (478-710 nt) with multiple upstream AUGs (3-13), and the potential to fold into stable structures. Injection studies suggested that the cloned PDGF-αR 5′UTR contains an intron. Splicing at potential 5' and 3' splice sites would result in a non-complex 5′UTR of 142 nt. The above mentioned 5′UTR characteristics are inhibitory for ribosomal scanning. Indeed, relative to the β-globin 5′UTR, the complex 5′UTRs strongly repressed initiation of protein synthesis in pre-MBT *Xenopus* embryos. However, later in embryogenesis, the inhibition was partly relieved. The results show temporal translational control by these 5′UTRs. Transgenic embryos showed that the 5′UTRs allowed translation throughout the embryo; spatial control could not be observed. Interestingly, a fragment in the PDGF-A 5′UTR highly similar to an element in the human PDGF-A 5′UTR is complementary to *Xenopus* 18S ribosomal RNA. None of these *Xenopus* 5′UTRs contains an IRES, as determined by injecting bicistronic constructs.

KEY WORDS: *5'UTR, translation regulation, Xenopus; TGF, PDGF.*

Introduction

The induction and patterning of mesoderm is a major event in early development of amphibian embryos. During gastrulation cells on the dorsal side of the embryo, the presumptive mesoderm, migrate between the ecto- and endoderm towards the anterior of the embryo. The migration, fate, and differentiation of these cells are controlled by extracellular signals, like polypeptide growth factors. Members of the transforming growth factor (TGF) family affect development of all three germ layers in *Xenopus laevis* embryos. They induce mesoderm in explanted tissue (Harland and Gerhart, 1997), and expression of mutant TGFβ-receptors in whole embryos results in ablation of mesoderm (Hemmati-Brivanlou and Melton, 1992). TGFβ signaling also plays a role in patterning the endoderm (Henry *et al.*, 1996). Given the expression patterns of the various TGFs in a wide variety of tissues in mice, multiple roles throughout development are suggested (Pelton *et al.*, 1991).

The platelet-derived growth factor (PDGF) system is also important during gastrulation and mesoderm specification. The PDGF-α receptor (PDGF-αR) is expressed in involuting cells, which migrate along ectodermal cells expressing PDGF-A. Furthermore, a mutant PDGF-αR results in abnormal gastrulation and loss of anterior mesodermal structures (Ataliotis *et al.*, 1995). It was recently suggested that PDGF itself can act as an inducer of dorsal mesoderm (Ghil and Chung, 1999). Later in embryogenesis, PDGF-αR mRNA is found in many mesenchymal tissues, whereas PDGF-A is present in adjacent ecto- or endodermal layers (Ho *et al.*, 1994). It is therefore proposed that PDGF signaling controls migration, specification, and differentiation of the mesenchyme.

The cDNAs of TGFβ5, PDGF-A, and PDGF-αR have been isolated from *Xenopus* embryos. It has not been established yet whether TGFβ5 is the amphibian homologue of mammalian TGFβ1 (Chimal-Monroy and Díaz de León, 1997), or is unique to amphibians (Vempati and Kondaiah, 1998). TGFβ5 is encoded by a 3 kb

Abbreviations used in this paper: bp, base pairs; CAT, chloramphenicol acetyl transferase; EMCV, encephalomyocarditis virus; GFP, green fluorescent protein; glob, β-globin; His-3, *Xenopus* Histon-3; IGF, insulin-like growth factor 2; IRES, internal ribosomal entry site; MBT, midblastula transition; nt, nucleotide(s); ORF, open reading frame; PDGF-A (PDGF), platelet-derived growth factor-A; PDGF-αR (PDGF-R), platelet-derived growth factor-α receptor; RACE, rapid amplification of cDNA ends; rRNA, ribosomal RNA; sAUG, start AUG; TGFβ5 (TGF), transforming growth factor-β5; u, upstream; UTR, untranslated region.

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CCUUUAAGUUACAGCACACAGGAGGAAGCCAAGAGGGGGG
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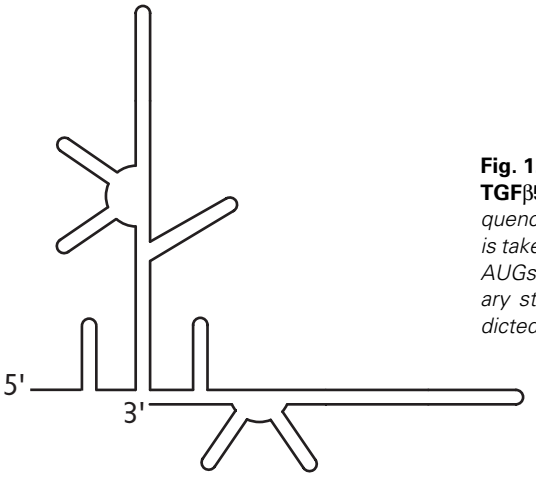

Fig. 1. The 5′UTR of the *Xenopus* TGFβ5 mRNA. *The nucleotide sequence (156 A, 130 U, 104 C, 113 G) is taken from Kondaiah et al. (1990). AUGs are underlined. The secondary structure of the 5′UTR is predicted by the Zucker algorithm.*

mRNA, present at low levels in the egg and early embryo. This maternal stockpile is supplemented by new transcription at the early neurula stage (Kondaiah *et al.*, 1990). PDGF-A mRNA, present as two transcripts of about 3 kb, is also maternally encoded and its zygotic transcription is initiated at the gastrula stage (Mercola *et al.*, 1988). A similar temporal expression is seen for the two 7 kb species of PDGF-αR mRNA (Jones *et al.*, 1993).

Transcription of zygotic genes in *Xenopus* embryos is initiated at stage 8-9, the midblastula transition (MBT). Before this stage, development is directed by maternal proteins and mRNAs stored in the egg. Therefore, all developmental decisions before MBT rely on control of translation rather than transcription. The translational control mechanisms during early development are diverse and complex. They involve the 3' and 5' untranslated region (UTR) of the mRNA, which influence mRNA stability, localization, and translational activation or repression (Curtis *et al.*, 1995; Wickens *et al.*, 1996). The regulatory potential of the 5'UTR is becoming increasingly apparent, especially during embryogenesis (Spirin, 1994; Van der Velden and Thomas, 1999).

The 5'UTR and 5' cap are the initial binding site for the 40S ribosomal subunit attached to the initiator Met-tRNA. According to the cap-dependent scanning model (Kozak, 1989a), this 43S complex scans along the 5'UTR searching for the AUG initiation codon, where the 60S subunit joins and protein synthesis begins. Scanning is severely hampered on a long 5'UTR, containing secondary structures formed by C-G, A-U, and G-U base pairing, and by upstream AUG codons (uAUGs); these characteristics reduce the translational efficiency of the mRNA (Pelletier and Sonenberg, 1985; Kozak, 1989b; 1991). Many growth factors, receptors, and proto-oncogenes are encoded by mRNAs with a complex 5'UTR (Kozak, 1987). It is shown that complex 5'UTRs can specifically regulate protein expression during embryonic development in a spatio-temporal way, and in adults during differentiation (Van der Velden and Thomas, 1999).

The described 5'UTRs of the *Xenopus* TGFβ5, PDGF-A, and PDGF-αR mRNAs are considerably longer and more complex than the average *Xenopus* 5'UTR of about 100 nt without uAUGs (TransTerm, Genbank databases). In this study, we describe the complete 5'UTRs after 5'RACE experiments, and investigated their temporal or spatial regulatory potential. Previously, we have shown that activity of the human insulin-like growth factor (IGF) 2 5'UTR becomes stimulated at MBT (Van der Velden *et al.*, 2000). Now we show that this is a general phenomenon for complex 5'UTRs, as translation initiation on the *Xenopus* TGFβ5 and PDGF-A 5'UTRs is also up-regulated during development.

Results

Characteristics of the Xenopus *TGFβ5, PDGF-A and PDGF-αR 5'UTRs*

Long 5'UTRs with secondary structures and uAUG codons are inhibitory for translation initiation, and reduce the translational efficiency of the message. The majority of *Xenopus* mRNAs contain a 5'UTR shorter than 150 nt devoid of AUGs, and will therefore support efficient protein synthesis. In this study we investigated the potential of architecturally complex *Xenopus* 5'UTRs in the embryo. A database search (Genbank) resulted in the TGFβ5 (503 nt, 7 AUGs), PDGF-A (407 nt, 3 AUGs), and PDGF-αR (408 nt, 4 AUGs) 5'UTRs.

GCUAUAAUCGCCACUCUCCCCAGAACAGGCCGUUGCUCUC
AGAGCUGCUGCGCUCAGUCCGCCAGAGUUUCGCAGCGUUG
UGGAUUGUUCGCAUUGGCCGAUCAGGUGCGA**CUGCAG**AGA
CGCCAGUGUGUUCCAUCUGCCGCAAAGGGAGCCGCUGUUC
GUGUUUGGAAAGUUCAUUCACCCUGCUCUUUGCCUGCUUU
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G

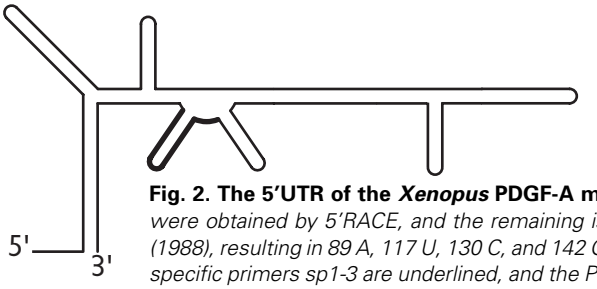

Fig. 2. The 5′UTR of the *Xenopus* PDGF-A mRNA. *The first 71 nt were obtained by 5′RACE, and the remaining is after Mercola et al. (1988), resulting in 89 A, 117 U, 130 C, and 142 G residues. AUGs and specific primers sp1-3 are underlined, and the PstI site is in bold. The 18S rRNA complementary sequence is in italic and is indicated by the thick line in the secondary structure prediction.*

GGGGAUUUACAGACUUUAACAGAUUGAAAGGUAAGAUGCA
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CGGUUCUCCCUUGGGCAGUUUCUCCUUUCUGUCGCUAUUC
ACUUUGCUGGAGCUGAGAGCUGGUACCUCUGGAAGAAUCU
AGGGCAAUCGCAGUUUUUUUUGCCAUUUUGGAUUGUAGAG
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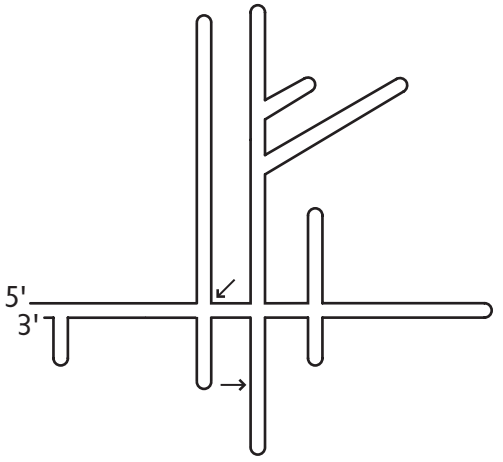


Fig. 3. The 5'UTR of the *Xenopus* PDGF-αR mRNA. *The first 302 nt were obtained by 5'RACE, and the 3' part is derived from Jones et al. (1993), giving 173 A, 205 U, 127 C, and 205 G residues. AUGs and specific primers sp1-3 are underlined, and the BamHI site is in bold. Potential 5' and 3' splice sites are indicated by arrows; note that a splicing event would remove and disrupt the majority of the secondary structure.*

The genomic sequence of TGFβ5 has been described (Vempati and Kondaiah, 1998), as well as the transcription start sites in neurula stage embryos. Therefore, the complete sequence of the longest 5'UTR could be deduced and cloned (Fig. 1). As no data were available on the completeness of the PDGF-A and PDGF-αR 5'UTRs, 5'RACE experiments were performed on embryonic RNA to obtain the full-length 5'UTRs. For the PDGF-A 5'UTR two populations of cDNAs were found, a short form with 51 additional nt as compared to the database sequence (Mercola *et al.*, 1988), and a longer form with 20 nt more (Fig. 2). The complete PDGF-αR 5'UTR appeared to be 302 nt longer than the described 5'UTR (Jones *et al.*, 1993), and contains an additional 9 uAUGs. The complete 5'UTR sequences as used in this study, and their secondary structure predictions are given in Figs. 1-3.

These three 5'UTRs share the characteristics of complexity. First their length, and second their uAUG content, respectively 7, 3, and 13. All uAUGs in these 5'UTRs are followed by in-frame stop codons, resulting in uORFs. The sequences surrounding the uAUG and stop codons were examined. In each 5'UTR, tolerable or good context sequences for initiation were found, followed by a stop codon surrounded by a sequence promoting ribosomal dissociation. This indicates that ribosomes will dissociate from these 5'UTRs before reaching the sAUG of the main ORF. Third, stable computer-predicted secondary structures were present in the 5'UTRs. The overall free energies, calculated for 20°C, were respectively -180, -230, and -290 kcal/mol. Furthermore, they all contained at least one separate structure with an energy lower than -50 kcal/mol, which has been shown to severely inhibit ribosomal scanning (Kozak, 1989b; 1991).

Besides inter-species homology in the coding region, also homology between 5'UTRs can be found. For example, the human and mouse TGFβ1 5'UTRs are 62% similar. The *Xenopus* 5'UTRs were aligned with the known parts (Genbank) of their human, mouse, and rat counterparts. Overall homologies between 18 and 35% were found, which is not considered as evolutionary homology.

Inhibitory effects of the 5'UTRs in vitro

To study the efficiency of translation initiation on the TGF, PDGF, and PDGF-R 5'UTRs, they were cloned upstream of the green fluorescent protein (GFP) ORF (Fig. 4). Capped 5'UTR-GFP mRNAs were transcribed *in vitro*, and translated in rabbit reticulocyte lysate. The efficiency of the complex 5'UTRs was compared to the efficient 60 nt *Xenopus* β-globin 5'UTR (Fig. 5). Translation of the complex 5'UTR-GFP mRNAs resulted in a 25- to 50-fold reduced amount of GFP as compared to glob-GFP RNA. This indicates that translation initiation on the complex 5'UTRs is severely impaired.

Efficiencies of the TGFβ5, PDGF-A and PDGF-αR 5'UTRs in Xenopus *embryos*

Translation in rabbit reticulocyte lysate indicated a very low initiation potential for the complex 5'UTRs. To determine their efficiency *in vivo* in the *Xenopus* embryo, DNA plasmids encoding the 5'UTR-GFP mRNAs were injected into the cytoplasm of fertilized eggs. Embryo extracts were prepared at developmental stages 13 and 19, and the amount of GFP, reflecting the initiation potential of the 5'UTRs was determined. The data in Fig. 6 indicate that in the embryo the TGF and PDGF 5'UTRs were less inhibitory than in the *in vitro* system, although they still inhibited about 9-fold as compared to the globin 5'UTR. The PDGF-αR 5'UTR seemed very inhibitory (34-fold), but an explanation for the very low amount of GFP is found in the Northern blot. The glob-, TGF-, and PDGF-GFP mRNAs had the expected sizes, and comparable levels were transcribed. However, transcription of PDGF-αR-GFP was se-

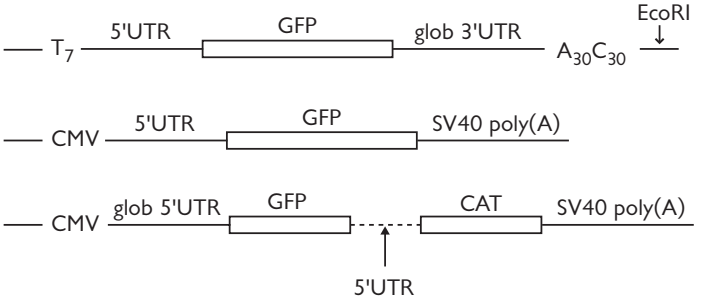


Fig. 4. Schematic representation of the reporter plasmids. *Plasmids for in vitro transcription contain one of the Xenopus 5'UTRs, upstream of the GFP reporter. The Xenopus β-globin 3'UTR, and a track of 30 A and C residues are present for mRNA stability. Monocistronic plasmids for injection and transgenesis contain the CMV promoter, one of the 5'UTR-GFP cassettes, and the SV40 polyadenylation signal. In the bicistronic plasmids the GFP and CAT ORFs are spaced by the Xenopus 5'UTRs, or by the EMCV IRES.*

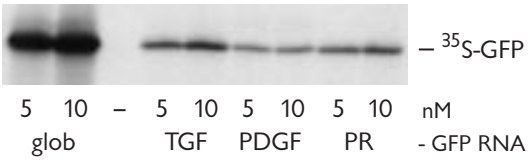


Fig. 5. Efficiencies of the *Xenopus* 5'UTRs *in vitro*. *In vitro synthesized capped glob-, TGF-, PDGF-, and PDGF-R (PR)-GFP mRNAs were translated in rabbit reticulocyte lysate, at a concentration of 5 and 10 nM. After SDS-PAGE, [35S]methionine-labelled GFP was visualized by autoradiography and was quantified.*

verely suppressed, and furthermore, at a longer exposure two mRNA species could be detected, one of the expected size, and one at about the same height as glob-GFP mRNA. These data prompted us to search for potential 5' and 3' splice sites in the 5'UTR, which were indeed found and will be outlined in the Discussion. This potential splicing event would result in a 5'UTR of 142 nt (Fig. 3), without stable structures; this 5'UTR is therefore not interesting for the rest of this study.

Regulation of translation initiation on the TGFβ5 and PDGF-A 5'UTRs during Xenopus *development*

Previously, we have shown that activity of the complex human IGF2 5'UTR is up-regulated around MBT in *Xenopus* development (Van der Velden *et al.*, 2000). *In vitro* synthesized TGF- and PDGF-GFP mRNAs were injected into embryos to study whether a similar temporal control is used by these *Xenopus* 5'UTRs. Embryo extracts were prepared before and after MBT, and the amounts of GFP were related to the amount synthesized from the non-regulated glob-GFP mRNA (Fig. 7). At stage 7, the TGF- and PDGF-GFP RNAs were almost completely silenced, resulting in barely detectable GFP. This low GFP expression did not result from a difference in stability of the injected mRNAs, as the Northern blot shows comparable levels of the full-length transcripts. These

DNA construct	GFP counts	background corrected
–	1200	0
glob-GFP	6300	5100
TGF-GFP	1800	600
PDGF-GFP	1800	600
PDGF-R-GFP	1350	150
PDGF-R-GFP		

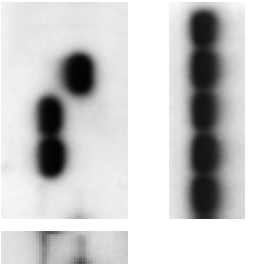


Fig. 6. Efficiencies of the TGFβ5, PDGF-A, and PDGF-αR 5'UTRs in post-MBT embryos. *DNA constructs encoding glob-, TGF-, PDGF-, or PDGF-R-GFP under control of the CMV promoter were injected into fertilized Xenopus eggs. Embryo extracts and total RNA were isolated at stage 13 and 19; as the efficiencies at both stages were similar, only stage 19 is shown here. The amount of GFP was measured with a spectrofluorometer and verified by Western blot analysis (not shown). GFP and Xenopus Histon-3 mRNA were visualized by Northern blotting using randomly primed 32P-labelled DNA probes. Their ratio, as determined after quantification with a Phosphor-imager, was used to calculate the translation efficiencies of the 5'UTRs. A longer exposure of the PDGF-R-GFP RNA containing lane is additionally shown.*

results indicate that the translational apparatus of pre-MBT embryos is incapable to efficiently recognize the TGF and PDGF 5'UTRs. At stage 12 however, efficiencies comparable with the DNA injection experiment (Fig. 6) were found; after correction for the amount of GFP RNA present in the embryos, it appeared that initiation on the TGF and PDGF 5'UTRs was respectively 15 and 9% relative to the globin 5'UTR. This indicates that the translational machinery gains the ability to initiate on complex growth factor 5'UTRs as development proceeds.

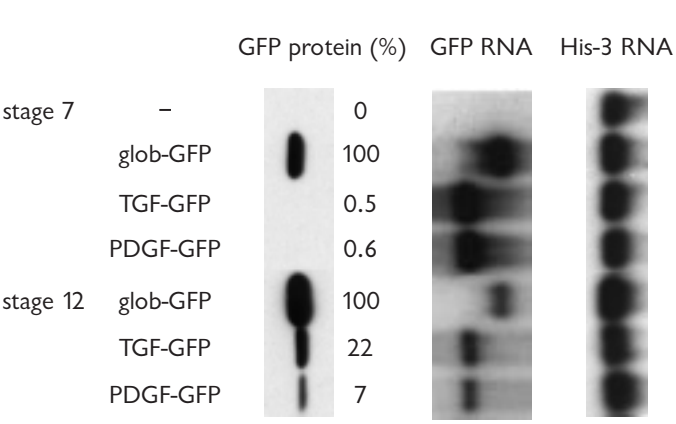
Tissue-specific initiation on the TGFβ5 and PDGF-A 5'UTRs?

By DNA and RNA injection experiments temporal control by the TGF and PDGF 5'UTRs was shown. We further determined whether these 5'UTRs can induce a spatial-specific expression pattern, by supporting initiation in some tissues, and at the same time preventing translation in other tissues. For this purpose, *Xenopus* embryos transgenic for the CMV-5'UTR-GFP DNA sequences were generated. Embryos were visualized throughout development for GFP-dependent green light emission, and stages 22, 27, 33/34 and 38-40 are shown in Fig. 8. GFP expression driven by the TGF and PDGF 5'UTRs was lower than by the globin 5'UTR, in agreement with Figs. 6 and 7. However, after enhancement of the signal the GFP expression pattern was similar as driven by the globin 5'UTR. As no major differences could be detected, neither in dissected embryos at earlier stages (not shown), it is concluded that the growth factor 5'UTRs do not influence the pattern of protein expression.

Internal ribosome entry on the Xenopus *TGFβ5 and PDGF-A 5'UTRs?*

Picornaviruses, for example encephalomyocarditis virus (EMCV), and some cellular 5'UTRs (fibroblast growth factor and antennapedia), use internal entry of the ribosome instead of 5' cap-dependent entry (Pelletier and Sonenberg, 1988; Vagner *et al.*, 1995; OH *et al.*, 1992). Ribosomes are able to directly recognize an internal ribosomal entry site (IRES) located in the 3' part of the 5'UTR. By using this alternative mechanism, secondary structures and uAUGs are circumvented. Internal entry can be demonstrated using bicistronic constructs. Two protein encoding ORFs are spaced by the potential IRES. After having translated the first cistron, ribosomes leave the RNA at the stop codon of this cistron. The start codon of the second cistron can only be reached by ribosomes if preceded by a 5'UTR promoting internal entry. A number of IRESs display a much higher potential *in vivo* than *in vitro*, which is also the case for the TGF and PDGF 5'UTRs. It was therefore investigated whether these *Xenopus* 5'UTRs use internal entry as initiation mechanism.

The 5'UTRs were cloned as intercistronic spacer between the GFP and CAT ORFs; translation of GFP is a measure for injection and transcription efficiency, whereas CAT synthesis determines IRES activity. These constructs, together with a positive control containing the EMCV IRES, and a negative control containing the non-IRES globin 5'UTR (Fig. 4), were injected into fertilized eggs. Embryo extracts were assayed for GFP and CAT activity (Fig. 9A). The amounts of GFP coincided very well with the amounts of bicistronic RNA shown on the Northern blot (Fig. 9B). This blot furthermore shows the expected sizes of the bicistronic transcripts. The CAT assay shows that the EMCV IRES strongly enhanced CAT synthesis, indicating that the translational machinery of *Xenopus* embryos can recognize an IRES. The TGF and PDGF 5'UTRs stimulate downstream CAT expression to the same extent as the globin 5'UTR, therefore the presence of an IRES is unlikely.



		GFP protein (%)	GFP RNA	His-3 RNA	$\frac{\text{GFP}}{\text{His-3}}$ RNA	% GFP corrected for RNA
stage 7	–	0			–	0
	glob-GFP	100			1.00	100
	TGF-GFP	0.5			1.06	0.5
	PDGF-GFP	0.6			1.17	0.5
stage 12	glob-GFP	100			1.00	100
	TGF-GFP	22			1.44	15
	PDGF-GFP	7			0.82	9

Fig. 7. Efficiencies of the TGFβ5 and PDGF-A 5'UTRs in pre- and post-MBT embryos. *Fertilized Xenopus eggs were injected with in vitro synthesized capped glob-, TGF-, or PDGF-GFP mRNA. Embryo extracts and total RNA were prepared at stage 7 and 12. The amounts of GFP were visualized by Western blotting and determined with the spectro-fluorometer (shown beside the Western blot). Total RNA was assayed by Northern blotting using a ^{32}P-labelled GFP probe, followed by a His-3 probe. Their ratio, as determined after quantification with a Phosphor-imager, was used to calculate the efficiencies of the 5'UTRs.*

Discussion

The complete 5'UTRs of the *Xenopus laevis* TGFβ5, PDGF-A, and PDGF-αR mRNAs were obtained by 5'RACE. At first sight, they all appeared to be long and complex (Figs. 1-3). However, after injection of the DNA construct encoding PDGF-αR-GFP mRNA a too short mRNA species was transcribed (Fig. 6). As the lysate contained GFP, the GFP ORF was intact and therefore the 5'UTR had to be truncated. We looked for potential splicing signals by comparing the 5'UTR sequence with the reported consensus splicing signals for higher eukaryotes (Green, 1991). The cloned PDGF-αR 5'UTR indeed contained potential 5' and 3' splice sites in good agreement with the consensus (Fig. 10A). The choice of the 5'RACE primers was based on the published 5'UTR sequence, and they hybridized with the putative intron; the PCR amplification steps therefore selected for the unspliced variant. Three observations support this splicing event *in vivo*. Splicing would result in a 5'UTR of 142 nt, which is in agreement with the mRNA size on the Northern blot (Fig. 6). Second, the length of the 5'UTR is comparable with the described mammalian PDGF-αR 5'UTRs. Furthermore, the sequence surrounding the ligation point of the connecting exons shows high similarity between *Xenopus* and mouse (Fig. 10B). Therefore, the sequence of 142 nt without AUG codons is most likely the full-length PDGF-αR 5'UTR. As this is an average 5'UTR, without scanning inhibiting elements, this 5'UTR was not further investigated.

The TGFβ5 and PDGF-A 5'UTRs do display a high degree of complexity and contain all elements inhibitory for ribosomal scanning. They contain respectively 503 and 478 nt, with 7 and 3

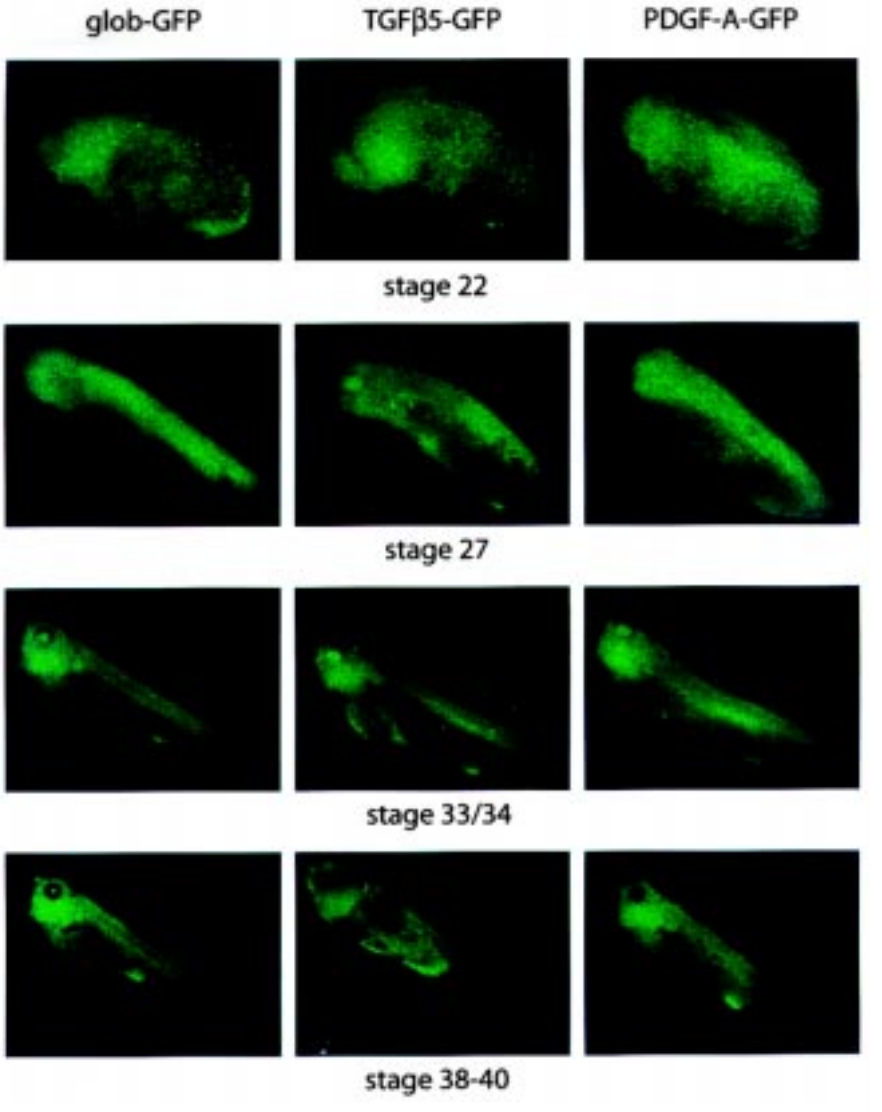


Fig. 8. GFP expression patterns directed by the globin, TGFβ5, and PDGF-A 5'UTRs in transgenic *Xenopus* embryos. *Mature Xenopus eggs were fertilized by injection of sperm DNA containing the linearized CMV-glob-GFP, CMV-TGF-GFP, or CMV-PDGF-GFP DNA construct. GFP fluorescence of MS222-anaesthetized embryos (stage 22, 27, 33/34 and 38-40) was visualized. The signals were 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). Northern blot analysis of RNA from transgenics showed similar levels of GFP RNA, of the expected sizes (not shown).*

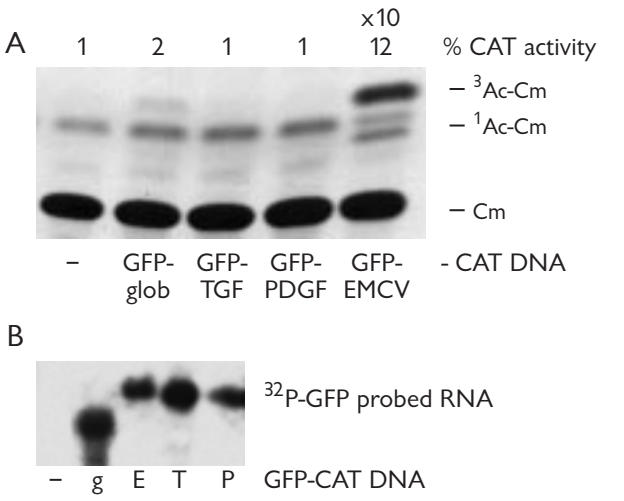


Fig. 9. IRES activity by the TGFβ5 and PDGF-A 5'UTRs in *Xenopus* embryos? *Four bicistronic DNA constructs (CS2-glob-GFP-5'UTR-CAT), containing the globin (glob/g), EMCV (E), TGF (T), or PDGF (P) 5'UTR were injected into fertilized Xenopus eggs. At stage 18, extracts were prepared and total RNA was isolated.* **(A)** *Extracts were first assayed for GFP, and equal amounts of GFP were subsequently tested for CAT activity; note that 10-fold less extract from CS2-glob-GFP-EMCV-CAT injected embryos was tested.* **(B)** *Total RNA was assayed by Northern blotting using a ^{32}P-labelled GFP probe.*

uAUGs, and the potential to fold into stable structures (Figs. 1,2). Translation of the 5'UTR-GFP reporter mRNAs in a reticulocyte lysate indeed showed that the 5'UTRs are potent inhibitors of translation initiation (Fig. 5). They share this characteristic with their human homologues; the TGFβ1 5'UTR of 840 nt strongly decreased protein expression, as well as the PDGF-A 5'UTR of 850 nt (Kim *et al.*, 1992; Takimoto and Kuramoto, 1994). Other isoforms, TGFβ3 and PDGF2/c-*sis*, are also encoded by mRNAs with an inhibitory 5'UTR of respectively 1100 and 1022 nt (Arrick *et al.*, 1991; Bernstein *et al.*, 1995). Specific translational control can be attributed to these highly complex 5'UTRs. The inhibitory effect of the TGFβ1 5'UTR was cell type specific (Kim *et al.*, 1992), whereas the activities of the PDGF-A and PDGF2/c-*sis* 5'UTRs changed upon differentiation signals (Wang and Stiles, 1993; Bernstein *et al.*, 1997).

A

5' splice site

C/A A G|G U A/G A G U	consensus
A A G|G U A A G A	PDGF-αR (28-36)

3' splice site

$(CU)_n$ N C/U A G|G/A	consensus
$(U)_8$ G C C A U U U U G G A U U G U A G|A	PDGF-αR (574-599)

B

A G A T T G A A A G|A G G A - T T C T	*Xenopus* (21-38)
A G A T T G - A A G|A G G G G T T C T	mouse (39-56)

Fig. 10. Homology of 5' and 3' splice sites, and ligation point in the PDGF-αR gene. (A) *The mammalian splice signal consensus sequences were aligned with the cloned PDGF-αR 5'UTR; cleavage sites are represented by vertical bars, and numbers refer to the non-spliced, cloned 5'UTR.* **(B)** *Alignment of the putative exon ligation point (bar) of Xenopus and mouse; numbers refer to the spliced 5'UTR.*

Numerous examples of complex 5'UTRs specifically controlling translation initiation were described, especially during embryonic development (Van der Velden and Thomas, 1999). The activities of the TGFβ5 and PDGF-A 5'UTRs also changed during *Xenopus* embryogenesis (Fig. 7). The reporter mRNAs were very poorly translated into GFP at pre-MBT stages, whereas a glob-GFP RNA was efficiently translated. This indicates that during early development the complex 5'UTRs are unable to efficiently compete with endogenous mRNAs for components of the translational apparatus. The DNA injection (Fig. 6), and the RNA injection scored at stage 12 (Fig. 7) showed that the translational efficiencies of the TGF and PDGF 5'UTRs were up-regulated. The same phenomenon was earlier observed for the human IGF2 5'UTR, which displays a similar degree of complexity as the 5'UTRs described here (Van der Velden *et al.*, 2000). These results imply that upregulation is not a 5'UTR specific phenomenon, but is a characteristic of the *Xenopus* translational machinery in combination with architecturally complex 5'UTRs found in regulatory genes. Two important questions arise from these results. First, what is the biological relevance of this 5'UTR mediated control, and second, what is the responsible mechanism?

TGFβ5 and PDGF-A are maternally encoded as low mRNA levels can be detected in pre-MBT embryos. Their zygotic transcription becomes initiated at respectively stage 14 and 12. As mentioned in the Introduction, TGF and PDGF play an important role during gastrulation, and in mesoderm induction and patterning, processes initiated prior to the neurula stage. These data can be combined in a three-step model. The poorly translated maternal store of mRNA results in a very low level of growth factors needed for rapid cell divisions. Around MBT this maternal stockpile is more efficiently translated, resulting in a higher growth factor level required for gastrulation and mesoderm induction events. Later in development, de novo transcription is initiated controlling the amount of mRNA in a tissue-specific way.

After MBT, an additional role for these 5'UTRs could be a temporal or spatial relief of their moderate inhibitory potential, as shown for the antennapedia and ultrabithorax 5'UTRs in *Drosophila* development (Ye *et al.*, 1997). With transgenic embryos constitutively expressing 5'UTR-GFP mRNA, it was determined whether the 5'UTRs could induce a specific expression pattern. Figure 8 shows that the complex 5'UTRs resulted in a similar general expression pattern as the non-regulated β-globin 5'UTR, indicating that the vast majority of tissues has the potential to initiate on the complex 5'UTRs. One can speculate about more subtle changes in expression levels which are hard to detect using this method: for example, uninhibited expression in specific cells within a tissue, or a transient stimulation added to the normal expression level during differentiation, or in a specific stage of the cell cycle. More research is needed to establish an additional function for complex 5'UTRs after MBT besides a general translational inhibition.

The second question dealt with the mechanism of initiation and regulation. Alignment studies with the *Xenopus* PDGF-A 5'UTR

A

C C C U **G** A G G G A U G **U** U A C U G A **C** U *Xenopus* (378-398)

C C C U **A** A G G G A U G **G** U A C U G A **U** U human (288-308)

B

5' C C U G A G G G A - - - - U G U U A C U G A C U *Xenopus* PDGF-A (379-398)

3' G G A U G C C U U U G G A A C A A U G - C U G A *Xenopus* 18S rRNA (1780-1802)

Fig. 11. Alignment studies with the *Xenopus* PDGF-A 5'UTR. (A) *The sequence surrounding uAUG3 is shown together with the uAUG2 surrounding part of the human PDGF-A 5'UTR. Non-identical nt are bold, and uAUGs are underlined.* **(B)** *The complementarity between part of the PDGF-A 5'UTR and Xenopus 18S rRNA.*

revealed some interesting aspects. The sequence surrounding the third uAUG shows similarity with the sequence surrounding the second uAUG in the human PDGF-A 5'UTR (Fig. 11A). Furthermore, this part of the *Xenopus* 5'UTR is complementary to nt 1780-1802 of *Xenopus* 18S ribosomal (r)RNA (Fig. 11B). Interactions between a 5'UTR and 18S rRNA have been implicated in translational control. Base pairing possibilities can inhibit translation due to stalling of ribosomes (Hu *et al.*, 1999; Verrier and Jean-Jean, 2000). In contrast, a 9-nt sequence complementary to 18S rRNA was sufficient to increase translation of the downstream ORF in bicistronic mRNA (Chappell *et al.*, 2000). The importance of 18S complementarity in IRES-driven translation has been known longer. The sequence surrounding the most 3' uAUG or sAUG of some picornavirus RNAs is complementary to rRNA (Scheper *et al.*, 1994), and the IRES-containing human PDGF2/c-*sis* 5'UTR also interacts with human 18S rRNA (Bernstein *et al.*, 1997). Interestingly, the *Xenopus* 18S rRNA fragment complementary to the *Xenopus* PDGF-A 5'UTR is identical to human 18S rRNA interacting with the picornavirus and PDGF2/c-*sis* 5'UTRs. The first part (nt 1781-1793) is exactly the same as nt 1826-1838 of human 18S rRNA complementary to the sequence preceding the sAUG of the PDGF2/c-*sis* 5'UTR (Bernstein *et al.*, 1997). The second part (nt 1793-1802) makes a perfect match to nt 1838-1847 of human 18S rRNA interacting with picornaviral RNA (Scheper *et al.*, 1994). These alignment studies indicate that the *Xenopus* PDGF-A 5'UTR could contain an IRES. However, neither the PDGF nor the TGF 5'UTR supported second cistron expression (Fig. 9), whereas the EMCV IRES was active in *Xenopus* embryos. These data deny internal entry on the PDGF-A and TGFβ5 5'UTRs. Interactions with 18S rRNA were also shown for the cauliflower mosaic virus, the late Adenovirus, and the hsp70 5'UTRs (Dolph *et al.*, 1990; Fütterer *et al.*, 1993; Yueh and Schneider, 2000). A ribosomal shunting process takes place, in which ribosomes start scanning but are discontinuously translocated to a ribosome acceptor site at the 3' end of the 5'UTR. A similar shunting mechanism could be possible for the PDGF-A 5'UTR. Ribosomal scanning through the complete TGFβ5 5'UTR is highly unlikely, internal entry was experimentally repudiated, and no 18S rRNA complementarity was found. Despite this difference, both 5'UTRs behaved very similar in the various assays, suggesting that they use a similar initiation mechanism. The TGFβ5 5'UTR could use a shunting-like mechanism where the ribosomal subunit moves to a specific conformation instead of a complementary sequence.

The translational up-regulation in *Xenopus* embryos suggests that this shunting or shunting-like mechanism becomes more efficient as development proceeds. It is proposed that in pre-MBT embryos the 18S rRNA complementary sequence inhibits initiation, whereas later in development it enhances shunting. For the TGFβ5 5'UTR a specific conformation could become altered, thereby promoting the ribosomal move. We suggest that a stage-specific activation of a trans-acting factor enhances the efficiency of these processes, thereby stimulating initiation on architecturally complex 5'UTRs.

TABLE 1

PRIMERS USED FOR CLONING PROCEDURES

TGF5'	GGCCC*AAG**CTT***TAAGTTACAGCAC
TGF3'	CGGGC*CCATGG***TGCAGTGTTCCAC**
PDGF5''	GGCCC*AAGCTT***GCTATAATCGCCACTCTCCCCAG**
PDGF5''	GGCCCAAGCTTCG**GCAGCGTTGTGG**
PDGF3'	CGGGC*CCATGG***CGTCCTGCTGCG**
PDGF-R5'	GGCCC*AAGCTT***GGGGATTTACAGACTTTAAC**
PDGF-R5''	GGCCCAAGCTTC**GGATGAGCAAGAGG**
PDGF-R3'	CGGGC*CCATGG***ACCGTAACAACTG**

The sequence of the primers is given in 5' to 3' direction. RE sites are in italic and 5'UTR complementary sequences are in bold.

Materials and Methods

5' Rapid amplification of cDNA ends

Total RNA was isolated using TRI-reagent (Sigma) from stage 22 and 33 *Xenopus* embryos as template for respectively the PDGF-A and PDGF-αR 5'UTR. Three specific primers (sp1-3, Figs. 2 and 3) were chosen which hybridize with known parts of the 5'UTRs (pO1, Mercola *et al.*, 1988; pKS-XaR, Jones *et al.*, 1993). The cDNA synthesis, adenylation, and two PCR amplifications were done according to the manufacturers instructions (5'/3' RACE kit, Boehringer Mannheim), with the addition that template RNA was heated for 10 min at 65°C prior to cDNA synthesis. The final PCR products were cloned and sequenced, resulting in 71 and 302 extra nt for the PDGF-A and PDGF-αR 5'UTR.

Cloning of the complete 5'UTRs in various vectors

Primers are shown in Table 1, and restriction sites in Figs. 2 and 3. *TGFβ5*: The 5'UTR was amplified from clone XCβ4 (Kondaiah *et al.*, 1990) using primer TGF5' including a HindIII site, and primer TGF3' with an NcoI site overlapping the sAUG; the resulting fragment was digested with HindIII/NcoI. *PDGF-A and PDGF-αR*: The 5' parts were synthesized from the final PCR-amplified 5'RACE fragments, using the PDGF5' or PDGF-R5' primer including a HindIII site, and their sp3 primers. The 3' parts were amplified from pO1 or pKS-XaR with the PDGF5'' or PDGF-R5'' primer, combined with the PDGF3' or PDGF-R3' primer containing an NcoI site overlapping the sAUG. The PDGF 5' fragment was cut with HindIII/PstI, and the 3' fragment with PstI/NcoI. The PDGF-R 5' and 3' fragments were cut with HindIII/BamHI, and BamHI/NcoI. The complete 5'UTRs were cloned as HindIII-NcoI fragments preceding the GFP ORF in the transcription vector pT_7TS and in the expression vector CS2, by removing the *Xenopus* β-globin 5'UTR from pT_7TS-glob-GFP and CS2-glob-GFP (Van der Velden *et al.*, submitted-a) by HindIII/NcoI digestion. Bicistronic constructs contain-

ing the TGF or PDGF 5'UTR as intercistronic spacer were made as follows. The 5'UTRs (blunt-ended HindIII-NcoI fragments), and the CAT ORF (NcoI-EcoRV fragment) were inserted into the SnaBI site behind the GFP ORF in pCS2-glob-GFP, resulting in the CS2-glob-GFP-*X*5'UTR-CAT plasmids. The control CS2-glob-GFP-glob/EMCV-CAT plasmids were described earlier. All reporter plasmids are shown in Fig. 4.

In vitro *transcription and translation*

T_7TS plasmids were linearized with EcoRI, and capped transcripts were made using the T_7mMessageMachine kit (Ambion). Translation in home-made rabbit reticulocyte lysate was reported earlier (Scheper *et al.*, 1992).

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 capped 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.

Embryos transgenic for the CS2-5'UTR-GFP constructs 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 in 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 (500 ng) of the PvuII-linearized plasmid. The extract mixture contained 0.5 Unit of PvuII. Embryos were cultured overnight in 0.1xMMR, 3% Ficoll, and 50 µg gentamycin/ml, and from then on in the same solution with 1% Ficoll, always at 16°C.

GFP in transgenic embryos was visualized with a mercury lamp-equipped microscope (Leica, MZ FLIII). 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 camera could be amplified (gain), and second, several frames could be superimposed using a home-made computer program.

Extract preparation, CAT, and GFP analysis

The preparation of embryo extracts was described elsewhere, as well as the CAT assay, and spectrofluorometric quantification of GFP protein (Van der Velden *et al.*, submitted-b). GFP protein levels were verified by Western blotting using a polyclonal anti-GFP serum (1:1000, Clontech), a goat-anti-rabbit-peroxidase secondary antibody, followed by ECL detection (Boehringer).

RNA isolation and Northern blotting

Total embryonic RNA was isolated with RNAzol, combined with the suggested NaCl/EtOH precipitation. After addition of RNAzol, embryonic debris was first pelleted, and chloroform was added to the supernatant. RNA (10 µg, glyoxylated in the presence of DMSO) was separated by electrophoresis in a 1.5% agarose gel containing 15 mM phosphate (7.5 mM of Na_2HPO_4 and 7.5 mM of NaH_2PO_4), and was transferred to a Hybond-N filter. After UV-cross linking, filters were hybridized overnight with a randomly primed ^{32}P-labelled GFP probe in 180 mM Na_2HPO_4, 70 mM NaH_2PO_4, 1 mM EDTA, 1% BSA (fraction V), and 7% SDS at 65°C. After autoradiography and quantification with a Phosphor-Imager, filters were stripped in boiling 0.1% SDS, and hybridized with a *Xenopus* Histon-3 probe, to correct the GFP-RNA data for the efficiency of gel loading.

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