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Mechanisms of Development 88 (1999) 15–31

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Localized XId3 mRNA activation in *Xenopus* embryos by cytoplasmic polyadenylation

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Received 4 May 1999; received in revised form 9 July 1999; accepted 9 July 1999

Abstract

In *Xenopus* development, during meiosis and cleavage, the extent of polyadenylation plays a central role in regulating the expression of transcripts and this is mediated by cis regulatory cytoplasmic polyadenylation elements (CPE) in the 3′-UTRs. We have identified a palindromic CPE in the mRNA of *Xenopus* Id3 which is conserved in the Id genes from other vertebrates. It promotes cytoplasmic polyadenylation and is negatively regulated by sequences further upstream in the 3′-UTR. This palindromic CPE promotes polyadenylation in both the epithelial and sensorial layers of the dorsal ectoderm in early embryos, but association with the upstream negative element blocks this effect in the epithelial layer. The asymmetric polyadenylation may be important for establishing a prepattern of transcriptional regulators.

Keywords: Xenopus development; Localization; Inhibition; Cytoplasmic polyadenylation; Palindrome CPE; Oocyte maturation; GVBD; Post-transcriptional regulation; Id2 sequence; Id3; Maternal mRNA; Galactosidase; In situ; Epithelial sensorial layer; Animal cap; Gasrtrula; Embryo; HLH bHLH transcription factor; 3′ UTR translation; Control prepattern axis

1. Introduction

In *Xenopus* the embryonic axes are set by cytoplasmic determinants localized in specific areas of the egg and activated at specific sites. To the asymmetry of egg, which is visible from the different pigmentations of the animal and vegetal hemispheres and which corresponds to an asymmetric distribution of maternal factors such as proteins and mRNAs, the dorso/ventral axis is added as a consequence of cortical rotation which is induced by sperm entry (Gerhart et al., 1989).

Cortical rotation causes a mixing of the outer layers of the egg and a modification of the boundary of vegetal and animal components and this rearrangement has been suggested to activate factors inducing gastrulation and consequently dorsal axis formation (Gerhart et al., 1989).

The precise molecular mechanism regulating the construction of the embryonic axes and the structural organization of the embryo during this early phase represents a major embryological question.

In *Xenopus*, during cleavage, when transcription is silent and until mid-blastula, events creating localized information and permitting specific gene transcription are activated by post-transcriptional or post-translational mechanisms. This is the case for example, of the Brachyury mRNA, which is activated around the blastopore (Latinkic et al., 1997) and Noggin mRNA which is activated at mid-blastula in the organizer region (Smith and Harland, 1992).

One of the mechanisms involved in regulating translation in the egg and during cleavage is cytoplasmic polyadenylation which depends on a nucleotide sequence called cytoplasmic polyadenylation element (CPE) (McGrew and Richter, 1990), located in the 3′-UTR part of various mRNAs, upstream from the AAUAAA polyadenylation signal.

Cytoplasmic polyadenylation can discriminate between different mRNAs depending on variations of the nucleotide sequence of the CPE and on the surrounding region (Jackson, 1993), but it has not been reported yet whether after fertilization, cytoplasmic polyadenylation may be restricted to specific areas of the embryo.

The Id proteins are negative regulators (Benezra et al., 1990) of transcription and differentiation factors of the bHLH family such as MyoD (Weintraub et al., 1991) and MASH (Johnson et al., 1990; Lo et al., 1991). Inhibition is obtained by dimerization of the negative HLH protein with

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0925-4773/99/$ - see front matter
PII: S0925-4773(99)00166-5

the positive bHLH factor. The resulting heterodimer is unable to bind to DNA and activate transcription.

The Id2 and Id3 genes from *Xenopus* exhibit in the 3′-untranslated part of the mRNA, upstream of the polyadenylation site, a CPE with a particular structure, since it is included in a palindrome. Interestingly the sequence is conserved in vertebrates and present for example in Id2 from trout (Rescan, 1997), mouse (Christy et al., 1991) and humans (Deed et al., 1993).

We were interested in the functional role of the CPE from Id3 during *Xenopus* embryogenesis wondering whether its function could correspond to a similar function in mammals which, contrary to frogs, develop from small eggs and embryos with the early activation of transcription during cleavage (Piko and Clegg, 1982).

The palindromic CPE from the *Xenopus* Id3 gene supports, -when isolated from the rest of the 3′-untranslated part of the mRNA-, cytoplasmic polyadenylation in oocytes and in embryos. In embryos of *blastula* stage translational activation is observed in the animal hemisphere where microinjected mRNA is predominantly localized. In *gastrulae* the activity is restricted to both epithelial and sensorial layers of the presumptive dorsal ectoderm.

But the activity of the pCPE is negatively affected by sequences located more upstream in the 3′-untranslated part of the mRNA. The negative effect is observed in oocytes and in gastrulae in the epithelial layer of the presumptive dorsal ectoderm.

A control galactosidase mRNA exhibiting the 3′-untranslated part of the rabbit globin mRNA, is translated in both ectoderm layers and not restricted to the dorsal side.

It has already been suggested (Simon et al., 1996) that cytoplasmic polyadenylation controls differentiation and pattern formation in early *Xenopus* development. The data presented here suggest that cytoplasmic polyadenylation during early embryogenesis is spatially regulated and represents therefore an additional molecular tool to mark or sustain asymmetries during development.

2. Results

2.1. The 3′-untranslated sequence of Id2 and Id3 mRNAs from Xenopus

The Id3 mRNA from *Xenopus*, now called XId3, previously XIdI (Zhang et al., 1995) or XIdX (Wilson and Mohun, 1995) exhibits the palindromic sequence ACUUUUUAUAAAAGU (Fig.1B) homologous to the cytoplasmic polyadenylation element UUUUUUAUAAAG (CPE) described by McGrew and Richter (McGrew and Richter, 1990) 23 bp upstream from the polyadenylation signal AAUAAA.

The Id2 mRNA from *Xenopus* (Wilson and Mohun, 1995) also presents a similar palindromic CPE like element (pCPE) 32 bp upstream from the polyadenylation signal (Fig.1A).

Analysis of the Id genes from other vertebrates reveals that the pCPE is also present in the Id2 mRNA from the trout (Rescan 1997), in Id2 and Id3 mRNAs from mouse and in Id3 mRNA from humans (Christy et al., 1991; Deed et al., 1993; Sun et al., 1991). Interestingly, in mouse Id2 mRNA it is located within the 3′-untranslated region but approximately 30 bp upstream from a cryptic polyadenylation signal.

The conservation of this element in different Id genes from a given species as well as in different organisms evolutionary as distant as fishes and mammals suggests a common function.

In mouse CPE activity (Huarte et al., 1987; Stutz et al., 1998) has been described in oocytes during the growing phase and meiotic maturation. In *Xenopus* the CPE (McGrew and Richter, 1990; Simon et al., 1996) functions when transcription is silent as during oocytes maturation, that can be induced in vitro by progesterone, and, in embryos, before mid-blastula transition. In each case, it is thought to permit regulation of gene expression in the absence of transcription.

The presence in the 3′-UTR of Id mRNAs of a conserved sequence element homologous to the CPE included in a palindromic structure is intriguing and suggestive of a specific function.

The Id genes are expressed in number of differentiating and proliferating cells in embryonic and adult organisms and encode an inhibitory HLH transcription factor (Benezra et al., 1990) which is able to retard determination and differentiation by bHLH differentiation factors.

We were puzzled over the activity of the palindromic CPE present in XId3 in regulating Id expression in *Xenopus* during meiotic maturation and cleavage, when the blastomeres cleave rapidly in the absence of transcription.

2.2. Regulatory activity of the pCPE from the XId3 gene during oocyte maturation

Low levels of maternal XId3 mRNA are detected in *Xenopus* oocytes and in cleaving embryos (Zhang et al., 1995) and zygotic transcription is activated at mid-blastula (Wilson and Mohun, 1995).

We speculated whether the pCPE from XId3 would be active during egg maturation and/or after fertilization.

During oocyte maturation induced in vitro by progesterone, germinal vesicle break down is accompanied by activation via cytoplasmic polyadenylation of a set of maternal mRNAs. This may be exemplified by the *c-mos* mRNA (Sheets et al., 1995) which is essential for meiosis and is activated by this mechanism.

To determine whether the XId3 pCPE enables cytoplasmic polyadenylation during meiosis, various CAT reporter sequences differing in the 3′-UTR but comprising the same XId3 promoter were tested after microinjection into

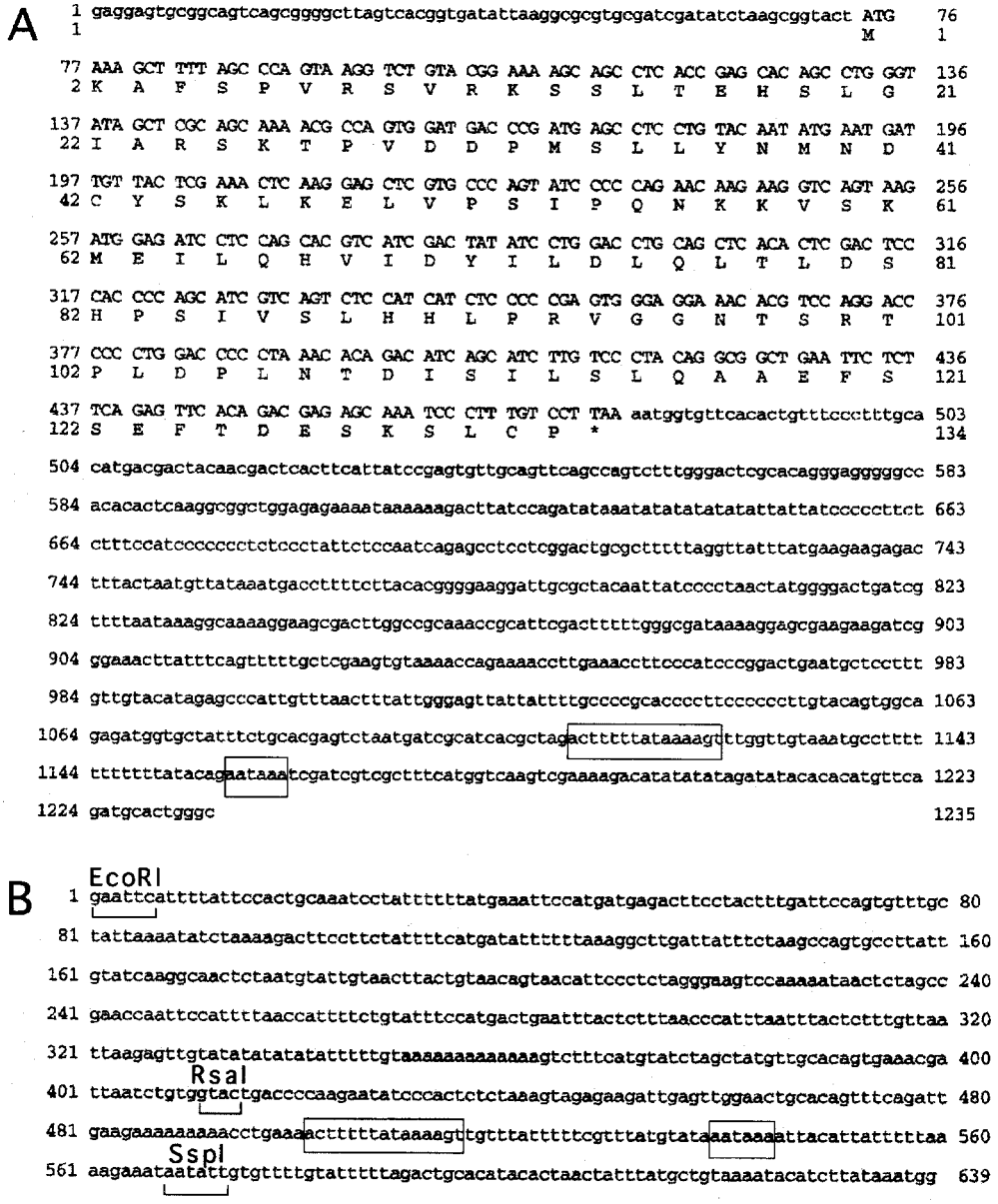

Fig. 1. Sequence and structure of Id2 and Id3 cDNAs from *Xenopus*. (A) DNA sequence of a *Xenopus* Id2 cDNA isolated from an oocyte library and amino acid sequence of the potentially encoded protein. The palindromic CPE and the polyadenylation signal AATAAA are boxed. (B) DNA sequence of the 3′-end of the *Xenopus* Id3 gene including the *Eco*RI and *Ssp*I sites bounding the 3′-UTR segment (PARS) studied in this report. The palindromic CPE and the polyadenylation signal AATAAA are boxed. The relevant restriction sites *Eco*RI, *Ssp*I and *Rsa*I are indicated by brackets. The sequence was obtained from a genomic clone (Zhang et al., 1995).

oocytes. We injected six different constructs depicted in Fig. 2A.

The PARS construct contains as 3′-UTR the last 570 nucleotides from the XId3 mRNA (Fig.1B). This sequence, which consists of an *Eco*RI/*Ssp*I fragment derived from the 3′-end of the *XId3* gene, contains pCPE and polyadenylation signal AAUAAA. The CPWS construct, contains the last 73 bp of the PARS sequence including the pCPE and polyadenylation signal. As a negative control we used a construct termed CPAS which contains the complete CPWS sequence inserted in the reverse orientation. It exhibits thus the palindromic pCPE element, but in a different environment and no polyadenylation signal. As an unrelated control mRNA we used a CAT-SV40 construct which contains the SV40 3′-UTR derived from the pA10Cat2 plasmid (Laimins et al., 1982), including the polyadenylation signal. The construct containing the CPMS sequence is identical to the CPWS except that one half of the palindromic sequence is mutated (see Section 4). The construct termed RSA, contains the last 156 bp from the 3′-side of the PARS construct and consequently includes the pCPE and polyadenylation signal.

Six hours after injection into the germinal vesicle of stage

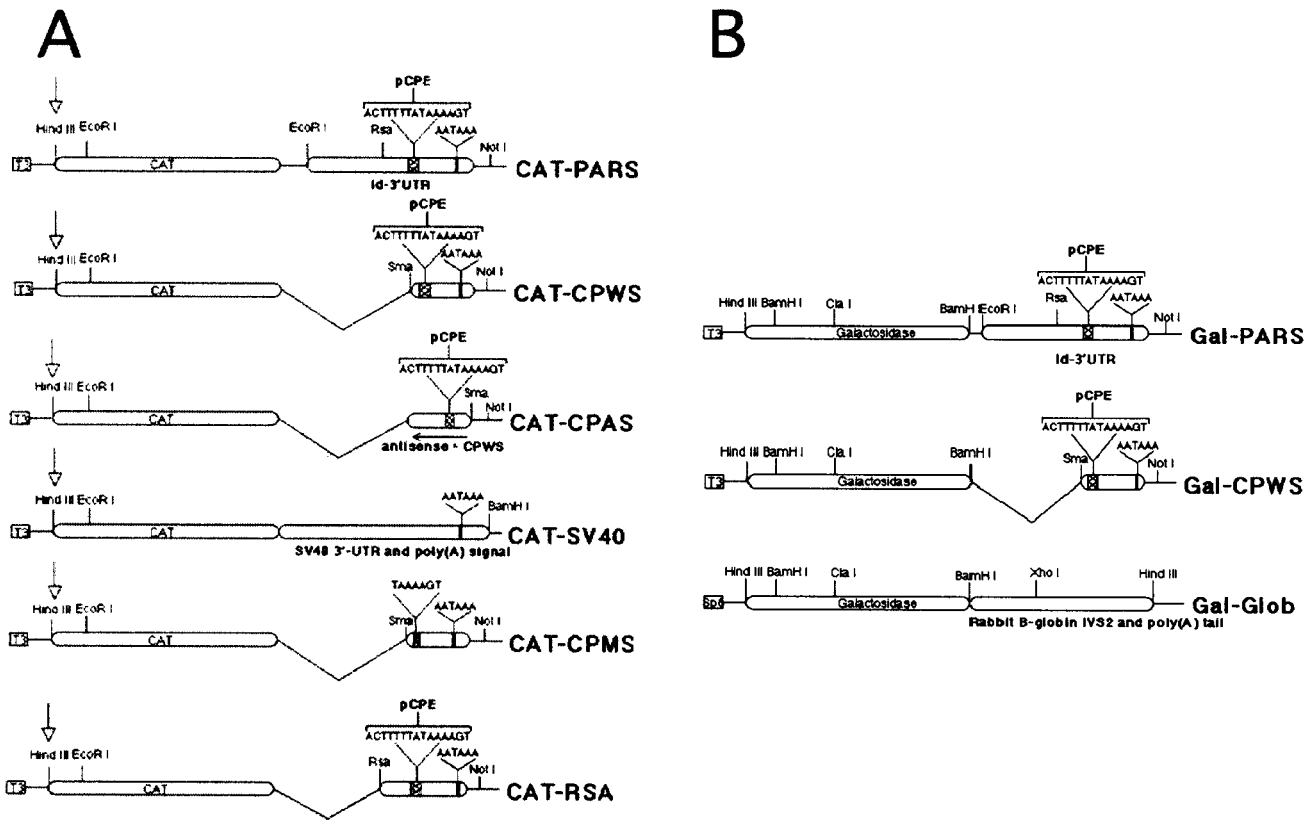

Fig. 2. CAT and Galactosidase constructs used to study the activity of the 3′-UTR from the *Xenopus Id*3 gene. (A) CAT constructs with various 3′-UTR sequences. The CAT encoding sequence, represented by a 772 bp fragment obtained by digesting with *Sau*IIIA and *Hin*dIII the plasmid pSV2CAT (Laimins et al., 1982), was inserted between the *Hin*dIII and *Bam*HI sites of the pVZ vector in which were previously inserted, at the *Sma*I site, the various 3′-UTR sequences. To synthesize RNA in vitro the constructs were linearized with *Not*I and transcribed with T3 polymerase. To study the activity after transcription in the embryos, a strong promoter derived from the *XId*3 gene (Reynaud-Deonauth, unpublished data) was inserted at the unique *Hin*dIII site indicated by an arrow and the obtained plasmid injected into fertilized eggs. (B) β-Galactosidase constructs with various 3′-UTR sequences. The Gal-Glob construct is the original pSP6nucβgal (Smith and Harland, 1991). The Gal-PARS and Gal-CPWS constructs were obtained by inserting in the pVZ vector, exhibiting at the *Sma*I site the PARS or CPWS sequence, between the *Hin*dIII and *Bam*HI sites, the *Hin*dIII-*Bam*HI and *Bam*HI-*Bam*HI fragments derived from Gal-Glob and encoding the β-galactosidase protein. To synthesize RNA in vitro, the Gal-Glob construct was linearized with *Xho*I and transcribed with Sp6 polymerase and the Gal-PARS and Gal-CPWS constructs were linearized with *Not*I and transcribed with T3 polymerase.

6 oocytes, progesterone was added. Extracts were prepared for determination of CAT activity another 12 h later, when germinal vesicle break down (GVBD) had occured.

The hormone addition resulted (Fig. 3A) in a reduction of the CAT activity of the CAT-PARS construct and in a stimulation of that of the CAT-CPWS construct. The CAT-CPMS construct was also activated by the hormone but, in some experiments, to a lower level (not shown). The negative control CAT-CPAS was unaffected by the hormone and the SV40 3′-UTR control is slightly stimulated. The data suggest that the pCPE can indeed promote stimulation of translation of mRNA stored in the oocyte, but the activity is silenced in the *XId3* gene context, by an inhibitory element located more upstream in the 3′-untranslated region. This inhibitory element, which manifests its activity in the oocyte, is located upstream from an *Rsa*I restriction site situated 82 bp from the pCPE (Fig. 1B).

To verify whether the stimulation of translation was caused by mRNA stability, we determined the quantity of CAT mRNA present in the oocytes after GVBD (Fig. 3B).

The mRNA was quantified by RT-PCR in an experiment similar to that described in Fig. 3A, using oligonucleotide primers complementary to the CAT sequence. A coinjected luciferase construct containing the SV40 3′-UTR sequence served as an internal standard. The result of the analysis, quantified by densitometric scanning of the analytical agarose gel (Fig. 3C), reveals that after GVBD, the mRNA quantity relative to the internal luciferase standard, is diminished to similar extents for all the constructs with the exception of the SV40 3′-UTR construct.

To determine whether the pCPE indeed promotes cytoplasmic polyadenylation we used two methods which gave comparable results. In a first more direct approach (Fig. 4A) radiolabelled RNA was synthesized in vitro and microinjected into oocytes. After induction of GVBD by progesterone the RNA was reextracted and its length determined by electrophoresis in polyacrylamide/8 M urea sequencing gels. We used RNAs copied from the CPWS-, CPAS-, CPMS- and PARS-sequence, without the CAT encoding part attached to it. As shown in Fig. 4A, CPWS RNA

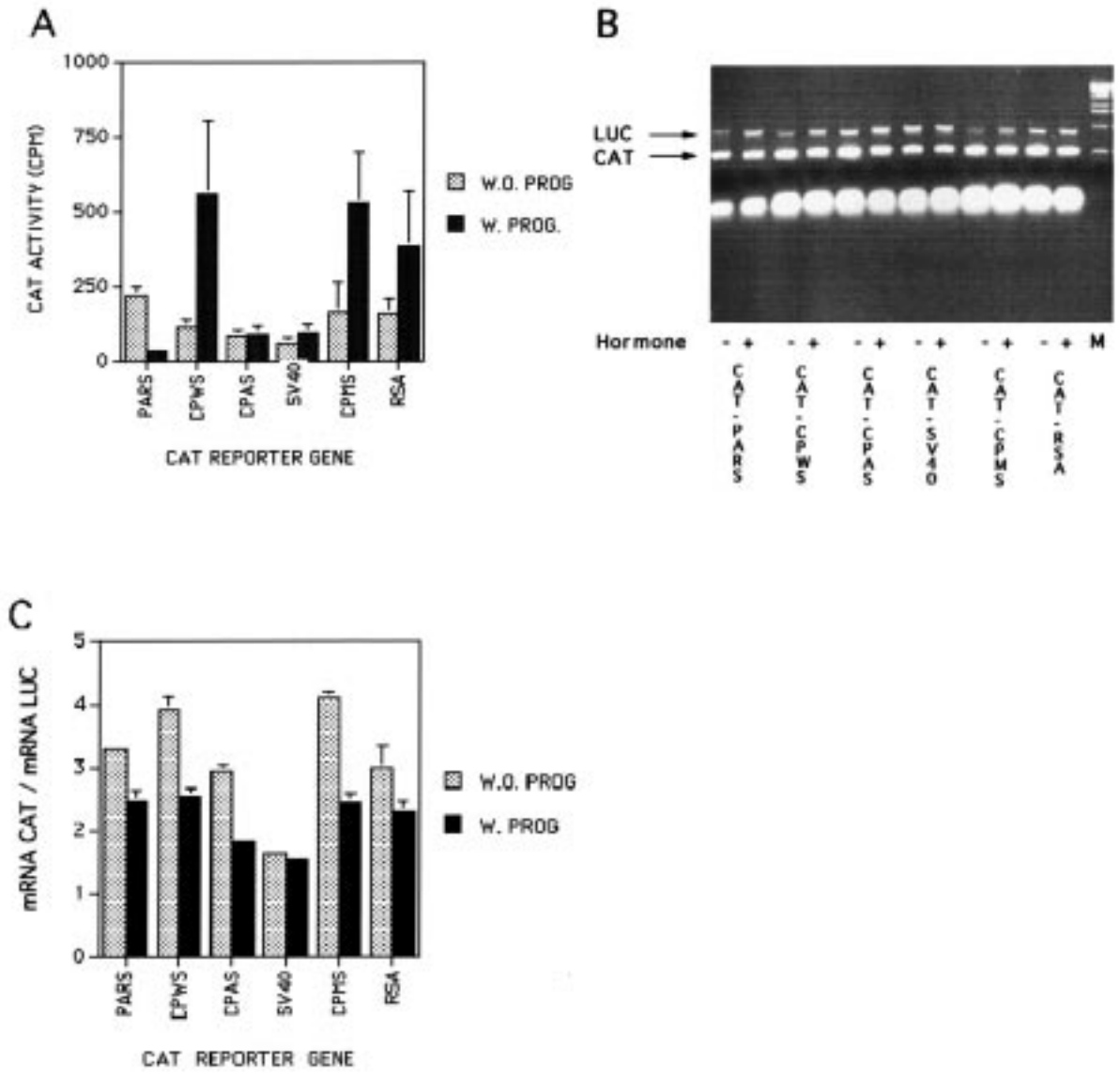

Fig. 3. Activity and stability of CAT mRNAs transcribed in the oocyte and differing in the 3′-UTR region during germinal vesicle break down. (A) Stage five to six oocytes were injected in the germinal vesicle with the reporter genes and incubated for 6 h at 21°C. Progesterone was then added to half of them and incubation continued for another 6–8 h, until germinal vesicle break down score had reached 50%. A lysate was then prepared in triplicated batches of 10–20 oocytes and CAT activity determined. (B) The CAT reporter genes were coinjected with a *luciferase* reporter gene controlled by the same XId3 promoter and exhibiting as a 3′-UTR sequence the SV40 small-t-antigen intron and polyadenylation signal (de Wet et al., 1987). The oocytes were incubated and exposed to progesterone as described under A, and then processed for RNA extraction. The Luciferase and CAT mRNA were determined by RT-PCR as described under Section 4 and the amplified product analysed by 1% agarose gel electrophoresis. The expected position of the Luciferase and CAT fragments are indicated. (C) The gel shown in (B) was recorded and analysed using a Kodak DC120 zoom digital camera and the Kodak Digital Science ID Image Analysis Software for Macintosh. Indicated is the amount of CAT mRNA relative to the amount of Luciferase mRNA.

injected into oocytes is elongated during maturation. The length of the added poly(A) tail is up to 150 residues. The CPMS-RNA is slightly elongated whereas the CPAS- and PARS-RNAs are not.

The result suggests that the pCPE is an active cytoplasmic polyadenylation element, but its activity is negatively regulated by an additional cis-element present the 3′-UTR, upstream from it.

In the second approach (Salles and Strickland, 1995) the poly(A) length of the RNA transcribed in the oocytes from microinjected CAT constructs was determined by an RT/PCR method called PAT and devised to copy and amplify the mRNA including its poly(A) tail (Fig. 4B). The length of the PCR product determined by agarose gel electrophoresis reveals then, by comparison with a reference, whether the RNA has become elongated. As a reference we used here RNA transcribed in oocytes which were not exposed to the hormone.

Again, we found that the CAT-CPWS (Fig. 4B) construct was elongated after hormone treatment. The elongation of

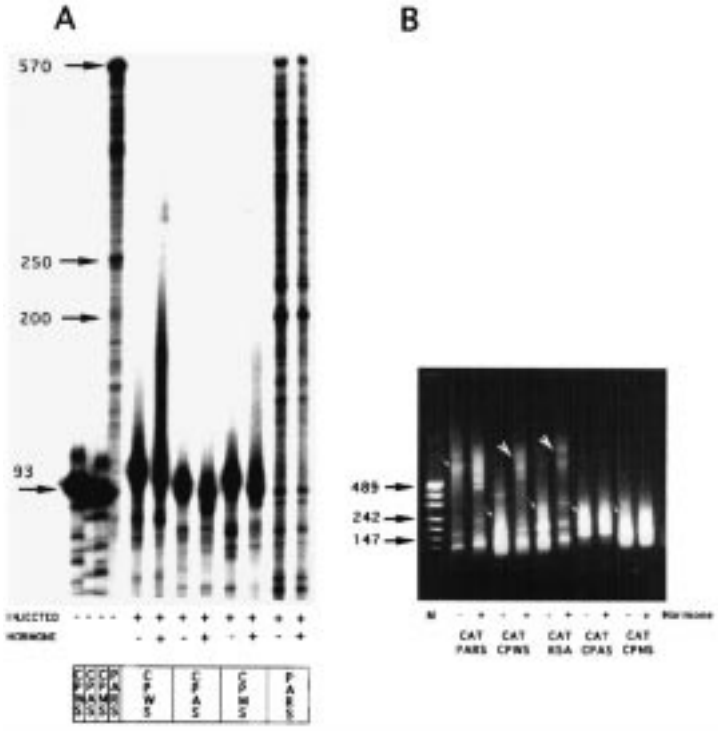

Fig. 4. (A) Elongation by cytoplasmic polyadenylation of RNA injected into oocytes. ^{35}S labelled CPWS-, CPAS-, CPMS- and PARS-RNA were synthesized in vitro and microinjected into stage 6 oocytes. The oocytes were then incubated for approximately 10 h in the presence of progesterone. When GVBD was evident in 50% of the oocytes exposed to the hormone, RNA was extracted and analysed after denaturation on 5% polyacrylamide/urea sequencing gels. (B) Elongation by cytoplasmic polyadenylation of RNA transcribed in the oocytes. The CAT-PARS-, CAT-CPWS-, CAT-RSA-, CAT-CPAS- and CAT-CPMS-DNA constructs were microinjected into stage 6 oocytes. After 8 h incubation at 21°C the various samples were divided into two batches and progesterone was added to one to induce maturation and GVBD and incubation pursued for an additional 8–10 h. When GVBD was evident in 50% of the oocytes exposed to the hormone, the oocytes were lysed, the RNA extracted and the length of the poly(A) tail determined by PAT (Salles and Strickland, 1995) and electrophoretic analysis on agarose gel. The arrows show the expected migration of the non-polyadenylated RNA and the arrowhead the one of the polyadenylated counterpart.

the CAT-CPMS RNA on the contrary, was too limited to be observable with this assay.

No elongation was observed in the case of the PARS construct. The CAT-RSA construct, on the contrary, becomes elongated suggesting that the element inhibiting polyadenylation must be located upstream from the *Rsa*I site.

Together, the data show that in oocytes stimulation of translation due to the pCPE element is likely to be the result of a polyadenylation event and is not caused by a modified mRNA stability, since the amount of CAT mRNA present in the oocytes is similar for all the constructs.

2.3. Regulatory activity of the pCPE from the XId3 mRNA in cleaving embryos

The cis-regulatory elements allowing cytoplasmic mRNA polyadenylation and translational activation respond to signals present in the oocyte (Barkoff et al., 1998; Richter, 1991) and also in the embryo (Simon et al., 1992). In the embryo cytoplasmic polyadenylation occurs during early cleavage stages that precede the onset of zygotic transcription.

To determine whether the pCPE may play a function during embryogenesis, we first determined whether the 3′-UTR from XId3 modulates expression of a CAT reporter gene transcribed in the embryo. CAT encoding DNA constructs placed under the control of an enhancer/promoter tandem derived from the *XId*3 gene (Reynaud-Deonauth, unpublished data) and exhibiting the PARS, CPWS, CPAS, CPMS or RSA sequence as 3′-UTRs, were injected into fertilized eggs. The Id promoter used is active from mid-blastula onward in proliferating cells of various origin including epidermis, neural crest cells, neural cells, somites and tailbud (Wilson and Mohun 1995; Zhang et al., 1995).

With the exception of the negative control CPAS, the CAT activity measured (Fig. 5A) in the embryos at neurula stage was similar for the various constructs tested, irrespective of the 3′-UTR present. This suggests that for mRNA transcribed after mid-blastula and polyadenylated in the nucleus, translational efficiency is unaffected by the pCPE element present in the 3′-UTR.

This is confirmed by the extent of polyadenylation (Fig. 5B). The PARS-, CPWS- and RSA-mRNA transcribed in the embryo are elongated when compared to the in vitro synthesized counterpart. One may remark that the electrophoresis bands observed are relatively broad. This is caused by the heterogeneity of the poly(A) tail length but also, and

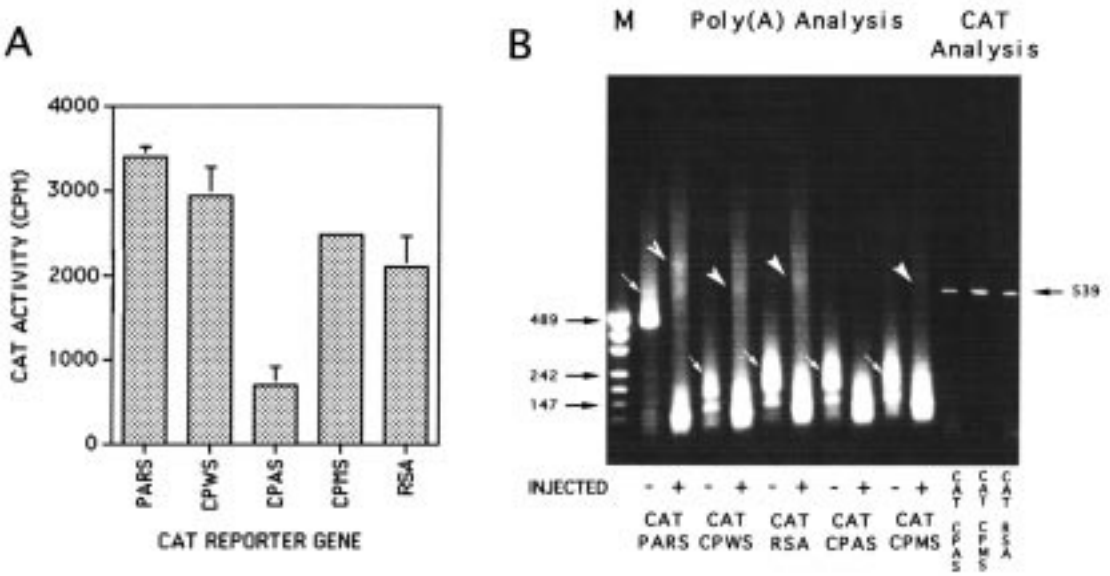

Fig. 5. CAT activity and polyadenylation efficiency of various CAT reporter genes differing in the 3′-UTR region after microinjection into embryos. (A) One to two cell stage embryos were injected with the CAT-DNA constructs indicated and incubated at 21°C until the neurula stage. The embryos were then lysed and CAT activity measured. (B) One to two cell stage embryos were injected and incubated as described under (A). At the neurula stage RNA was extracted and poly(A) elongation measured by PAT and comparing with non-polyadenylated RNA synthesized in vitro. The quantity of CAT mRNA extracted was determined by RT-PCR. The amount of the PCR products analysed by electrophoresis corresponds to constant amounts of rRNA. The amplified fragment obtained is shown for the CPAS-, CPMS-, and RSA-constructs. The arrows show the expected migration of the non-polyadenylated RNA and the arrowhead that of the polyadenylated counterpart.

particularly for the in vitro synthesized RNA, which has no poly(A) tail, by internal priming during the RT reaction, on oligo(A) stretches present in the 3′-UTR. The negative control, represented by the construct CPAS that lacks the polyadenylation signal, shows no mRNA elongation and only a low translational activity. Elongation of the CPMS-mRNA is weak.

To find out whether the function of the palindromic CPE and of the inhibitory element present in the 3′-UTR of the XId3 mRNA affects the maternal mRNAs deposited in the egg, CAT-PARS-, CAT-CPWS-, CAT-CPAS-, CAT-CPMS- and CAT-RSA-mRNAs were injected at the one or two cell stage and embryos subsequently harvested for CAT activity determination and RNA extraction at the blastula stage.

Quantification of CAT-RNA by RT/PCR (Fig. 6C) revealed that the 3′-untranslated part had no measurable effect on the mRNA stability during this developmental period, since the RNA injected were found to be present, at blastula stage, in similar amounts.

Determination of CAT activity (Fig. 6A) revealed that the CAT-CPWS- and CAT-CPMS-RNA are efficiently translated and the CAT-PARS and CAT-RSA mRNA much less, suggesting the presence of an inhibitory element in these last two constructs. Surprisingly, relatively high translational efficiency was observed with the CAT-CPAS mRNA which lacks the polyadenylation signal.

Determination of the mRNA elongation by polyadenylation (Fig. 6B) revealed furthermore that the CAT-PARS-, CAT-CPWS- CAT-CPMS- and CAT-RSA-RNA were all elongated though to variable extents, whereas the control CAT-CPAS-RNA was not. This suggests that the factor(s) and (or) the cis-regulatory elements, which inhibit polyadenylation in oocytes are inactive during cleavage. Nonetheless the translational efficiency is not raised to the same level for all the polyadenylated constructs. The CAT-PARS RNA and CAT-RSA RNA are approximately 6× less efficiently translated than the CPWS construct (Fig. 6A). The effect depends on cis-elements present downstream from the *Rsa*I site, in the last 156 nucleotides of the PARS sequence, and should involve a mechanism regulating translation independently of poly(A) addition. This mechanism is different from the one observed in the oocytes whose target is located upstream from the *Rsa*I site.

This observation is also helpful in understanding the activity of the CPMS construct which presents half of the palindromic CPE and lacks the sequence element located upstream from the *Rsa*I site which inhibit polyadenylation as well as those located downstream from it which inhibit translation. The low level of polyadenylation which it exhibits (Figs. 4A,6B) is apparently sufficient to explain its intermediate activity in supporting translation.

It cannot be excluded however, that polyadenylation of the PARS and RSA constructs occurs only in selected areas of the cleaving embryo. This also would reduce their global translational activity, compared to the CPWS.

The RNA elongation pattern (Fig. 6B) is not inconsistent with this assumption. A careful observation of the electro-

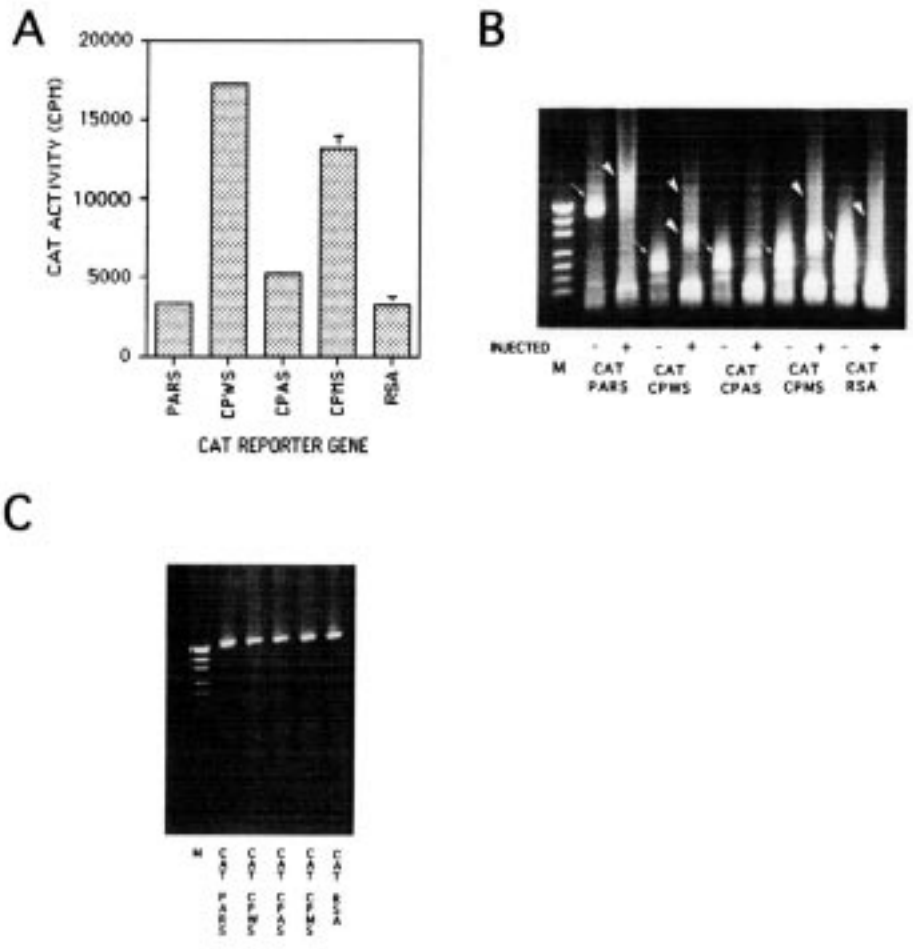

Fig. 6. Activation by cytoplasmic polyadenylation of in vitro synthesized RNA microinjected into fertilized eggs. (A) The various in vitro synthesized RNA constructs were microinjected into one to two cell stage embryos. At mid-blastula, embryos were lysed and CAT activity measured. (B) RNA was extracted from embryos microinjected as described under (A), and poly(A) elongation measured by PAT and comparing with the unpolyadenylated RNA synthesized in vitro. (C) The amount of RNA extracted as described under (B) was amplified by RT-PCR using oligos specific for the CAT sequence, and the amplified products compared after electrophoresis on agarose gel. Up to mid-blastula, the various RNAs injected are similarly stable. The amount of the PCR products analysed by electrophoresis corresponds to constant amounts of embryonic rRNA. The arrows show the expected migration of the non-polyadenylated RNA and the arrowhead that of the polyadenylated counterpart.

phoretic profile suggests that the CAT-CPWS RNA is quantitatively more elongated than the CAT-PARS RNA.

On the other hand, a different degradation rate is unlikely to cause the effect observed at blastula, since the total amount of CAT-RNAs present (Fig. 6C) is similar for the various constructs.

2.4. Localized cytoplasmic polyadenylation in early embryos

The pCPE mediated translational activation in cleaving embryos is negatively regulated by sequences located more upstream in the 3′-UTR.

Since CAT-PARS-RNA was polyadenylated after injection into the embryo but not translated as efficiently as CAT-CPWS-RNA, we wondered whether the inhibitory activity is equally distributed in all the tissues, or whether it is concentrated in part of the embryo, similar to a maternal factor such as Nanos, for example (Curtis et al., 1995).

We therefore prepared galactosidase reporter genes exhibiting as 3′-untranslated sequence the PARS or the CPWS element (Fig. 2D). These constructs were used in comparison with RNA transcribed from the plasmid pSP6nucβgal (Smith and Harland, 1991) which we called Gal-Glob for simplicity and which exhibits the 3′-UTR and poly(A) signal of the rabbit beta globin mRNA.

Synthetic RNAs were microinjected into embryos of the one, two or four cell stage and the embryos that developed to blastula, gastrula or neurula stage were fixed and stained for β-galactosidase.

The staining profile of embryos injected with the Gal-Glob RNA reveals that at the blastula stage (Fig. 7A1,2) the mRNA is translated exclusively in the animal cap cells. At the gastrula stage as a consequence of convergent extension, galactosidase activity is widespread (Fig. 7B1),

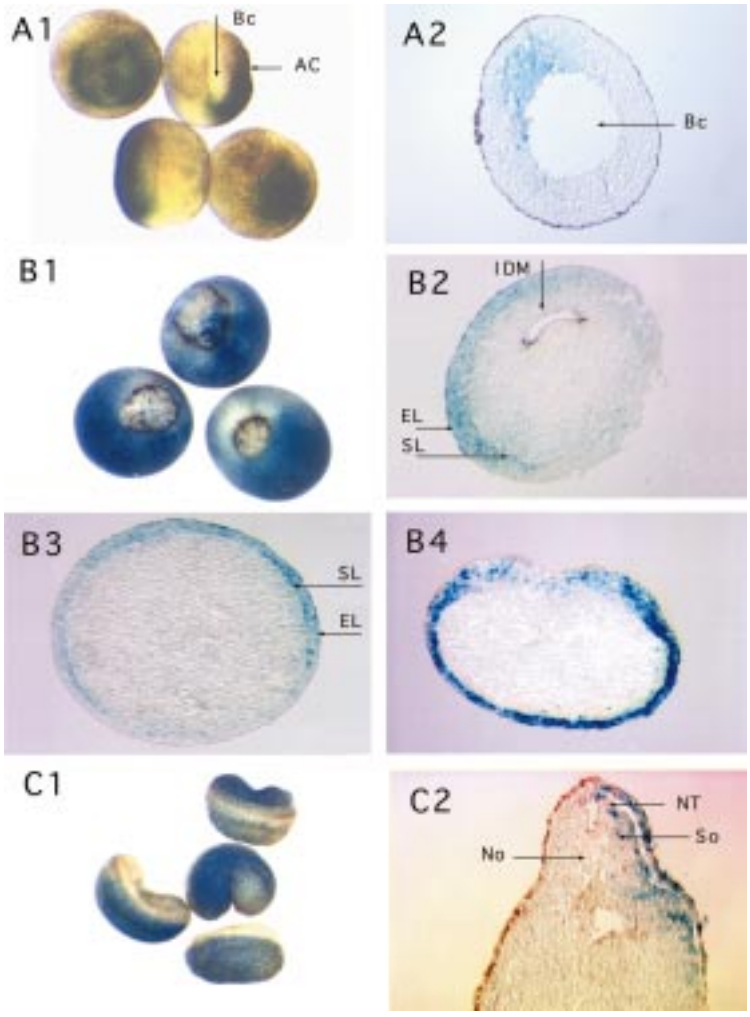

Fig. 7. Localized expression of Gal-Glob mRNA microinjected into fertilized eggs. Gal-Glob RNA was synthesized in vitro and microinjected into embryos of one to two cell stage. Embryos were incubated at 21°C until bastula (A), gastrula (B) and neurula (C) stage, stained for β-galactosidase and observed after making them transparent (A1) or as that in (B1, C1). Selected embryos were embedded in paraffin and observed in sections (A2, B2–B4, C2). A2 represents a lateral section cut in the blastocoel area. B2 and B3 represent transversal sections from the blastopore area and from the central part of the embryo respectively. B4 represents a transversal section obtained from a more advanced embryo in which neurulation had already been initiated. C2 represents a transversal section from the central part of the neurula. Animal cap (AC), blastocoel (Bc), epithelial layer (EL), sensorial layer (SL), involuting dorsal mesoderm (IDM), neural tube (NT), notochord (No) and somites (So) are indicated.

being present in both the epithelial and sensorial layers of the ectoderm (Fig. 7B3), but not in the cells involuted through the blastopore (Fig. 7B2). When neurulation starts (Fig. 7B4) galactosidase activity is still evident in both epidermal layers, all around the embryo.

At the neurula stage one still observes galactosidase activity in the epidermis (Fig. 7C1,2), a derivative of the epithelial layer of the ectoderm and in the neural tube which derives from the sensorial layer by neural induction, but also, surprisingly, in the somites. The lineage of these last cells is difficult to establish. However in several sections we have observed that cells from the sensorial layer seem to migrate into the mesoderm. One such example is depicted in Fig. 8B4, where the dorsal mesoderm contains galactosidase positive cells located close to the sensorial layer of the ectoderm and probably derived from it.

Such a distribution is consistent with translation of galactosidase mRNA in the animal cap cells of blastulae and later in their derivatives, but silencing of the transcript in the cells that involute through the blastopore (Keller, 1984).

The galactosidase-CPWS RNA injected into the one to two cell stage embryos gave an activity pattern which was similar to that of the Gal-Glob mRNA in early blastulae (Fig. 8A1,2) but evolved differently during gastrulation. At this stage galactosidase is detected only on the side of the blastopore (Fig. 8B1) which corresponds to the future dorsal side (Fig. 8B4). Sections through such embryos (Fig. 8B3,4) reveal that the epithelial and the sensorial layers of the ectoderm express the CPWS RNA including the tissue involuting through the blastopore (Fig. 8B2). This suggests that silencing of this mRNA construct occurs later than the Gal-Glob mRNA.

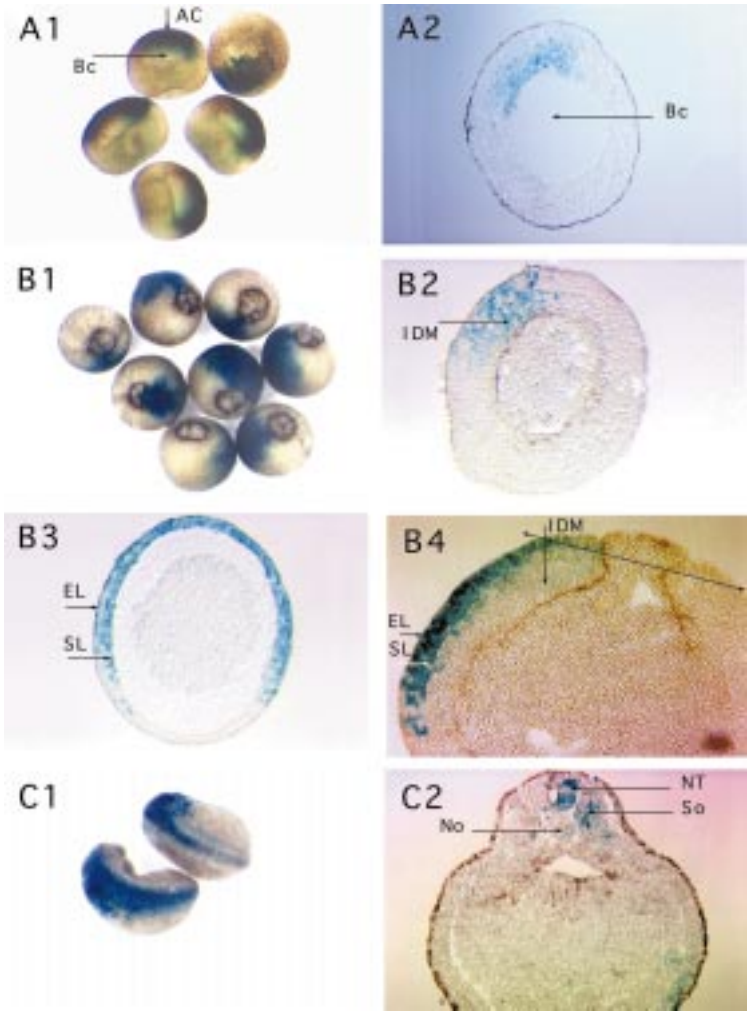

Fig. 8. Localized expression of Gal-CPWS mRNA microinjected into fertilized eggs. Gal-CPWS RNA was synthesized in vitro and microinjected into fertilized eggs of one to two cell stage. Embryos were incubated at 21°C until blastula (A), gastrula (B) and neurula (C) stage, stained for β-galactosidase and observed after making them transparent (A1) or as that (B1, C1). Selected embryos were embedded in paraffin and observed in sections (A2, B2–B4, C2). A2 represents a lateral section cut in the blastocoel area. B2 and B3 represent transversal sections from the blastopore area and from the region opposite to it, respectively. B4 is a sagittal section through the blastopore and the line shown represent the cut used to obtain sections as the one represented in B2. C2 represents a transverse section from the anterior half of the neurula. Animal cap (AC), blastocoel (Bc), epithelial layer (EL), sensorial layer (SL), involuting dorsal mesoderm (IDM), neural tube (NT), notochord (No) and somites (So) are indicated.

This localized expression is confirmed by analysis of the lineages of these precursors cells. At the neurula stage galactosidase activity is present in the epidermis (Fig. 8C1), neural tube, somites (Fig. 8C2) and neural crest cells derivatives (not shown).

Like the CPWS-RNA, the Gal-PARS RNA is expressed in the animal cap cells (Fig. 9A1,2) and on the dorsal side of the gastrulating embryo (Fig. 9B1), between the blastopore and the future anterior region. Analysis of sections (Fig. 9B3,4) reveals that this expression is only in the sensorial layer of the ectoderm.

Sections through the blastopore area (Fig. 9B2) confirm that the PARS-RNA activity is limited to the cells derived from the sensorial layer, whereas the CPWS-RNA (Fig. 8B2) is active in both layers originating from the ectoderm, including the involuting dorsal mesoderm and the internalized cells which will form the notochord and the archenteron roof.

The results are consistent with a Gal-PARS-RNA expression restricted to a subset of cells compared to the Gal-CPWS-RNA. The inhibitory cis-element present in the PARS sequence mediates inhibition in the epithelial layer of the ectoderm.

Galactosidase activity persists and can be measured till neurulation. This allows to trace the lineage of the identified embryonic cells, but it may also indicate where in the embryo the maternal XId3 mRNA -from where the PARS sequence was isolated- is activated to fulfill its function.

The galactosidase translated from the PARS mRNA (Fig. 9C1,2) finally ends in the neural cells, neural crest cells derivatives and in the somites. In these tissues also the endogenous XId3 gene is transcribed at high rate (Wilson

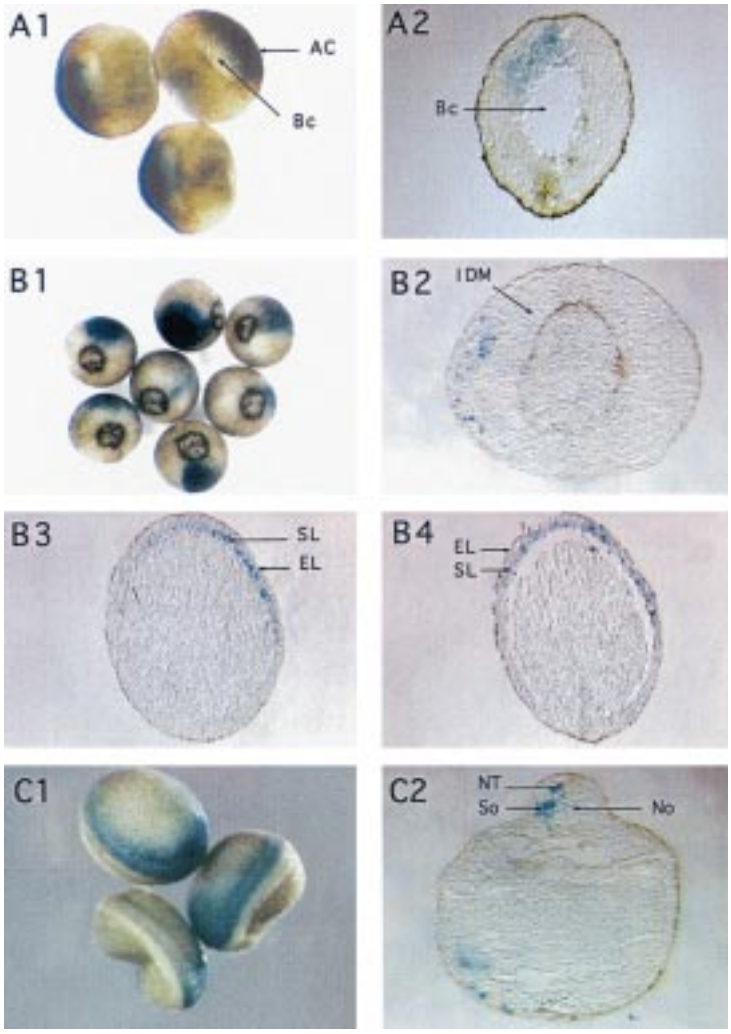

Fig. 9. Localized expression of GAL-PARS mRNA microinjected into fertilized eggs. Gal-PARS RNA was synthesized in vitro and microinjected into fertilized eggs of one to two cell stage. Embryos were incubated at 21°C until blastula (A), gastrula (B) and neurula (C) stage, stained for β-galactosidase and observed after making them transparent (A1) or as that (B1, C1). Selected embryos were embedded in paraffin and observed in sections (A2, B2–B4, C2). A2 represents a lateral section cut in the blastocoel area. B2, B3 and B4 represent sections from the blastopore area, from the central part of the embryo and from the area opposite to the blastopore respectively. C2 represents a transversal section through the central part of a neurula. Animal cap (AC), blastocoel (Bc), epithelial layer (EL), sensorial layer (SL), involuting dorsal mesoderm (IDM), neural tube (NT), notochord (No) and somites (So) are indicated.

and Mohun 1995; Zhang et al., 1995). The galactosidase translated from the Gal-Glob mRNA is distributed differently and is localized predominantly in the ectoderm.

2.5. Localization of microinjected RNA by in situ hybridization

The microinjected RNA is translated in the blastulae with a pattern which is similar for the three constructs tested: the translation product is present in the animal hemisphere and absent from the vegetal one. To find out whether this is due to preferential distribution of the injected RNA or whether the translation machinery is activating the mRNA selectively in this area, we determined the distribution of the injected RNA by in situ hydridization using a probe derived from the galactosidase encoding sequence.

To our surprise the hybridization pattern, which is similar for the three constructs (Fig. 10A–C), shows that the microinjected mRNAs accumulate, at the blastula stage, in the animal hemisphere, and colocalizes thus with the translated protein. We do not think that the selective localization is a consequence of the microinjection technique that we used, since we have concentrated our effort in injecting the material into the center of the egg. It is more likely the RNA moves to this part of the embryo as a consequence of a transport mechanism active during cleavage on this type of RNA.

In embryos of the gastrula stage the RNA distribution pattern is different for the various constructs and roughly reflects the distribution of the translation product. The CPWS mRNAs distribution (Fig. 10B2), similar to the Gal-Glob mRNA distribution (Fig. 10A2), extends from the future dorsal side more ventrally than the correponding protein synthesis profile (Fig. 8B1). The PARS mRNA (Fig.

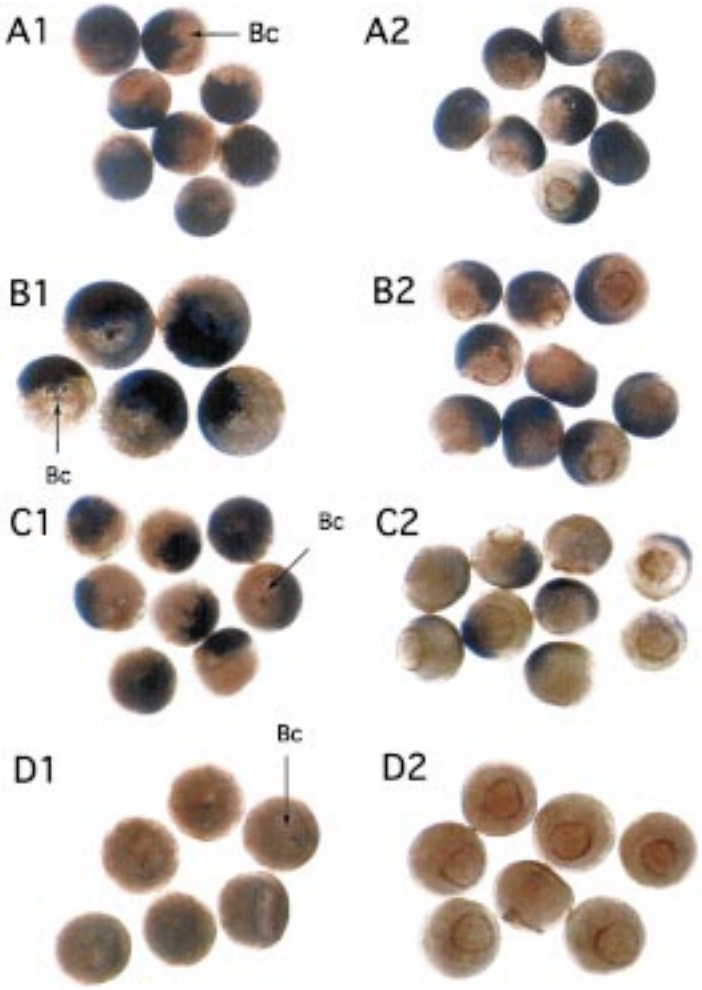

Fig. 10. Localization of mRNA microinjected into fertilized eggs determined by in situ hybridization. Gal-Glob-, Gal-CPWS- and Gal-PARS-mRNA (A,B and C, respectively) were microinjected into fertilized eggs of 1-2 cell stage. Embryos were incubated at 21°C and processed for whole mount in situ hybridization as described by Harland (1991). To prepare an RNA antisense probe, the central *Bam*HI fragment from the Gal-Glob construct was first subcloned into the pVZ vector and then in vitro transcribed. Embryos were fixed at blastula (1) or gastrula stage (2). Removal of vitelline envelope was skipped. The control panel D1 and D2 show the pattern obtained with uninjected embryos hybridized with the same Gal-Glob probe. The blastocoel, below the animal cap, is indicated (Bc).

10C2) is found in the dorsal area but in reduced amounts, consistent with a reduced translational activity.

At this stage the translational activity and mRNA stability of the PARS construct could both affect total protein synthesis.

The background hybridization profile obtained with non-injected control embryos is shown in Fig. 10D1,2.

3. Discussion

The maternal mRNA synthesized in the oocyte contains genetic information necessary for development from oocyte maturation until mid-blastula transition. During this period important metabolic changes occur (Simon et al., 1992; Barkoff et al., 1998) and molecular events take place which produce or sustain the asymmetries culminating in the embryonic axes.

During these phases regulation is based on post-transcriptional and post-translational mechanisms (Curtis et al., 1995; Hake and Richter, 1997). These occur despite the absence of any transcription and result instead from post-transcriptional events such as cytoplasmic polyadenylation (Bachvarova, 1992; Stebbins-Boaz and Richter, 1994). Previously synthesized mRNAs can be activated at various times during oocyte maturation and early embryonic development. Several classes of mRNAs have thus been identified (Stebbins-Boaz and Richter, 1994; Ballantyne et al., 1997). They are characterized with respect to the activity of the cytoplasmic polyadenylation element (CPE) present in the vicinity of the polyadenylation signal which can be modulated by sequences located more upstream in the 3′-UTR. The cdk2 mRNA (Stebbins-Boaz and Richter, 1994), for example, is polyadenylated and activated during oocyte maturation but silenced, via deadenylation, after fertilization. The C12 mRNA (Simon et al., 1992) on the other hand, is activated by polyadenylation after fertilization.

3.1. Activity of the palindromic CPE from the XId3 gene in the oocytes

The palindromic CPE is present in the Id2 and Id3 mRNAs from *Xenopus* and from higher vertebrates (Christy et al., 1991; Deed et al., 1993), suggesting a conserved regulatory function specific to these mRNAs.

The activity of the XId3 pCPE, as described here, resembles that of C12 mRNA. Both function in fertilized eggs but are inhibited in oocytes by sequence elements present in the 3′-UTR.

However the CPE of the C12 mRNA consists of a dodecauridine and differs from the pCPE of the XId3 which exhibits, more similarity to the G10 CPE, UUUUUUAUAAAG (Richter, 1991) on a sequence level.

The pCPE is potentially able to promote cytoplasmic polyadenylation and as a consequence stimulate translation in oocytes after maturation and in eggs after fertilization. The activity however is masked, in the oocyte, by sequence elements present in the 3′-UTR which inhibit cytoplasmic polyadenylation. The inhibitory element has been localized in the 3′-untranlated region, upstream from an *Rsa*I site. Comparison with other mRNAs exhibiting an inhibitory element did not reveal to us significant sequence homologies which could allow the recognition of a common regulatory element. One possible mechanism (Stumpf et al., 1996), as has already been suggested could rely on the rich secondary structure (not shown) which can be constructed with the PARS sequence. In this RNA, sequence elements upstream from the pCPE and downstream of it can interact with each other to generate a closed, 'ring shaped' secondary structure sealing in its core the pCPE. In the case of the shorter constructs RSA or CPWS, the RNA/RNA interactions are reduced and the palindrome is not included in a larger stabilized structure.

This explanation would take account of the fact that in the case of the C12 mRNA (Simon et al., 1992) sequences upstream as well as downstream of the CPE are necessary to obtain the inhibitory effect.

The data however are also consistent with the existence of more than one cis-regulatory elements recognized by trans-acting factors.

3.2. Activity of the pCPE from the Xenopus Id3 gene in cleaving embryos

The galactosidase mRNA used here as a control, which exhibits the 3′-untranslated part of the rabbit globin mRNA has already been used (Smith and Harland, 1991) as a lineage marker after microinjection into animal, marginal or vegetal blastomeres. In the experiments described here it was injected into the one to four cell stage embryos. We have not studied the question of whether this in vitro synthesized RNA is polyadenylated in the embryo or whether it is translated as it is. The RNA and the protein were detected at the blastula stage predominantly in the animal cap cells. This was surprising, since this mRNA has been used previously as a lineage tracer also for endodermal structures (Smith and Harland, 1991), which are not derivatives of the animal cap cells. In those experiments however the RNA was injected in larger amounts (2.5×) in individual blastomeres of embryos of approximately 16 cells so that the final concentration per blastomere was approximately 10 times higher. In gastrulae the protein is present in both ectodermal layers and, differently from the Gal-CPWS RNA, not restricted to the future dorsal part of the embryo. In the larger cells derived from the vegetal pole which subsequently form the endoderm, the mRNA is absent. In this last respect it behaves like the CPWS- and PARS-RNAs.

Translation of the Gal-PARS and the Gal-CPWS RNA constructs is restricted to the dorsal side of the embryo, the PARS RNA translation being further restricted to the sensorial layer.

This suggests that the machinery necessary to polyadenylate preexisting mRNAs exhibiting the pCPE is present in the animal cap cells. In gastrulating embryos the activity is detected in both cell layers of the dorsal ectoderm, but inactivated in the epithelial layer by the inhibitory cis-element present in the PARS sequence.

The inhibitory mechanism, inefficient in the animal cap cells, is apparently activated in the epithelial layer during its formation.

Our data show that PARS-, and CPWS-RNA are polyadenylated during cleavage, yet PARS RNA expression is reduced with respect to the CPWS RNA when measured by CAT assay in the whole embryo (Fig. 6) and also with respect to the spatial distribution of the produced protein since it is limited to the sensorial layer of the ectoderm (Fig. 9B3). The polyadenylation profile (Fig. 6B) suggests furthermore that the CPWS construct is polyadenylated to a larger extent than the PARS construct. This is consistent with the assumption that the inhibition mediated by the 3′-UTR is restricted to the epithelial layer.

To our surprise, the RSA construct behaves in embryos similarly to the PARS construct: it is polyadenylated but its translational activity is reduced. It would be interesting to find out whether the expression of a GAL-RSA construct is also locally restricted in the embryo. In this case the inhibition of pCPE would relay on at least two different mechanisms, one mediated by sequences located upstream from the *Rsa*I site and active on cytoplasmic polyadenylation in the oocyte and the other mediated by sequences located downstream from the *Rsa*I site and active on translation after fertilization.

3.3. Functional role of the XId3 pCPE

In the area of the blastopore, the CPWS RNA is active in both epidermal layers of the presumptive dorsal side in the precursor cells of the skin, neural plate, neural crest cells, chordal plate, paraxial mesoderm and lateral mesoderm. These lineages are confirmed by the staining profile of

early tailbud embryos. Galactosidase activity is detected in the skin, neural crest cell derivatives, neural tube, somites and weakly in the notochord (not shown).

The translation product of the PARS-RNA, the activity of which is limited in gastrulae to the sensorial layer, is detected at the early tailbud stage in the neural crest cells, eye precursors, neural tube and somites but not in the skin. At this stage the staining is weaker than the one obtained with the CPWS construct suggesting that during invagination, it is silenced or degraded more rapidly.

The Id gene is transcribed in numerous proliferating cells of the embryo and by dimerizing with bHLH transcription factors, the synthesized protein prevents the activation of genes necessary for differentiation (Benezra et al., 1990).

XId3 mRNA exhibits a palindromic CPE adjacent to additional cis-regulatory elements, which allows activation and translation of maternal messenger RNA selectively in the sensorial layer of the ectoderm.

This may play a role during embryogenesis by maintaining these cells temporarily refractory to differentiation induced by bHLH transcription factors. Differentiation of neural and neural crest cells is based on the activation of the bHLH factors MASH-1 and MASH-2 (Johnson et al., 1990; Lo et al., 1991) and differentiation of somites relies on the activation of the bHLH factors of the MyoD family (Weintraub et al., 1991). Activation of translation of maternal Id3 mRNA in these tissues before mid-blastula may be necessary to retard determination and differentiation in the absence of sufficient zygotic XId3.

The XId2 gene also exhibits a pCPE element 32 nucleotides upstream from the polyadenylation signal. We do not know whether the XId2 3′-UTR also contains an inhibitory element active in specific areas of the embryo. Sequence comparison with the XId3 3′-UTR do not reveal any evident homology. It will be interesting to find out whether also this mRNA is regulated via the 3′-UTR and in which manner.

It is surprising to us that the pCPE sequence is conserved in all Id3 genes from vertebrates described so far, despite the relatively large evolutionary distance, and important differences in embryonic development amongst vertebrates. It would be interesting to determine whether the pCPE from mice confers tissue specific regulation also in mouse early embryos.

The mechanism described here and allowing localized polyadenylation of maternal mRNA deposited in the egg could activate also other mRNAs and represent a more general tool to generate molecular asymmetries contributing to the building of a complex structured organism.

4. Materials and methods

4.1. Cloning, sequencing and DNA constructs

The Id3 gene from *Xenopus*, XId3, previously called XIdx or XIdI (Zhang et al., 1995) has already been isolated and described (Wilson and Mohun, 1995) and the XId2 cDNA was isolated from an oocyte library and sequenced using standard procedures.

The various 3′-UTR sequences were either prepared from a genomic clone containing the 3′-end of the XId3 gene or obtained by synthesis.

The PARS sequence consists of the 570 bp bounded by the *Eco*RI and *Ssp*I sites and the RSA sequence of the 156 bp located between the *Rsa*I and *Ssp*I site of the 3′-UTR sequence of *XId3* shown in Fig. 1B. The CPWS, CPMS, and CPAS were obtained by synthesis.

The sequence of the CPWS element, GGG AAC TTT TTA TAA AAG TTG TTA TTT TTC GTT TAT GTA TAA ATA AAA TTA CAT TAT TTT TAA AAG AAA TAA T, corresponds to the 3′-part of PARS.

The sequence of the CPMS element, GGG AAC GTC GAC TAA AAG TTG TTA TTT TTC GTT TAT GTA TAA ATA AAA TTA CAT TAT TTT TAA AAG AAA TAA T, is identical to the CPWS except in the palindrome.

The sequence of the CPAS, ATT ATT TCT TTT AAA AAT AAT GTA ATT TTA TTT ATA CAT AAA CGA AAA ATA ACA ACT TTT ATA AAA AGT TCC C, is the complementary strand of the CPWS and contains thus the same palindrome but no polyadenylation signal.

These elements were inserted by blunt end ligation into the *Sma*I site of the Blue Script derived pVZ vector (Hevikoff modified Blue Script).

The CAT encoding sequence was isolated as a 772 bp *Hin*dIII/*Sau*IIIA fragment from the pSV2CAT plasmid (Laimins et al., 1982) and inserted between the *Hin*dIII and *Bam*HI site of the pVZ constructs containing the various 3′-UTR.

For transcription in vivo a strong promoter/enhancer combination (Reynaud-Deonauth, unpublished data) derived from the *XId3* gene (Zhang et al., 1995) was inserted into the unique *Hin*dIII site (indicated with an arrow in Fig. 2A).

The Gal-Glob construct is the original pSP6nucβgal (Smith and Harland, 1991). To obtain the constructs Gal-PARS and Gal-CPWS the two fragments *Hin*dIII/*Bam*HI and *Bam*HI/*Bam*HI encoding the galactosidase were isolated and inserted between the *Hin*dIII and *Bam*HI sites of the pVZ derivatives containing the PARS and CPWS element described before.

The *luciferase* reporter gene was similarly obtained by inserting in the pVZ vector, the luciferase encoding sequence attached to the SV40 small intron and polyadenylation signal into the *Bam*HI site and the XId3 enhancer/promoter system into the *Hin*dIII site.

4.2. Manipulation of oocytes and embryos

Xenopus laevis oocytes and embryos were staged and described according to Hausen and Riebesell (Hausen and Riebesell, 1991), and prepared for microinjection according to the method of Gurdon (Gurdon and Wakefield, 1986) and

as described (Modak et al., 1993). Microinjection was carried out as described (Etkin and Maxson, 1980; Yisraeli and Melton, 1989; Modak et al., 1993). Needles were made from Drummond micropipettes and pulled using an Inject + Matic devise (Gabay, Geneva). Needles were calibrated by making 1 mm distant marks. A calibrated needle was then attached to an Inject + Matic microinjector.

For injecting RNA into oocytes, 0.1 ng of synthesized RNA in 5 nl of water were microinjected into the cytoplasm of stage five to six oocytes. After 6–8 h incubation at 21°C, progesterone (Sigma) was added to the medium containing gentamycin (10 μg/ml) to a concentration of 10 μg/ml, to induce maturation. The oocytes were then incubated for another 8–10 h and oocytes were collected when maturation had occured in 50% of the treated oocytes. The oocytes were washed in NaCl 0.15 M and sonicated in ice cold 0.25 M Tris (pH 7.5). CAT activity was determined with 5–10 μl of supernatant by the non-chromatographic assay as described (Sleigh, 1986). To obtain a standard deviation, for every point, three or more batches of 10–20 oocytes were collected.

For DNA injection 0.05 ng of circular DNA in distilled water were injected into the nucleus of stage five to six oocytes. Oocytes incubation was as described above.

In the case of embryos, RNA or DNA were injected into a single blastomere up to the four cell stage, in 5–10 nl of water at a concentration 10 ng/μl. When injecting galactosidase mRNA, a concentration of 20 ng/μl and 10 nl total volume were used. For in situ hybridization the mRNA was injected at a concentration of 0.1 ng/nl.

During the injection procedure, embryos were maintained in modified barth saline, MBS (Gurdon and Wakefield, 1986). Two hours after injection the MBS was replaced by aquarium water containing gentamycin (10 μg/ml). Embryos were grown at 21°C until the desired stage and lysed to determine CAT activity or fixed to determine galactosidase activity. For each time point and construct approximately 50 embryos were injected. The experiments were usually repeated five times with some parameters modified. In situ hybridization was repeated twice. The staining profiles shown in Figs. 7–10 were usually observed in more than 90% of the surviving embryos, the survival rate depending on the batch of eggs and on the amount of RNA injected. Only experiments with a survival rate of more than 50% were considered.

4.3. Histology

Embryos were washed twice in PBS and fixed for 1 h in phosphate buffered saline (PBS) containing formaldehyde at 1% and glutaraldehyde at 0.2%. After fixation they were rinsed in PBS, and stained with X-gal (Vize et al., 1991). Embryos were rinsed twice in PBS, twice in 100% methanol and cleared with a mix of benzyl alcohol/benzyl benzoate (1/2). To prepare sections (7 μm) they were embedded in paraplast.

In situ hybridization was carried out as described by Harland (1991).

4.4. In vitro RNA synthesis

Capped RNA was synthesized in vitro (Yisraeli and Melton, 1989) using T3 or Sp6 RNA polymerases depending on the constructs. Template DNA was removed by DNAseI digestion. The RNA was phenol extracted, phenol/chloroform extracted twice, ethanol precipitated. After precipitation RNA was resuspended in distilled water, assayed for integrity, quantified by formaldehyde agarose/ethidium bromide gels electrophoresis and by comparison with known quantities of RNA.

4.5. RNA extraction

RNA was extracted as described previously (Modak et al., 1993). Small scale RNA preparation for RT/PCR analysis was slightly modified.

Briefly, a batch of 5–10 oocytes or embryos were washed in 0.15 M NaCl and lysed by vortexing in 100 μl of SDS proteinase K buffer (0.1% proteinase K, 0.3 M NaCl, 2% SDS, 10 mM EDTA and 0.1 M Tris pH 7.6), incubated for 30 min at room temperature, extracted twice with phenol/chloroform and once with chloroform and precipitated with ethanol. The pellet was resuspended, digested with 130 u of DNAseI, RNAse free (Gibco/BRL), reextracted as before and precipitated with ethanol.

4.6. RT-PCR and PAT analysis

For mRNA quantification the RNA extracted from 5–10 oocytes or embryos was analysed by electrophoresis under denaturing conditions to determine quality and quantity. Equivalent amounts, based on the rRNA electrophoresis profile and representing approximately five oocytes or embryos, were reverse transcribed with 200 u of MMLV-RT, from Gibco/BRL in a volume of 20 μl containing 15 pmoles pd(N)6 random primer (Pharmacia), 1 mM dNTPs, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mg/ml gelatin and 10 mM Tris–HCl (pH 9.0). The mixture was incubated for 10 min at room temperature and 15 min at 42°C. The reaction was stopped by heating for 5 min at 100°C and chilled for 5 min on ice.

The PCR was carried out in a final volume of 50 μl containing the 5 μl RT product, 1 μl 10 mM dNTPs, 50–100 pmol of each pair of CAT and Luciferase primers, 2.5 u Taq DNA polymerase (Appligene), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mg/ml gelatin and 10 mM Tris–HCl (pH 9.0).

As CAT primers we used 5′-TTT GAG GCA TTT CAG TCA GTT GC 3′- (sense) and 5′-CAT GGA AGC CAT CAC AGA CGG C-3′ (antisense), and as Luciferase primers, 5′-GTC GCT CTG CCT CAT AGA ACT GCC-

3′ (sense) and 5′-TTC CGT CAT CGT CTT TCC GTG C-3′ (antisense).

The optimal temperature conditions and number of cycles were determined experimentally. Usually we used 93°C (5 min), 30 cycles of 93°C (1 min), 52°C (2 min), 72°C (5 min) plus 72°C (10 min). After amplification, an aliquot of 5 μl was analysed by electrophoresis in a 1.5% agarose/ethidium bromide gel and the DNA visualized by UV light. For quantification, the gels were scanned and intensity of the bands measured using Kodak Digital Science 1D software from Macintosh-Apple.

To measure the length of the poly(A) tail we used the PAT procedure (Salles and Strickland, 1995). Briefly, the amount of RNA representing five oocytes or embryos was first annealed with oligo(dT) and the anchor/primer 5′-GCG AGC TCC GCG GCC GCG TTT TTT TTT TTT-3′ in the presence of T4 DNA ligase. MMLV-RT was then added and cDNA synthesized. The PCR was carried out in a final volume of 50 μl containing 5 μl of the PAT product, 1 μl 10 mM dNTPs, 100–200 pmol of each, the oligo previously used as anchor/primer and the sense oligo 5′-GCC TGG TGC TAC GCC TGA ATA AGT G-3′, 2.5 units Taq DNA polymerase (Gibco/BRL), 5 μl of the 10× buffer supplied by the manufacturer. The optimal temperature conditions and number of cycles were determined experimentally to allow a quantitative evaluation. Usually amplification was carried out for 40 cycles at 93°C (1 min), 62°C (2 min), 72°C (5 min). Before the cycles the samples were heated at 93°C for 5 min and at the end maintained at 72°C for 10 min.

The size of the PCR product was determined by electrophoresis in 1.5% agarose/ethidium bromide gel.

To confirm the specificity of the amplification of the target DNA, a PCR was carried out with 5 μl of PAT product using the pair of primers 5′-TTT GAG GCA TTT CAG TCA GTT GC-3′ and 5′-CAT GGA AGC CAT CAC AGA CGG C-3′ that are specific for the CAT gene using the conditions: 93°C (5 min) 30 cycles of 93°C (1 min), 52°C (1 min) and 72°C (2 min) plus 72°C (10 min).

4.7. EMBL nucleotide sequence database submission number

The accession number for the *Xenopus laevis* mRNA for Id2 protein is AJ133647 and for the *Xenopus laevis* 3′-UTR of mRNA for Id3 protein is AJ133648.

Acknowledgements

We are grateful to Mrs. Franca Chatelain for expert technical assistance. This work was supported by the State of Geneva and the Swiss National Science Foundation (Grants 3100-040787.94 and 31-50873.97).

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