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Posttranscriptional regulation of c-myc RNA during early development of *Xenopus laevis*

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The remarkable stability of c-myc during oogenesis contrasts with its degradation during the early developmental period in *Xenopus laevis* Three evolutionary conserved motifs found in the 3′-untranslated region of Xenopus c-myc RNAs have been analyzed for a possible role in c-myc RNA degradation No specific degradation was observed when these sequences were cloned downstream of a reporter gene and the corresponding RNAs were injected into fertilized eggs The relation between polyadenylation and degradation of c-myc mRNA has been examined during early development c-myc is adenylated during early oogenesis, and a dramatic de-adenylation occurs in full grown oocytes Consequently, the de-adenylation of c-myc mRNA that occurs in eggs might be a requirement for its degradation after fertilization, but is not sufficient to trigger its degradation

C-myc, mRNA stability, Polyadenylation, *Xenopus laevis*

1 INTRODUCTION

c-myc may be an important regulator of cell proliferation and differentiation (see [1] for review) The c-myc gene is found in the genomes of vertebrates [2] and is highly conserved.

Two classes of c-myc transcripts have been found in *Xenopus laevis* [2,3]. They are transcribed from two distinct genes. Both c-myc transcripts are accumulated to high levels during oogenesis, which is likely due to their unusual stability in the oocytes At the end of oogenesis, c-myc I mRNA represents 90% of total c-myc transcripts. In contrast, fertilization triggers the rapid degradation of c-myc mRNAs until the gastrulation stage From the neurula stage, only c-myc I was further expressed in the zygotic embryo [3]

The mechanisms which control c-myc mRNA stability during early development in *Xenopus laevis* are not understood. Cytoplasmic c-myc RNA is usually very unstable [4,5] although its turnover can vary depending on physiological conditions [4,6]. The short half-life of c-myc mRNA is mainly directed by sequences located within the 3′-untranslated region (UTR) [6,7] We have recently compared the 3′-UTR of 7 different vertebrate c-myc cDNAs [8]. The size of this region is heterogeneous but it contains three completely conserved motifs, which could play a role in c-myc RNA decay

Abbreviations UTR, untranslated region, CAT, chloramphenicol acetyltransferase

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In this work, we have examined the influence of the 3 conserved motifs within the 3′-UTR in the stability of c-myc RNA during early development We have also investigated the relationship between polyadenylation and degradation of c-myc RNA

2 MATERIALS AND METHODS

2 1 *Biological materials*

Oocytes, eggs and embryos from *Xenopus laevis* were obtained and selected as previously described [2]

2 2 *RNA extraction and analysis*

Total RNA was extracted from a large number of oocytes and embryos (100–200 per experimental point) as previously described [2] Poly(A)$^+$ mRNAs were purified by oligo dT cellulose, according to Sambrook et al [9] RNA quantities were evaluated by absorbance at 260 nm and verified by gel analysis Poly(A)$^+$ mRNAs represent 1 to 2 5% of total RNAs except in oocytes of stages I and II (20%) in agreement with the results of Cabada et al [10] RNAs were analysed by Northern blots with the complete coding sequence of c-myc I as a probe [2]

2 3 *Plasmid constructions and synthesis of corresponding RNAs*

All the constructs used are shown in Fig 1 CAT A$^-$ plasmid contained the coding sequence of chloramphenicol acetyl transferase (CAT) It was obtained from BTCAT [11] The minimal sequence containing the 3 c-myc conserved motifs (*SphI-EcoRI* fragment) was isolated from the 3′-UTR of human c-myc in pMC41 [12,13, Fig 1A] The motifs were inserted into CAT A$^-$ at *HindIII-HincII* sites (CAT MA$^-$). Poly (T) was isolated from pSP65 CAT A [13], inserted at *XbaI* from CAT A$^-$ and CAT MA$^-$ and respectively gave CAT A$^+$ and CAT MA$^+$. RNAs were synthesized and capped from the constructs using a mRNA capping kit (Stratagene) The poly(A) tail was 27 A long A poly(A) of 20–30 nucleotides is the mean length of the poly(A) tail in poly(A) mRNA from full grown oocytes [14] The integrity of RNAs was checked by electrophoresis in a 3 5% acrylamide-urea gel and by in vitro translation using a rabbit reticulocyte lysate (BRL)

2.4 *Microinjections into Xenopus eggs*

Microinjections into eggs were done 45–60 min after fertilization 200 pg of each synthetic RNA in 25 nl (unless otherwise indicated in figure legends) were injected per egg and batches of 15–20 eggs were used for each experimental point At appropriate times during development, the embryos were frozen in dry ice RNAs were extracted and analysed by Northern blotting

3. RESULTS

3.1. *Relationship between conserved sequences in the 3'-untranslated region and decay of c-myc RNA*

We have recently compared the 3'-UTR of c-myc RNA in vertebrates and found 3 entirely conserved motifs (A=CACAACCUUGGC, B=AACUGCCUA and C=UUUGUAUUUAA) [8, Fig. 1A] We suggested that two of them (A and B) may be involved in the specific decay of c-myc mRNA Sequence C corresponds to an extension of the AUUUA sequence which was demonstrated to be involved in mRNA decay [15]

Since c-myc RNA is degraded after fertilization [2,16,17], we assayed for a possible involvement of the 3 conserved motifs during c-myc decay in the early development of *Xenopus laevis* The 3 motifs were cloned downstream of the chloramphenicol acetyl transferase

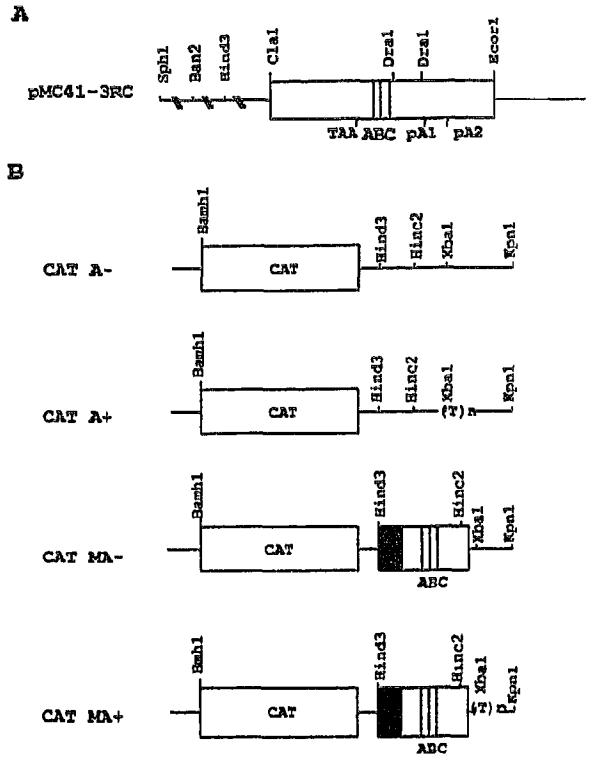

Fig 1 Fusion gene constructs A pMC41-3RC contained the three conserved motifs (A,B C) of human c-myc A=CACAACCTTGGC, B=AACTGCCTA, C=TTTGTATTTAA B CAT was the reporter gene used for the other constructions Hatched boxes represent pBR322 (52 nucleotides), stippled boxes correspond to the minimal sequence containing the 3 motifs (295 nucleotides) (T)n is 27 1 pA1 and pA2 correspond to polyadenylation site 1 and polyadenylation site 2, respectively TAA is the stop codon

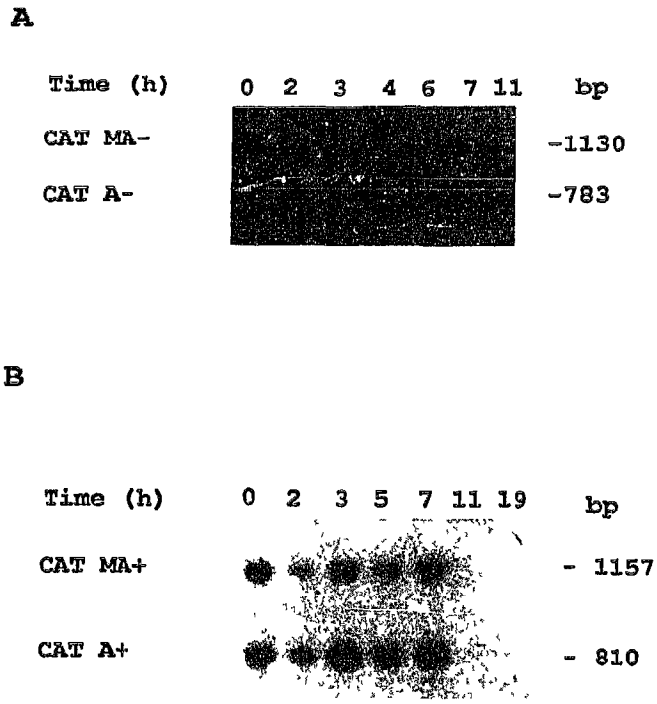

Fig 2 mRNA stability after RNA injection into fertilized eggs mRNAs transcribed (see section 2) from the constructs were injected into fertilized eggs Total RNAs were extracted from a batch of 15–20 embryos at different times after injection 10 μg of RNAs were analysed by Northern blotting and hybridized with the CAT probe A poly(A)$^-$ mRNAs 100 pg of CAT A$^-$ and 100 pg of CAT MA$^-$ were injected into fertilized eggs B poly(A)$^+$ mRNAs 200 pg of CAT A$^+$ and 200 pg of CAT MA$^+$ were injected in fertilized eggs The abscissa represents time after fertilization, injections were done 45–60 min later 2 h, 4 cells, 3 h 32 cells, 4 h, morula, 6 h, blastula, 7 h, late blastula 11 h gastrula, 19 h, neurula

(CAT) reporter gene, encoding an mRNA previously shown to be stable during early development of Xenopus [11] The constructs are shown in Fig 1 and the corresponding RNAs either polyadenylated or not, were synthetized

The effect of the conserved motifs on RNA stability were checked by injection of adenylated (CAT MA$^+$) or non-adenylated (CAT MA$^-$) RNAs into fertilized eggs and incubated various times CAT RNAs which do not contain the conserved motifs (CAT A$^-$, CAT A$^+$) were simultaneously injected in each case and used as internal controls These mixed injection experiments avoid any artifactual effects due to experimental manipulation Saturation of the endogenous enzymatic system for RNA degradation was avoided by injecting low amounts of RNA (100–200 pg of each RNA per egg) RNAs from embryos were extracted and analysed by Northern blotting using a CAT probe. As shown in Fig 2A, the 3 conserved motifs do not influence the stability of non-adenylated RNAs at least until gastrula stage Since a role of adenylation in the differential stability of c-myc mRNA in culture cells was shown by Swartwoot and Kinnburg [18], we have also injected

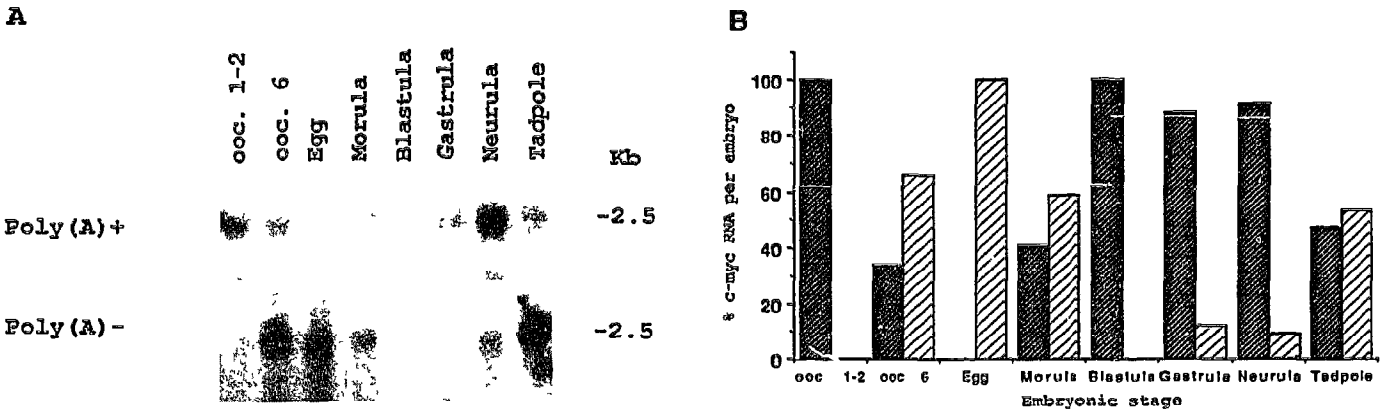

Fig 3 Changes in adenylation of c-myc RNA during development of *Xenopus laevis* A Northern blotting of poly(A)$^+$ and poly(A)$^-$ RNAs isolated from different stages of development Poly(A)$^+$ and poly(A)$^-$ RNAs were isolated from oocyte stages I–II (ooc 1–2), oocyte stage VI (ooc 6), eggs, morula (stages 6–6 5), blastula (stages 7–9), gastrula (stages 10 5–12), neurula (stages 13 20) and tadpole (stages 37–38) Each lane contained 150 ng poly(A)$^+$ or 15 μg poly(A)$^-$ (except ooc 1–2 1 5 μg) of RNAs The membrane was hybridized with a probe corresponding to the total coding region of c-myc I B Relative percent of poly(A)$^+$ and poly(A)$^-$ per oocyte and embryo at each stage Autoradiographs of different exposure times were densitometrically scanned RNA amounts were quantitated and expressed per oocyte or embryo The sum of poly(A)$^+$ and poly(A)$^-$ at each stage corresponds to 100%

the poly(A)$^+$ RNAs Fig. 2B indicates that adenylation does not affect stability of injected RNAs until gastrula stage. After this stage both RNAs are no longer detected This is in agreement with the degradation of numerous maternal mRNAs observed at mid-blastula/ gastrula stage in *Xenopus laevis* [19]

3 2 *Changes in adenylation of c-myc RNA through oogenesis and early development of* Xenopus laevis

c-myc RNA is highly synthetized and accumulated during the oogenesis period and it was shown to be stable in the egg After fertilization a dramatic degradation of c-myc is observed [2,16,17] To determine whether the c-myc RNA decay is related to its adenylation, poly(A)$^+$ and poly(A)$^-$ RNAs from oocytes and embryos were analysed by Northern blotting hybridization (Fig 3A) The relative poly(A)$^+$ and poly(A)$^-$ fraction of c-myc RNA was determined from densitometer scans of autoradiograms with different time exposures (one is shown in Fig 3A) and expressed per embryo (Fig 3B)

At early oogenesis, c-myc mRNA is exclusively accumulated as the adenylated form De-adenylation of c-myc RNA is first detected in late oogenesis, in full grown oocytes (stage VI) All of the c-myc mRNA pool is deadenylated in eggs Thus c-myc is de-adenylated shortly before start of early development, and the de-adenylated form is the substrate for degradation. At morula and blastula stages a minimum amount of c-myc RNA is detected ([3] and Fig. 3) and part of it is adenylated At latter stages until neurula, c-myc mRNA is mainly polyadenylated

4 DISCUSSION

We and others have observed post-transcriptional regulation of c-myc involving degradation of c-myc RNA after fertilization [2,16,17] The results presented here show that the 3 conserved motifs in 3'-UTR and de-adenylation of RNA are not sufficient for c-myc RNA degradation during early development However, we cannot exclude that the mechanism of RNA degradation during early development is different from that used in somatic cells Jones and Cole [7] and Bonnieu et al. [5] have shown that in cultured human cell, 3'-UTR of c-myc RNA was involved in RNA decay Deletion experiments in this region [7] indicated the involvement of our B motif in the process The C-motif, AAUAAAUA, which targets mRNAs for rapid degradation in mammalian cells [15] is not sufficient to confer instability to an RNA The relationship between poly(A) tail degradation and mRNA degradation is still far from clear but it is assumed that the de-adenylation of mRNA leads to its destabilisation [20] This relationship is observed for c-myc RNA in somatic cells [18,21] but does not apply to the early development of *Xenopus laevis*

c-myc RNA is adenylated during most of the oogenesis period [3], a period during which it is stable During maturation of the oocytes into eggs, c-myc RNA is de-adenylated, and in the egg the c-myc RNA remains still stable [2,16,17]. Thus removal of the poly(A) tail per se is not sufficient for its degradation. Then, after fertilization most of the c-myc RNA is degraded to its poly(A)$^-$ form Taken together these observations indicate that although deadenylation of c-myc RNA is not sufficient for degradation, it might be a requirement for the progression of c-myc RNA from a stable form to its degradation after fertilization.

We conclude that the 3 motifs found in the 3'-UTR and de-adenylation of c-myc mRNA do not appear to be solely responsible for the degradation of the maternal

RNA pool during early development Other *cis*-acting regulatory elements might be involved independently or via interaction with these motives, these regulatory elements may be absent or inactive in early embryos

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