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Fertilization of *Xenopus* eggs imposes a complete translational arrest of mRNAs containing 3′UUAUUUAU elements

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Abstract

The early embryonic development of *Xenopus* is mainly governed by post-transcriptional regulations until the mid-blastula transition. In this report, we present evidence demonstrating that fertilization of *Xenopus* eggs triggers a complete translational arrest of mRNAs containing UA-rich elements in their 3′-untranslated region. This control is maintained at least until the mid-blastula transition. Neither maturation nor pseudo-fertilization of the egg is sufficient for triggering this control, suggesting that components originating from the male gamete are involved in the mechanism. Moreover, this control is exerted whether the mRNA is polyadenylated or not.

Key words: Translational control; AU-rich region; Embryogenesis

1. Introduction

Xenopus early embryonic development relies mainly on post-transcriptional regulation since, from maturation to the mid-blastula transition, no detectable transcription occurs and all proteins synthesized are the products of maternal mRNAs [1,2]. One major mechanism regulating maternal mRNA expression during early development involves cytoplasmic polyadenylation and deadenylation. Several maternal mRNAs which are translationally silent in oocytes or eggs become polyadenylated and enter polysomes in response to oocyte maturation or fertilization. Alternatively, other polyadenylated mRNAs that are polysomal in oocytes or eggs, are deadenylated and no longer translated during maturation or fertilization [3,4]. McGrew et al. showed that poly(A) elongation of the mRNA G10 during *Xenopus* oocyte maturation is required for translational recruitment and is mediated by a short element present in its 3′ UTR [5]. Other cytoplasmic polyadenylation elements (CPE) have been identified in different maternal mRNAs undergoing translational activation at oocyte maturation and at least two CPEs have been shown to interact with two different factors [6,7]. Aside from cytoplasmic polyadenylation and deadenylation, no other posttranscriptional mechanism affecting maternal mRNAs has been identified thus far. Messenger RNAs encoding many cytokines and oncoproteins contain UA-rich sequences in their 3′ UTR. These elements can mediate either mRNA destabilization [8,9] or translation inhibition [10]. We previously demonstrated that in *Xenopus* oocytes, mRNAs containing UA-rich (UAR) sequences in their 3′ UTR (GM-CSF, IFN-β and c-*fos*) are poorly translated as compared to mRNAs lacking these sequences [11]. We also showed that this translation inhibition is observed with heterologous coding regions and that the UAR sequences exert their effect independently of their position downstream from the stop codon [12].

By using CAT (chloramphenicol acetyl transferase) reporter mRNAs containing UAR sequences, we demonstrate here that these sequences mediate a complete translational arrest after fertilization of the *Xenopus* egg.

2. Materials and methods

2.1. Construction of the different plasmids used for in vitro transcription of corresponding RNAs

CAT 3′ IFN mRNA, CAT 3′ IFNUA$^-$ mRNA and the CAT 3′ G mRNA, polyadenylated or not, were obtained by in vitro transcription of a *Bam*HI (polyadenylated) or *Sst*I (non-polyadenylated) linearized pSP64poly(A) vector (Promega) containing the chloramphenicol acetyl transferase (CAT) coding sequence and the different 3′ UTRs.

The plasmid pSV2CAT [13] was cut with *Hin*dIII and *Bam*HI. The resulting 1634 bp fragment was then cut by *Sau*3AI which cleaves 86 bp after the CAT stop codon. The 784 bp fragment was then inserted into the pSP64 plasmid (Promega), previously cut with *Hin*dIII and *Bam*HI. The resulting pSP64CAT plasmid was linearized by *Hin*dIII. The extremities were filled and the plasmid was cut by *Bam*HI. The 788 bp resulting fragment was then inserted in the previously described pSP65IFN and pSP65IFN3′ G plasmids [14] cut by *Xba*I, filled in and cut again by *Bgl*II. Ligation of the two plasmids with the 788 bp CAT fragment generated the pSP65CAT3′ IFN and pSP65CAT3′ G respectively where the CAT coding sequence was followed either by the 3′ UTR of IFN-β or that of globin. The CAT3′ IFN and the CAT3′ G fragments from pSP65CAT3′ IFN and pSP65CAT3′ G were then in-

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Abbreviations: UAR, UA-rich; UTR, untranslated region; A, adenosine; U, uridine; MBT, mid-blastula transition; CAT, chloramphenicol acetyl transferase; IFN, interferon; TNF, tumor necrosis factor; GM-CSF, granulocyte macrophage-colony stimulating factor; CPE, cytoplasmic polyadenylation element; MMR, Marc's modified Ringers solution.

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serted in the pSP64poly(A) plasmid. The pSP64poly(A) was first linearized with *Eco*RI, filled in and ligated in the presence of a *Bam*HI linker to generate a unique restriction site after the poly(A) tail. The resulting plasmid was then cut with *Xba*I and *Sma*I and ligated with the *Xba*I/*Bam*HI(blunted) CAT3′ IFN or CAT3′ G fragments obtained from the pSP65CAT3′ IFN and pSP65CAT3′ G constructs.

pSP64CAT3′ IFNUA⁻ poly(A) was obtained from the pSP64CAT3′ IFN poly(A) construct. The 100bp UA-rich region of the IFN-β 3′ UTR was deleted from the pSP64CAT3′ IFN poly(A) construct by cutting with *Nde*I and *Sst*I. A *Sst*I linder (CGAGCTCG) was inserted in the plasmid to generate the pSP64CAT3′ IFNUA⁻ poly(A) construct.

The CAT reporter plasmids containing the human TNF-α 3′ UTR are derived from CAT constructs inserted in the pSVL plasmid and are described elsewhere [15]. The CATTNF fragments from constructs IV and V were subcloned in pGEM3 for in vitro transcription. The resulting CAT3′ TNF construct contains the last 420 nucleotides of the human TNF-α 3′ UTR and the CAT3′ TNFUA⁻ the last 250 nucleotides. Only the first of the two bears the UA-rich sequence (50 nucleotides). Both plasmids were linearized with *Sma*I before transcription.

2.2. In vitro transcription with SP6 RNA polymerase

All the RNAs were synthesized by in vitro transcription of the constructs described above according to Kruys et al. [14]. The Cap structure was incorporated during the transcription reaction. The polyadenylated mRNAs contained all a stretch of exactly 30 adenylic residues which is above the minimal length required for the poly(A) to exert a stabilizing effect [16].

2.3. Preparation of Xenopus oocytes and eggs

Full-grown *Xenopus laevis* oocytes (stage VI) were dissected from *Xenopus* female ovaries. Maturation of the oocytes was induced in vitro by progesterone treatment (10 μg/ml, 1 h).

Xenopus eggs were obtained from females injected 3 days before use with 100 units of pregnant mare serum gonadotropin (Intervet) and 1 day before with 1000 units of human gonadotropin. The eggs were fertilized with *Xenopus* sperm and the jelly coat was removed with 2% cysteine-HCl (Sigma), and adjusted to pH 7.8 with NaOH. The eggs were then washed several times with Marc's modified Ringer's solution (MMR 1 ×) [17], then transferred to MMR containing 3% Ficoll 400 (Sigma) and incubated for 5 min at room temperature before injection.

2.4. Injection into oocytes and eggs

Oocytes and eggs were injected with 40 nl of mRNA dissolved in water and adjusted at a concentration of 0.1 mg/ml except for the CAT3′ TNF and CAT3′ TNFUA⁻ mRNAs which were injected at a concentration of 0.01 mg/ml. The injection procedure and the incubation of the oocytes or eggs were the same as described by Gurdon et al. [18] except for the use of MMR instead of Barth's medium.

2.5. Analysis of mRNA stability

RNA extraction and Northern blot analysis were performed according to the procedure described by Kruys et al. [12]. Five μg of total RNA was used for each sample. The filters were hybridized with an antisense riboprobe corresponding to the CAT coding region [13].

2.6. CAT assay

A thin-layer chromatography procedure was used to assay CAT activity [13]. In most cases, one tenth of an oocyte was used to assay CAT activity. However, for the samples containing higher amounts of CAT, only one fiftieth of an oocyte was used in the CAT assay. For the extracts of eggs injected with the CAT3′ TNF and CAT3′ TNFUA⁻ mRNAs, only one-eightieth of an egg was used to assay CAT activity.

3. Results

3.1. Translation of CAT mRNAs containing or not UAR sequences in fertilized eggs

Fertilization of the *Xenopus* egg is accompanied with major changes in mRNA translation: some mRNAs associate with ribosomes while others are released from the translational machinery. The mechanisms governing these translational controls are largely unknown. Since UA-rich sequences have been shown to be translational inhibitory elements [10], we investigated whether these sequences migh affect mRNA translation during early embryonic development.

Therefore, we synthesized four different reporter constructs containing or not UAR sequences. All the mRNAs code for CAT; the two first mRNAs contain the human IFN-β 3′ UTR downstream from the CAT sequence and the two others contain the human TNF-α 3′ UTR (Fig. 1). The CAT3′ IFN mRNA contains the complete IFN3′ UTR whereas the CAT3′ IFNUA⁻ construct specifically lacks the UAR sequences. Similarly, the CAT3′ TNF and the CAT3′ TNFUA⁻ constructs differ by the presence or absence of the UAR sequences. Messenger RNAs were transcribed from these constructs and injected into *Xenopus* eggs 30 minutes after fertilization. Sets of 7 to 13 eggs were used to measure CAT accumulation at different times after injection. As shown in Fig. 2, CAT accumulation in embryos injected with the UA⁻ mRNAs increases up to 7–8 h after fertilization, which corresponds in *Xenopus* to the onset of the midblastula stage. Remarkably, in embryos injected with

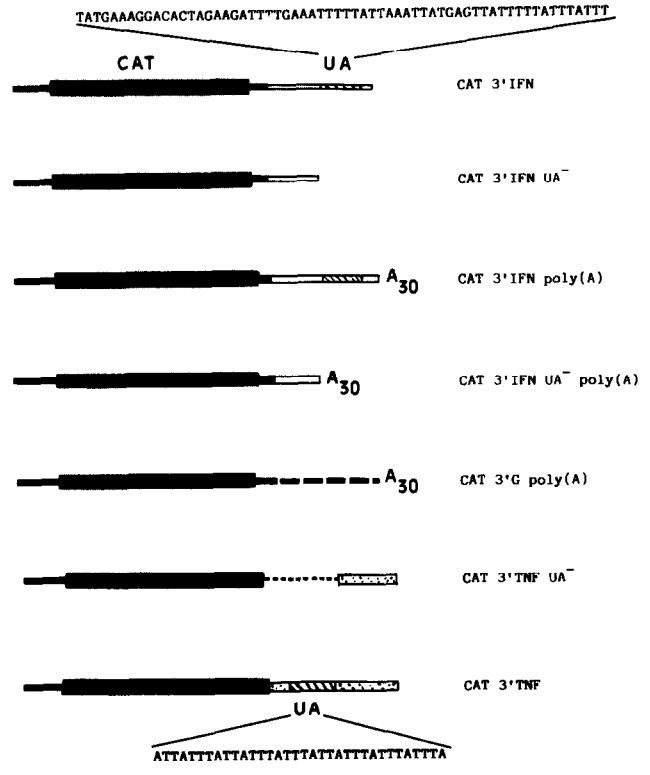

Fig. 1. Schematic representation of the different mRNAs constructs. Black boxes indicate CAT sequences except for the discontinued box which specifies *Xenopus* globin sequences. Open boxes correspond to the human IFN-β sequences with the hatched box indicating the UAR sequences. Dotted boxes indicate human TNF-α sequences.

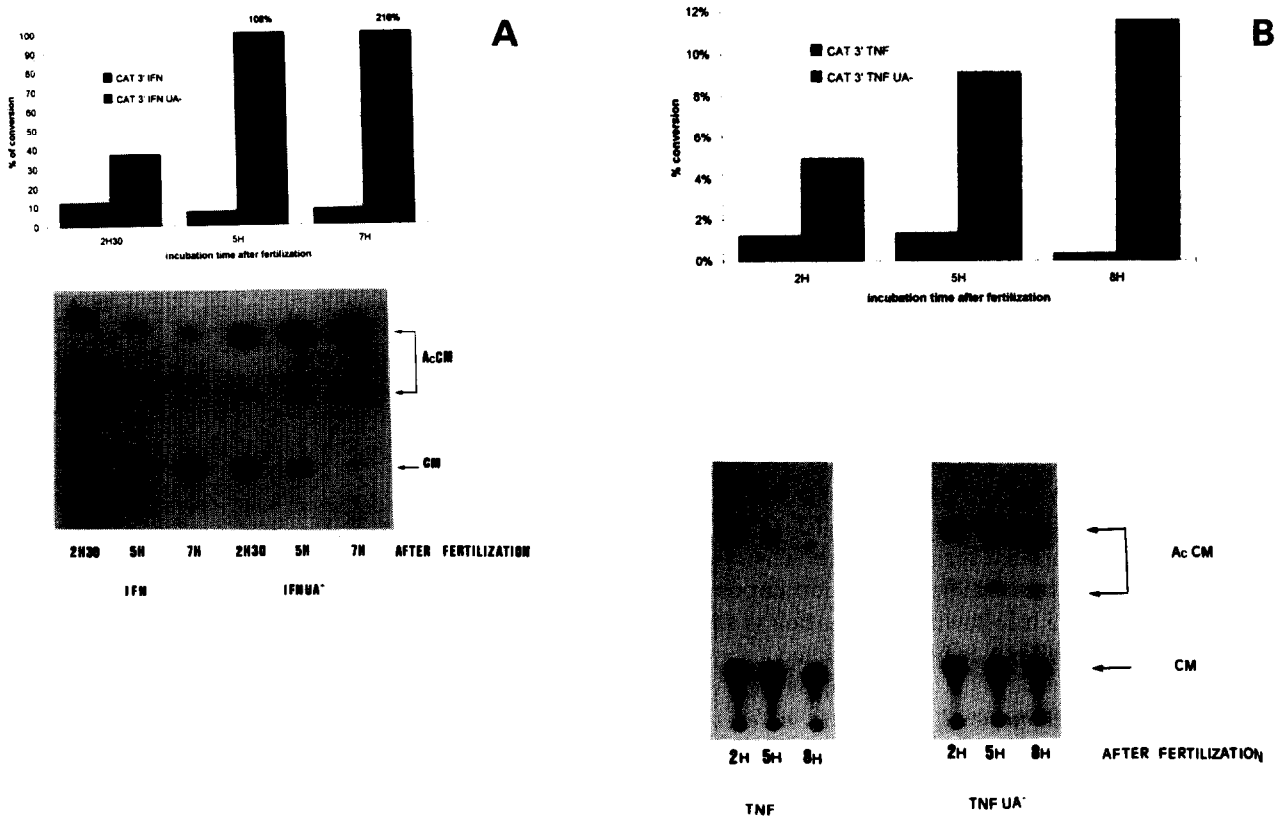

Fig. 2. Analysis of CAT accumulation in fertilized eggs injected with CAT chimeric mRNAs containing or not UAR sequences. Four different reporter mRNAs (CAT3′ IFN, CAT3′ IFNUA$^-$, CAT3′ TNF and CAT3′ TNFUA$^-$) were injected into eggs 30 min after fertilization. Batches of 7 to 13 eggs were homogenized at different times after fertilization and CAT activity was measured as described in section 2. Quantification of chloramphenicol conversion in its acetylated forms was performed on non-satured assays by measuring the ^{14}C counts of the corresponding spots from the thin-layer plates. For samples containing high quantities of CAT, quantification was performed on CAT assays made with a higher dilution of oocyte extract. Therefore, the rate of conversion is expressed in relative values. Values beyond 100% are written on top of each row. (A) The upper panel illustrates the quantitative analysis of the CAT assays shown on the lower panel. CM, chloramphenicol used as substrate in the CAT assay; AcCM, 1-acetyl, 3-acetyl forms of chloramphenicol. Formation of the acetylated products indicates expression of CAT. (B) CAT assays performed with fertilized eggs injected with the two CAT3′ TNF mRNAs. Time course of CAT accumulation.

CAT3′ IFN or CAT3′ TNF mRNA, some CAT protein is produced during the first two hours, but the enzyme level remains constant thereafter.

This suggests that translation of the CAT3′ IFN and CAT3′ TNF mRNAs is blocked shortly after fertilization. To test this possibility, fertilized eggs were treated with cytochalasin-B in order to prevent segmentation of the embryos. The embryos remain as unique coenocytic cells containing an increasing number of nuclei, allowing microinjection at different times after fertilization. Two-hour pulses were performed at different times after fertilization and CAT production was assayed. No CAT accumulation was observed in embryos injected with the CAT3′ IFN or CAT3′ TNF mRNA 3, 5, 7 and 9 hours after fertilization, (CAT3′ IFN: see Fig. 3; CAT3′ TNF: data not shown). Yet, CAT accumulation was systematically observed during the first 2.5 h that followed fertilization. In contrast, the translation rate of the CAT3′ IFNUA$^-$ and CAT3′ TNFUA$^-$ mRNAs remained high for up to 7–9 h after fertilization, and declined thereafter due to decreased viability of cytochalasin-B treated eggs (CAT3′ IFNUA$^-$: see Fig. 3; CAT3′ TNFUA$^-$: data not shown).

Treatment of the eggs with cytochalasin-B alone did

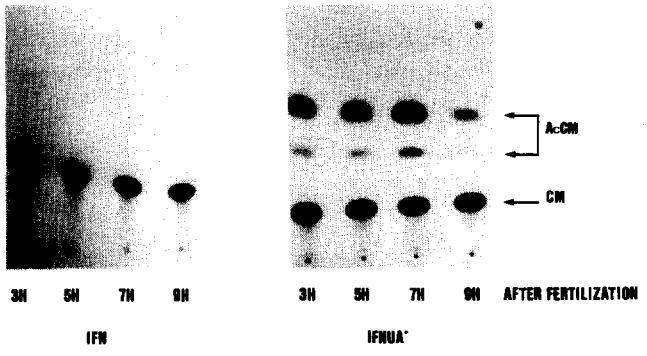

Fig. 3. Pulse analysis of the translation of the CAT chimeric mRNAs containing or not UAR sequences in fertilized *Xenopus* eggs. Fertilized eggs were treated with cytochalasin B and were injected with the CAT3′ IFN or CAT3′ IFNUA$^-$ reporter mRNA at different times after fertilization. Two hours after injection, eggs were homogenized and CAT activity was measured as described in section 2. Abbreviations are as in Fig. 2.

not interfere with CAT production. Indeed, the difference in CAT accumulation in fertilized eggs injected with the two mRNAs (CAT3′ IFN and CAT3′ IFNUA$^-$) was observed whether the eggs were treated or not with cytochalasin-B (data not shown).

We also examined CAT accumulation following injection of polyadenylated transcripts. In addition to the two CAT3′ IFN mRNAs, we injected another reporter containing the *Xenopus* globin 3′ UTR (see Fig. 1). This 3′ UTR was chosen as a reference since it confers a high translation efficiency to different heterologous coding regions in *Xenopus* oocytes [14], and its length is in the same range as the IFN3′ UTR.

As shown in Fig. 4, CAT accumulation in fertilized eggs injected with the polyadenylated CAT3′ IFN mRNA ceases rapidly following fertilization whereas the polyadenylated CAT3′ IFNUA$^-$ and CAT3′ G mRNAs are continuously translated for at least 6 h. Therefore, it seems that complete translational inhibition imposed by UAR sequences 2.5–3 h following fertilization is exerted whether or not the mRNA is polyadenylated.

3.2. *Stability of the chimeric CAT mRNAs in developing embryos*

The complete arrest of CAT production in embryos injected with the CAT3′ IFN or CAT3′ TNF mRNAs could result from rapid and selective degradation of these mRNAs due to the presence of the UAR sequences in their 3′ UTR. To test this possibility, we analyzed the CAT mRNA remaining in the embryos at different times after fertilization on Northern blot. As shown in Fig. 5, the level of all four mRNAs remained constant during the first 5 hours after fertilization. It dropped markedly 8 h after fertilization at the mid-blastula transition. When a poly(A) tail is added to the 3′ end of the CAT3′ IFN construct, the stability of the mRNA is greatly enhanced and almost no degradation occurs at least up to the mid-blastula transition (Fig. 5B).

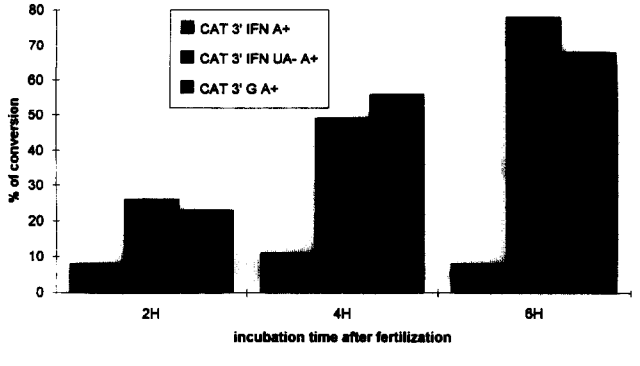

Fig. 4. Quantitative analysis of CAT accumulation in fertilized eggs injected with polyadenylated CAT mRNAs. Polyadenylated transcripts were injected into fertilized eggs 30 min after fertilization. Batches of 10 eggs were homogenized at different times after fertilization and CAT activity was measured. The CAT assays and the quantification were performed as described in Fig. 2.

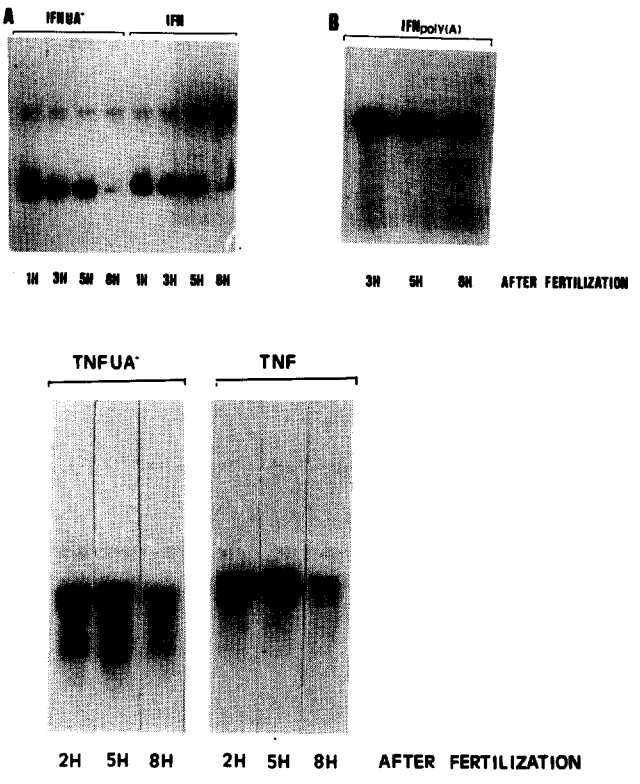

Fig. 5. Northern blot analysis of the CAT chimeric mRNAs containing or not UAR sequences remaining in eggs at different times after fertilization. Fertilized eggs were injected with CAT reporter mRNAs and total RNA was extracted from batches of 10 eggs at different times after fertilization. Five micrograms of total RNA was denatured by glyoxal dimethylsulfoxyde treatment and separated on a 1.4% agarose gel before northern blot analysis (see section 2). (A) Non-polyadenylated CAT3′IFNUA$^-$ and CAT3′ IFN mRNAs. (B) Polyadenylated CAT3′ IFN mRNA. (C) Non-polyadenylated CAT3′ TNFUA$^-$ and CAT3′ TNF mRNAs.

3.3. *Translation of the CAT chimeric mRNAs in oocytes undergoing maturation*

Maturation of *Xenopus* oocytes is accompanied by several metabolic modifications, one being an overall increase in translation. In addition, several stored mRNAs become polyadenylated and translated during maturation, while others are released from polyribosomes. This phenomenon is associated with a shortening of the poly(A) tail [19].

In order to see whether the UAR sequences already impose complete translational arrest at the earlier stage of development, we injected CAT chimeric transcripts with or without UAR sequences into oocytes and subsequently induced their maturation by incubating them for one hour in progesterone (10 μg/ml). Fig. 6 illustrates the CAT activity in maturing oocytes injected with the CAT3′ IFN or CAT3′ IFNUA$^-$ mRNAs. CAT accumulation is lower in maturing oocytes injected with the CAT3′ IFN mRNA as compared to maturing oocytes injected with the CAT3′ IFNUA$^-$ mRNA. However, no translational arrest of the UA$^+$ mRNA occurs during this earlier stage.

3.4. Translation of the CAT reporter mRNAs in activated eggs

We examined whether activation of mature oocytes would be sufficient to trigger the translational arrest imposed by UA-rich sequences observed at fertilization. Activation by pricking releases the metaphase block in which mature oocytes are arrested and induces the oocytes to start the early embryonic cell cycle. However, cleavage does not take place because the centrosomes are missing [17].

Fig. 7 illustrates an experiment similar to that shown in Fig. 3 except that mature oocytes were activated to start the cell cycle by an electric shock and were injected at 3, 5, 7, 9 h after activation. After two hour pulses for each time point, CAT activity was measured. Under these conditions, no translational arrest of the CAT3′ IFN mRNA was observed. However, the rate of CAT accumulation was 3 to 4 times lower in activated eggs injected with the CAT3′ IFN mRNA than in the eggs injected with the CAT3′ IFNUA⁻ mRNA.

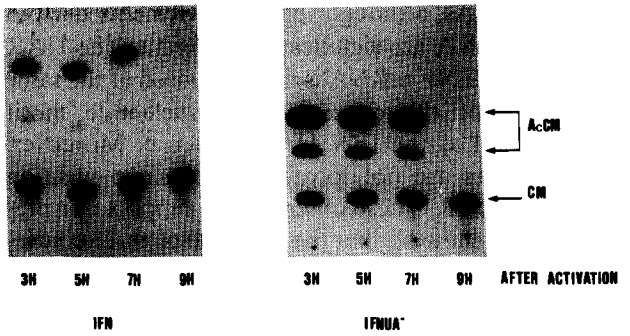

Fig. 7. Pulse analysis of the translation of the CAT chimeric mRNAs containing or not UAR sequences in activated *Xenopus* eggs. Mature oocytes were activated by an electric shock and were injected with the CAT3′ IFN or CAT3′ IFNUA⁻ at the specified times after activation. Two hours after injection, batches of 10 oocytes were homogenized and CAT activity was measured.

4. Discussion

We report that fertilization of the *Xenopus* egg is accompanied by a marked change in the translational status of two chimeric mRNAs containing the CAT sequence followed by the 3′ UTR of the IFN or TNF mRNAs. The translation of these two mRNAs is completely blocked two hours after penetration of the male pronucleus into the egg. This inhibition of translation is mediated by the UAR sequences present in the 3′ UTR. The translational arrest observed after fertilization is independent of a poly(A) tail in the mRNA and is not due to an accelerated degradation of the mRNA containing UAR elements.

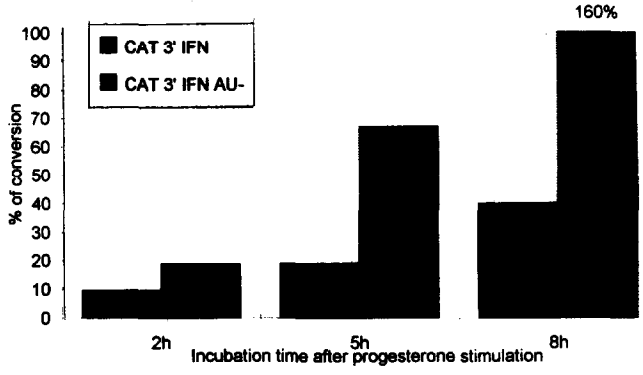

Fig. 6. Translation of the CAT chimeric mRNAs containing or not UAR sequences in maturing oocytes. Progesterone treated oocytes were injected with the CAT3′ IFN or CAT3′ IFNUA⁻ mRNAs and batches of 10 activated oocytes were homogenized at different times after progesterone treatment. CAT activity was measured quantitatively as described in Fig. 2. For samples containing high amounts of CAT, quantification was performed on CAT assays made with a higher dilution of oocyte extract. Therefore, the rate of conversion is expressed in relative values. Values beyond 100% are written on the top of the row.

Many recent studies [3–7,19] demonstrated that translational activation and inactivation of several mRNAs during *Xenopus* early development are tightly correlated with cytoplasmic adenylation and deadenylation, respectively. Translational arrest mediated by UAR sequences after fertilization does not seem to involve any poly(A) modification. Northern blot analysis of the CAT3′ IFN mRNA before and after injection does not indicate any increase in mRNA size, which would reflect the addition of adenylic residues to the mRNA after injection (data not shown). On the other hand, deadenylation of the polyadenylated CAT3′ IFN transcript after injection seems unlikely since the polyadenylated and the nonpolyadenylated CAT3′ IFN mRNAs have very different stabilities, the polyadenylated form being substantially more stable 8 h after fertilization than the non-polyadenylated form (Fig. 5A,B). Indeed, this marked difference of stability at the MBT between the poly(A)⁺ and poly(A)⁻ CAT3′ IFN mRNAs suggests that the polyadenylated mRNA is not deadenylated after injection. Moreover, it seems unlikely that the CAT3′ IFNUA⁻ mRNA undergoes cytoplasmic polyadenylation since it does not contain any AAUAAA polyadenylation signal.

The translational arrest mediated by UAR sequences is also observed when the fertilized eggs are treated with cytochalasin-B. Segmentation of the egg is thus not required for this control. Artificial activation of the egg by puncture does not induce any change in translational rate. In activated eggs, some translation inhibition mediated by UAR sequences occurs but it is similar to that observed in maturing oocytes. Taken together, these data suggest that the complete arrest of translation observed after fertilization probably requires components provided by the male gamete.

It is worth noting that in fertilized eggs, the translational arrest is observed for both the IFN and the TNF

chimeric mRNAs, although the UAR elements derived from each mRNA are significantly different. The UAR sequence from TNF mRNA is not composed of the same combination of the consensus octanucleotide motif UUAUUUAU as the IFN UAR sequence. Moreover, these sequences are not located at the same position within the 3′ UTR. These observations support our previous findings showing that the presence of three contiguous octanucleotides UUAUUUAU in the 3′ UTR is all that is required to inhibit mRNA translation regardless to their position relative to the STOP codon [11,12].

The mechanism by which the UAR sequences suppress mRNA translation has not yet been elucidated. However, the analysis of polyribosome assembly with mRNAs with or without UAR sequences in a reticulocyte lysate, showed that the presence of these sequences decreases the ability of the mRNA to form polyribosomes [10].

It has been shown by Grafi and Galili [20] that oocyte cytoplasmic extracts contain two proteins which specifically bind the UAR sequence derived from IFN-β mRNA. Following fertilization, the binding ability of the proteins markedly increases and a third binding protein can be detected. It is tempting to speculate that these modifications mediate the translational suppression observed after fertilization.

At this point, *Xenopus* mRNAs that are controlled by this mechanism have not yet been identified. However, the complete arrest of translation, that we observe after fertilization strongly points to the existence of mRNAs containing UAR motifs in *Xenopus*. Interestingly, *Xenopus* c-*myc* mRNA has been shown to contain UA-rich sequences [21]. Although the c-*myc* UA-rich motifs are not interleaved and are dispersed within the 3′ UTR, it is worth noting that the synthesis of the c-*myc* protein stops between fertilization and the mid-blastula transition. The c-*myc* protein accumulates mainly during stage IV of oogenesis and no further synthesis of this protein is observed after fertilization [22]. Possibly, translation of the remaining c-*myc* mRNA is blocked after fertilization due to the presence of UAR elements within the 3′ UTR.

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