Identification of post-transcriptionally regulated *Xenopus tropicalis* maternal mRNAs by microarray

Antoine Graindorge, Raphaël Thuret[1], Nicolas Pollet[1], H. Beverley Osborne and Yann Audic*

CNRS UMR 6061, IFR 140, Functional Genetics, Agricultural and Health Sciences, Faculté de Médecine, Université de Rennes 1, Rennes, France and [1]CNRS UMR 8080, Université Paris Sud, Orsay, France

ABSTRACT

Cytoplasmic control of the adenylation state of mRNAs is a critical post-transcriptional process involved in the regulation of mRNAs stability and translational efficiency. The early development of *Xenopus laevis* has been a major model for the study of such regulations. We describe here a microarray analysis to identify mRNAs that are regulated by changes in their adenylation state during oogenesis and early development of the diploid frog *Xenopus tropicalis*. The microarray data were validated using qRT–PCR and direct analysis of the adenylation state of endogenous maternal mRNAs during the period studied. We identified more than 500 mRNAs regulated at the post-transcriptional level among the 3000 mRNAs potentially detected by the microarray. The mRNAs were classified into nine different adenylation behavior categories. The various adenylation profiles observed during oocyte maturation and early development and the analyses of 3′-untranslated region sequences suggest that previously uncharacterized sequence elements control the adenylation behavior of the newly identified mRNAs. These data should prove useful in identifying mRNAs with important functions during oocyte maturation and early development.

INTRODUCTION

Regulation of mRNA translation or stability is crucial to the production of a specific level of protein both in transcriptionally active and inactive cells. In conditions where transcription is shut down, post-transcriptional controls are the main regulators of gene expression. In *Xenopus laevis*, stored maternal mRNAs are translationally recruited at specific times during development and this translational regulation is often linked to a change in the adenylation state of the mRNAs (1). Although it is not always clear whether the change in adenylation is a cause or a consequence of changes in translational status, both occur concurrently. *X.laevis* development is particularly suited to studying changes in adenylation status of specific mRNAs, as from oocyte maturation to the mid-blastulean transition (MBT, 4000 cells embryo), transcription is practically silent (2,3). In addition, and in contrast to what occurs in somatic cell, mRNAs without a poly(A) tail are stable up to the MBT (4,5). Changes in the level of poly(A)$^+$ mRNAs can therefore generally be attributed to changes in the length of the poly(A) tail rather than to changes in the overall quantity of the mRNA.

Since the mid 1970s, it is known that changes in the adenylation status of maternally stored mRNAs occur at specific times in development (6–10). From early experiments that examined the global adenylation status of mRNAs with no reference to specific sequences (11,12) to now, identification of post-transcriptionally regulated mRNAs during early Xenopus development was mainly performed on a mRNA candidate approach. However, a screen performed by Paris *et al*. (13) identified the Eg mRNAs as being specifically deadenylated following fertilization in *X.laevis*. Most of these mRNAs encode proteins with important functions: cdk2 (Eg1) (14), Aurora A (Eg2) (15), a cell cycle regulated protein (Eg3) (16), a kinesin (Eg5) (14) and CAPD2 (Eg7) a protein of the condensin complex (17).

Oocyte maturation (resumption of meiosis) and fertilization are two key events that can be considered as switches for the control of adenylation. Specific mRNAs are polyadenylated after the onset of oocyte maturation while other mRNAs are deadenylated. Similarly, after fertilization some mRNAs are specifically polyadenylated while others are specifically deadenylated.

Work by many laboratories has led to the identification of *cis*-sequences required for specific deadenylation or

polyadenylation at precise times of development [(1,18) and references therein]. These *cis*-sequences are usually located in the 3′-untranslated regions (3′-UTRs) of mRNAs. Several sequences elements have been shown to drive poly-adenylation of maternal mRNAs. First, cytoplasmic poly-adenylation elements (CPEs) with nuclear polyadenylation signal (NPS) (19,20) and polyadenylation response element (PRE) trigger the early cytoplasmic polyadenylation of several mRNAs during oocyte maturation (21,22). Second, poly(C) sequences identified in the phosphatase 2Ac mRNA (23) and Embryonic CPE (eCPE) poly(U) sequences (24,25) can induce cytoplasmic polyadenylation following fertilization. mRNAs devoid of CPEs in their 3′-UTR are deadenylated during oocyte maturation (26,27), a process termed default deadenylation.

Similarly, specific deadenylation activities have been described. The embryonic deadenylation element (EDEN) (18), and AU-rich elements (AREs) (5), can both trigger specific deadenylation in post-fertilization embryos. Later, when the rapid division of the embryo slows down and transcription resumes, a deadenylation activity requiring the onset of zygotic transcription is activated (28,29).

X.laevis maternal mRNAs with known adenylation behaviors have been grouped into four classes but only a limited number of representative mRNAs are known for each class (30). Hence, despite the relatively detailed knowledge of some of the *cis*-acting elements and *trans*-acting factors controlling cytoplasmic polyadenylation and deadenylation during oocyte maturation and after fertilization, there is no global information concerning how many mRNAs are differentially adenylated between these stages. In addition, new adenylation or translation control elements probably remain to be discovered.

With the advent of the *Xenopus tropicalis* genome sequencing project (http://genome.jgi-psf.org/Xentr4/Xentr4.home.html), it has become possible to run a large-scale screen using *X.tropicalis* microarrays to search for mRNAs with differential adenylation profiles. *X.tropicalis* diploid genome, as opposed to the allotetraploid *X.laevis*, facilitates the design of oligonucleotides for microarrays and the analysis of regulatory elements in mRNAs.

Here, we describe a screen using a *X.tropicalis* microarrays that allowed us to determine the changes in the adenylation status of over 2000 maternally expressed mRNAs during late oogenesis and early development. The microarray data were validated by quantitative RT–PCR (qRT–PCR) analysis of representative mRNAs. Also, we directly visualized the adenylation status of some endogenous mRNAs with a poly(A) test to determine both the change in adenylation and the distribution of the poly(A) tail size of selected mRNAs. From these analyses, we have classified the mRNAs into nine categories according to their adenylation changes during maturation and following fertilization. The classification of the numerous mRNAs studied into functional adenylation behavior classes should enable the identification of motifs or structures common to co-regulated RNAs. As was the case for the previous screen performed by Paris and Philippe (13). This data should also prove useful for identifying mRNAs encoding protein with important functions in early development and cell cycle progression.

MATERIALS AND METHODS

Microarray design

A set of 3000 50mer oligonucleotides was designed from 2898 *X.tropicalis* gene sequences and spotted in duplicate (Supplementary Table 1). Oligonucleotides were spotted in 16 blocks of 14×14 spots, each containing an *Arabidopsis thaliana* probe, as well as blank and empty buffer controls. MWG Biotech performed oligonucleotide design, synthesis and spotting. *X.tropicalis* gene sequences were derived from the assembly of public and in-house expressed sequence tags (R. Thuret and N. Pollet, personal communications).

Oocytes and embryos

X.tropicalis adults were obtained from the CNRS Bioresource Center in Rennes. *X.tropicalis* stage VI oocytes (StVI) were harvested according to *X.laevis* procedures (31) by treating ovarian follicles with dispase (0.4 mg/ml in OR2 1×, 1 mM $CaCl_2$) and collagenase (Clostridium type I collagenase 333 U/ml in OR2 1× without $CaCl_2$). *X.tropicalis* unfertilized eggs (UFE) were obtained 4 h after injecting females with 100 U of Human Chorionic Gonadotropin. Fertilization was performed with testis lysate (in 0.1× F1). 64-cell embryos (64C) or UFE were dejellied in 2% cysteine (pH 7.8 in F1 buffer (HEPES/NaOH 10 mM, pH 7.6, NaCl 31.25 mM, KCl 1.75 mM, $CaCl_2$ 1 mM and $MgCl_2$ 60 µM)). Collection of fifty StVI oocytes, UFE or 64C embryos was independently performed from three females. The samples were treated in parallel until the final analysis on microarrays.

Total RNA preparation and poly(A)$^+$ RNA labeling

Total RNA was extracted using Tri-reagent (Molecular Research Center) and resuspended in water. RNA quality was assessed on a Bioanalyzer (Agilent) and quantified by spectrophotometry on a Nanodrop-1000 (Agilent).

A reference sample (REF) was prepared that consists of equal amounts of each RNA sample to be analyzed. RNAs (500 ng) were reverse transcribed into double-stranded cDNAs by MMLV-RT using an oligo(dT)-T7 promoter primer at 40°C. Then, cRNAs were transcribed from the cDNAs by T7 RNA polymerase with direct incorporation of Cy5-CTP or Cy3-CTP (REF), using the Agilent Low Input Fluorescent Linear Amplification Kit. Probes were purified using the RNAeasy kit (Qiagen). Label incorporation and probe yield was controlled on a Nanodrop-1000.

Probing of cDNA microarrays

QMT Epoxy Slides were blocked 15 min at 50°C, in 50 mM ethanolamine and 0.1% SDS in 0.1 M Tris, pH 9.0. Fluorescent probes (700 ng each of tested and reference cRNAs) were mixed with Hybridization Buffer (MWG). The mixture was denatured 3 min at 95°C and applied to the microarray. Hybridizations were performed at 42°C overnight and followed by a 15 min wash in 2× SSC with 0.2% SDS at 30°C, then two 15 min washes in 1× SSC at 30°C and two 15 min washes in 0.5× SSC at 20°C. Microarrays were scanned using a GenePix 4000B scanner (Axon Instruments) and GenepixPro was used to acquire spot information.

Table 1. qRT–PCR oligonucleotides

mRNA		Sequences 5′–3′	Accession number
Xt_ODC	F	AAAATGGATGACTGTGAGATGGG	CX441537
	R	AATGAAGATGCTGACTGGCAATAC	
CapD2(Eg7)	F	CCGATCTGGAGGCTGAGCT	DR877301
	R	CGTCTGATGGGCGTTGG	
cdk2 (Eg1)	F	TAGCATTGTTGAAATAGGTTAGGATGC	CR436302
	R	TTTTGTTGTAGAACTTCCTCTTAATAC-TTTTATC	
Eg3	F	AAGTGACTATGCAATTTGAGCTAGA-AGTAT	CR589439
	R	CTTGCAGCTGGAGAGAATGTCTT	
DBD21429	F	AGACTCACAATGACTCTTTAGCAT-AAACC	CX497491
	R	AAAAAGAACAATATATGGACAGCAACA	
DRB1	F	CCTGACTGGAGACAGCTTTTATGTACT	CR576027
	R	CAATGCAGACCATGTCACTTTTTT	
HETE1	F	TTGATACTCGTGCTCCAAAGGA	CR437328
	R	TTAACAGCAACATACAGTTTGATAA-GTAAAA	
Aurora A(Eg2)	F	GGGTCACAATCTGGCAACTCA	CN106813
	R	AGGCAGTCTAGTCCCATTCACAA	
HR1B	F	GGAGACTTCCGTGTCCCTATAGACT	CX747663
	R	TCCTCTGTGCCTGCGTGAC	
BC061284	F	CAGCCTCTTCTTGACAACCAAATT	BC061284
	R	CTCTCAGCAGCAGATATGAACACA	
Kinesin (Eg5)	F	AGAGCAAATACAACACTTTTGGAGAGA	CR575787
	R	TCGACTTGGGAAGGGACTGT	

For each mRNA (left column), are indicated the sequences of the forward (F) and reverses (R) primers. GenBank accession numbers are indicated in the right column.

Microarray analysis

Microarray data were median-normalized in Cy5 and Cy3 channels. Normalized Cy5/Cy3 ratios (six values for each gene at each stage) were used to perform a significance analysis of microarray (SAM) using the TIGR Multi Experiment Viewer (TMEV V3.0.3). Criteria for the SAM were chosen so that up or down regulated genes would vary by at least 33% and the delta value was chosen as small as possible with a number of false significant (median) equal to 0 (32,33).

qRT–PCR

Aliquots containing 5 µg of each of the 9 RNA samples (StVI oocyte, UFE and 64c embryos in triplicate) were reverse transcribed with oligo(dT)-12 primer and SuperScript II Reverse Transcriptase (Invitrogen). qRT–PCR was performed in triplicate using SybrGreen PCR Master Mix in an ABI Prism 7000 apparatus (Applied Biosystems). Primers are shown in Table 1. The relative quantity of poly(A)$^+$ mRNA was determined for each stage using ornithine decarboxylase (ODC) mRNA as reference (34).

Poly(A)tail analysis

RNA Ligation mediated PAT (RL-PAT) was performed on *X.tropicalis* total RNA. An aliquot of 1 µg of total RNA was ligated with a 3′ amino 5′ phosphorylated oligonucleotide P1 (Proligo) according to Rassa *et al.* (35) (Figure 2). Reverse transcription was performed with Superscript II (Invitrogen) and primer P′1. Primers are shown in Table 2. PCR was

Table 2. RL-PAT oligonucleotides

Name	Sequence 5′–3′	Enzymes
P1	P-GGTCACCTTGATCTGAAGC-NH2	NA
P′1	GCTTCAGATCAAGGTGACCTTTTT	NA
xtP53BP2_F	TTGGACAACAGACTGGTTACTAACACTATT	NT
xtEmi1_F	GAATTGCTTTTCTCATTGAGGGA	BsaAI
xtODC_F	AAAATGGATGACTGTGAGATGGG	AluI
XtHETE1_F	TTGATACTCGTGCTCCAAAGGA	DraI
xtEg3_F	AAGTGACTATGCAATTTGAGCTAGAAGTAT	PvuII
xtEg5_F	AGAGCAAATACAACACTTTTGGAGAGA	HindIII
XtCAPD2_F	CCGATCTGGAGGCTGAGCT	DraI
xtHR1B_F	GGAGACTTCCGTGTCCCTATAGACT	HindIII

The sequences of the ligated primer (P1), the complementary RT/PCR primer (P′1) RNA and the gene-specific primers are given. The restriction enzymes used to cleave the PCR products are also indicated (right column). NT, not tested; NA, applicable.

Table 3. Regular expressions

Sequences searched	Regular expressions
NPS (a)	((AAT)|(ATT)|(TAT)|(AGT)|(AAG))AAA[AGCT]{1,30}$
CPE (b)	((TTTTATT)|(TTTTAAT)|(TTTTTAT))[AGCT]{1,200}$
Poly(C) (c)	C{14,}
eCPE (d)	T{12,}[AGCT]{1,200}$
PRE (e)	[ACG][CG]T[CT][ACT][AT][AT][ACT][CT][AGCT][AT][AGT][ACT][AT][ACT][AG]T[ACT][ACT][AGT][GT][GCT]T[AT]
AU-rich elements (f)	(TTATTTAT)|(TATTTATT)
EDEN (g)	(T[GA]){4,}[AGCT]{1,8}(T[GA]){4,}

The *in silico* search for specific sequence element used the indicated regular expressions that were defined according to the EMBOSS DREG nomenclature. (a) (38,39); (b) (61); (c) (23); (d) (24); (e) (21,22); (f) (62); (g) (40,63).

performed with *Taq* gold DNA polymerase (Invitrogen) and ^{32}P-labeled gene-specific primer and unlabeled primer P′1. PCR conditions were as follows: 7 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 56°C and 45 s at 72°C. PCR products were analyzed by electrophoresis on a native 10% polyacrylamide gel run in 1× TBE buffer and visualized by phosphorimaging (Hewlett-Packard). For restriction analysis, radiolabeled PCR products were incubated in restriction buffer and the restriction enzymes indicated in Table 2. Digestion products were analyzed as above.

3′-UTR sequence recovery and analysis

Due to the increase in genomic information between the conception and use of the microarray, for each candidate gene the spotted oligonucleotide sequence was blasted on *X.tropicalis* assembly V4.1 (http://genome.jgi-psf.org/Xentr4/Xentr4. home.html). 3′-UTRs were selected according to JGI annotations. To search for regular expressions the DREG software from the EMBOSS suite was used (http://www.infobiogen.fr/ docs/EMBOSSdoc/dreg.html). Regular expression searches are shown in Table 3.

RESULTS

We used a microarray-based analysis to classify maternally expressed genes according to the adenylation behavior of

their mRNAs. Oligonucleotides were designed to hit *X.tropicalis* sequences encoding genes implicated in numerous developmental, cell cycle and metabolic processes (Supplementary Table 1).

To have a strong statistical background three microarrays were used for each stage with total RNA from three females. Total RNA was isolated from StVI oocytes, UFE and 64-cell embryos. Selection of poly(A)$^+$ mRNA and labeling were performed simultaneously using a reverse transcription approach. We determined that in the conditions used, mRNAs with a poly(A) tail of 5 As or less where not reverse transcribed while mRNAs bearing 10 or more As were reverse transcribed as efficiently as mRNAs bearing 30 As (see Supplementary Figure 1). Therefore, the threshold for detection of a poly(A)$^+$ mRNAs lies between 5 and 10 As.

Twenty-four percent of identified maternal mRNAs are differentially adenylated during meiosis and early development

A mixture of Cy5-labeled sample and Cy3-labeled references was hybridized to the microarrays (complete results are available upon request to the corresponding author). Signals corresponding to less than three times the median background signal were considered as unexpressed genes. About two-thirds (2089) of the oligonucleotides correspond to expressed genes and were analyzed for changes in adenylation of the corresponding mRNAs.

Data were median-normalized and Cy5/Cy3 ratios submitted to a Significance Analysis of Microarrays (SAM) as a two-class unpaired study (32,33) in order to determine the differentially adenylated genes between StVI and UFE (Maturation) and between UFE and 64C (Fertilization). At each transition (StVI to UFE, and UFE to 64C) each mRNA was attributed one of three behaviors: polyadenylated, deadenylated or not significantly changed. Only signals with at least 33% variation between the two studied stages were considered significant. During maturation, 142 mRNAs are polyadenylated, 294 are deadenylated and 1653 remain unchanged. After fertilization, 114 are polyadenylated, 122 are deadenylated and 1853 unchanged. For 1572 mRNAs, no change in adenylation was observed. Therefore 24% (517/2089) of the expressed mRNAs are differentially adenylated during early development.

Maternally expressed mRNAs were next assigned to one of nine conceptual categories depending on adenylation changes at each transition (see Table 4). No mRNAs were found in category 7. The correspondence between these categories and the four classes previously described (30) is also shown in Table 4.

Existing deadenylation and polyadenylation data validates microarray data

An initial confirmation of the microarray results was obtained by analyzing the data for mRNAs with known behaviors (see Table 5). Eg mRNAs are deadenylated after fertilization in *X.laevis* embryos (13,36). Our microarray analysis classifies Aurora A (Eg2), the kinesin Eg5, Eg3 and cdk2(Eg1) as deadenylated after fertilization in *X.tropicalis* embryos. Other mRNAs provide controls for polyadenylation. Survivin mRNA is polyadenylated during *X.laevis* oocyte maturation

Table 4. Adenylation behavior categories

Categories	Described classes	Mat	Fert	N	% genes
1	I	↓	–	188	9
2	II	↑	–	93	4.5
3	III	–	↑	28	1.3
4	IV	↑	↓	49	2.3
5		–	↓	53	2.5
6		↓	↑	86	4.1
7		↑	↑	0	0
8		↓	↓	20	1
9		–	–	1572	75.3
Total				2089	100

The adenylation changes of mRNAs during oocyte maturation (Mat) and after fertilization (Fert) are indicated by arrows: polyadenylated (upward arrow), deadenylated (downward arrow) or unchanged (−). The number (*N*) and percentage of mRNAs in each category is indicated. The previously described classes (30) are indicated.

Table 5. mRNA microarray data

mRNAs	Maturation Fold change	Score	Fertilization Fold change	Score
Eg3	1.01	−0.08	2.30	−12.21
CAPD2 (Eg7)	1.14	1.67	1.25	−2.7
Eg5	1.04	0.5	1.39	−4.38
Emi1	1.89	−8	1.04	0.37
P53BP2	1.41	−3.8	1.58	6.6
HR1B	2.72	−5.8	3.24	10.5
Aurora A(Eg2)	1.03	−0.36	1.66	−7.15
Cdk2(Eg1)	1.04	0.54	1.62	−7.78
Survivin	1.34	3.76	1.35	−2.96
HETE1	2.94	−11.4	2	11.45

For any mRNA, the fold change between two stages represents the ratio between the average poly(A)$^+$ mRNA level in the higher level developmental stage versus that in the lower level developmental stage. mRNAs with bold lettering correspond to mRNAs with some previously known adenylation behavior in *X.laevis*. The score corresponds to an unpaired *t*-test. For example, a score of 2.228 corresponds to a probability of 95% that the differences between the two stages are significant, the higher the absolute value of the score, the better the probability. Negative score value indicate that the RNA is deadenylated. The gray background indicates significant changes.

(37) and was observed to be polyadenylated at this time by the microarray analysis. Microarray analysis also showed that this mRNA was deadenylated after fertilization; published information (37) indicated that this mRNA is degraded after MBT, a behavior compatible with a post-fertilization deadenylation (4,5).

Quantitative RT–PCR validates microarray results

To confirm that the changes observed by microarray analysis correspond to real changes in the abundance of poly(A)$^+$ mRNAs, we performed qRT–PCR analysis on representative mRNAs. Reverse transcription was primed with oligo(dT) to specifically detect poly(A)$^+$ mRNAs. Overall, about 75% of the changes predicted by the microarray analysis are qualitatively confirmed by the qRT–PCR analysis (Figure 1, compare panels A and B). In general, the magnitude of the changes detected by qRT–PCR is more important than those seen with microarray analysis. Notable exceptions to the agreement between the two methods are HR1B and DRB1 mRNA.
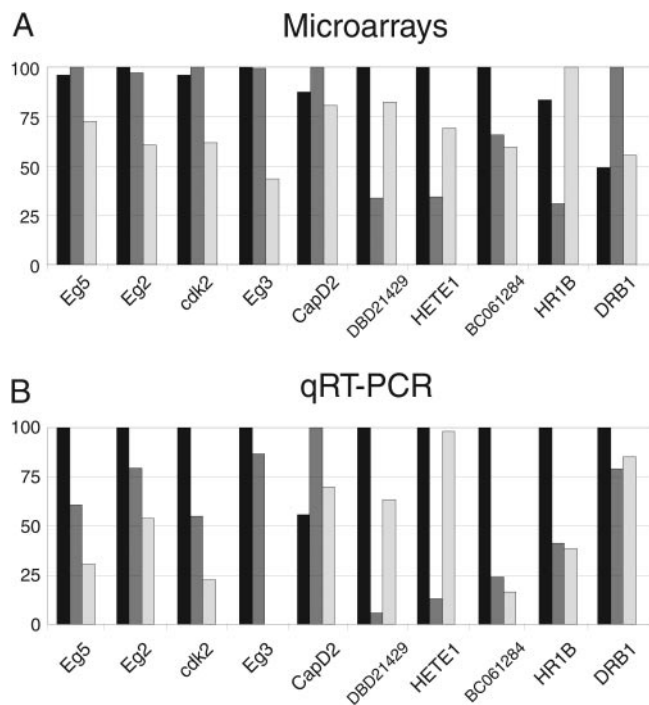
Figure 1. Comparison of microarray and qRT–PCR results. Relative amounts of poly(A)$^+$ mRNA of the indicated gene products in stage VI oocytes (black bars), UFE (gray bars) and 64-cell embryos (open bars), as measured by (**A**) microarrays and (**B**) qRT–PCR. Relative levels for microarray data are given with the maximal normalized intensity as 100%, and for qRT–PCR, the maximal poly(A)$^+$ mRNA amount is considered as 100%.

Figure 2. Sequence analysis of mRNAs classified according to their adenylation behaviors. Regulatory sequences were searched for in putative 3'-UTR of mRNAs in each category. For each category, the percentage of sequences containing the regulatory element is indicated. The number of sequences analyzed and the total length of the sequence in each category are indicated. The percentage of each regulatory sequence elements in the totality of the 3'-UTR sequences of categorized mRNAs (cat UTR) and of all the UTR's recovered from the genome annotation (whole 3'-UTR) is indicated.

Do regulatory elements match adenylation categories?

The *X.tropicalis* genome assembly (V 4.1) is annotated with gene models and predicted or mapped protein and transcripts sequences based upon cDNAs sequences, protein homology and *ab initio* methods. We recovered potential mRNA 3'-UTR sequences from *X.tropicalis* genome annotation by aligning predicted protein sequence with predicted mRNAs sequences and selecting the nucleotidic sequences downstream from the stop codon. Overall, 7474 putative 3'-UTRs were recovered, of which 1496 corresponded to mRNAs in categories 1–9.

3'-UTRs were analyzed for the presence of known regulatory elements. *In silico* searches were built that represents known information regarding each particular regulatory element (Table 3) and the number of 3'-UTRs bearing the indicated regulatory elements was assessed in each category (Figure 2 and Supplementary Table 2). First, as a confirmation of the selected 3'-UTR, the presence of a NPS was assessed. We used the five most abundant NPS (together they represent about 80% of the NPSs) in human and mouse located at a maximum of 30 nt from the 3' end (38,39). When considering the totality of the 3'-UTRs, 4616 (62%) of the 7474 UTRs contained one of the five most abundant NPS at <30 nt from the 3' end. CPEs were present in 2162 (29%) mRNAs while eCPEs in 254 (3%). Deadenylation elements (ARE and EDEN) were detected in, respectively, 17 and 5% of the sequences. Practically no poly(C) (1%) or PRE (1%) were found. However, the PRE consensus is ill defined and of weak predictive value. When the same analysis was performed on the 3'-UTR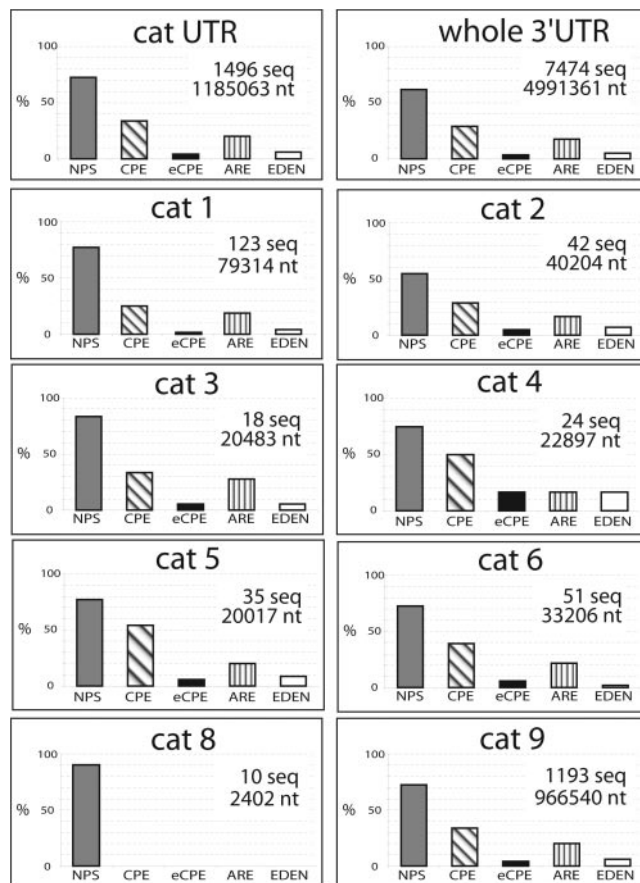s of mRNAs belonging to categories 1–9 as a whole, similar results were obtained (compare Figure 2, 'cat UTR' to 'Whole 3'-UTR').

When analyzed by category, CPEs are present in more than 50% of the mRNAs of categories 4 and 5 that correspond to mRNAs being respectively polyadenylated during oocyte maturation or substrates for post-fertilization deadenylation [and therefore having a poly(A) tail in unfertilized eggs]. Notably, CPE sequences are under-represented (25%) in category 1 mRNAs that are deadenylated during maturation. This is in agreement with these mRNAs being predominantly deadenylated by a default process that requires the absence of a CPE. When the search was extended to atypical CPEs, such as those present in the D7 mRNA 3'-UTRs (UUUUAACA, UUUUUUAA or UUUUUACA) (22), such sequences were mainly found in categories 3 and 6 mRNAs that are polyadenylated after fertilization (Supplementary Table 2).

Globally, AU-rich elements are present in 1299 3'-UTR of 7474 mRNAs (17%). When analyzed by category, there is no clear over or under representation in any category.

EDENs are globally present in 384/7474 mRNAs (5%) and are over-represented in category 4 (4/24, 17%) mRNAs that

are polyadenylated during maturation and deadenylated after fertilization.

Interestingly, EDENs are under-represented in categories 6 mRNAs that are polyadenylated after fertilization, in agreement with previous data (40) showing that EDENs override CPEs in embryos. Both CPEs and deadenylation elements can be found in category 4 mRNAs that are polyadenylated during oocyte maturation and deadenylated after fertilization. However, the limited proportion of mRNAs deadenylated after fertilization that contains deadenylation sequences (as defined here) suggests either that the search strings used are too restrictive or that elements other than EDENs and AREs trigger deadenylation after fertilization. Category 8 mRNAs contained no known regulatory elements.

Visualization of the poly(A) tail size of endogenous maternal mRNAs

One potential caveat with the validation of our microarray analysis by qRT–PCR is that both microarray and qRT–PCR rely on the specific detection of poly(A)$^+$ mRNAs selected with oligo(dT). To measure the length of the poly(A) tail on endogenous mRNAs with an oligo(dT)-independent approach, we used an RNA ligation-mediated poly(A) test (RL-PAT) (35). As shown in Figure 3A, this technique enabled us to directly determine the change in the length of the poly(A) tail and not the mere presence or absence of a poly(A) tail.

To determine whether RL-PAT signals were sequence specific, two different tests were used. First, we verified that digestion of the radiolabeled PCR product by a specific restriction enzyme gave only one radiolabeled digestion product whose size was compatible with the size predicted from the 3′-UTR sequences (Figure 3B). This further validates the 3′-UTR sequences used in the sequence analysis we performed. Due to the particular size heterogeneity of the poly(A) tail of ODC mRNA (see below), the RL-PAT/restriction enzyme test was performed on RNA samples pretreated with oligo(dT) and RNase H. Second, we determined that oligo(dT) directed RNase H digestion reduced the size of the amplified product to that expected for the deadenylated mRNA (Figure 4A and B, RH lanes). For all the tested samples, in RNase H-treated samples, the amplified signals migrated faster and were much sharper than in untreated samples. This indicates that the poly(A) tail is of heterogeneous size for any given mRNA.

Figure 4A presents *X.tropicalis* adenylation changes for mRNAs with known behaviors in *X.laevis*. ODC mRNA is known to be poly(A)$^+$ during early development (41). Here, we show that this mRNA is also consistently poly(A)$^+$ during *X.tropicalis* development. This mRNA shows the highest heterogeneity in poly(A) tail size with a length ranging from 30 to more than 100 As.

The kinesin Eg5, CAPD2(Eg7) and Eg3 mRNAs are deadenylated after fertilization during *X.laevis* development (36). Figure 4A shows that *X.tropicalis* kinesine Eg5, CAPD2(Eg7) and Eg3 mRNAs are efficiently deadenylated after fertilization. Particularly, Eg3 mRNAs seems to be deadenylated beyond the 3′end defined by oligo(dT) directed RNase H digestion. The 3′ end of Eg3 mRNAs (GenBank accession no. CR589439) is very A-rich making it a possible substrate for the poly(A) nuclease; however, this A-rich sequence
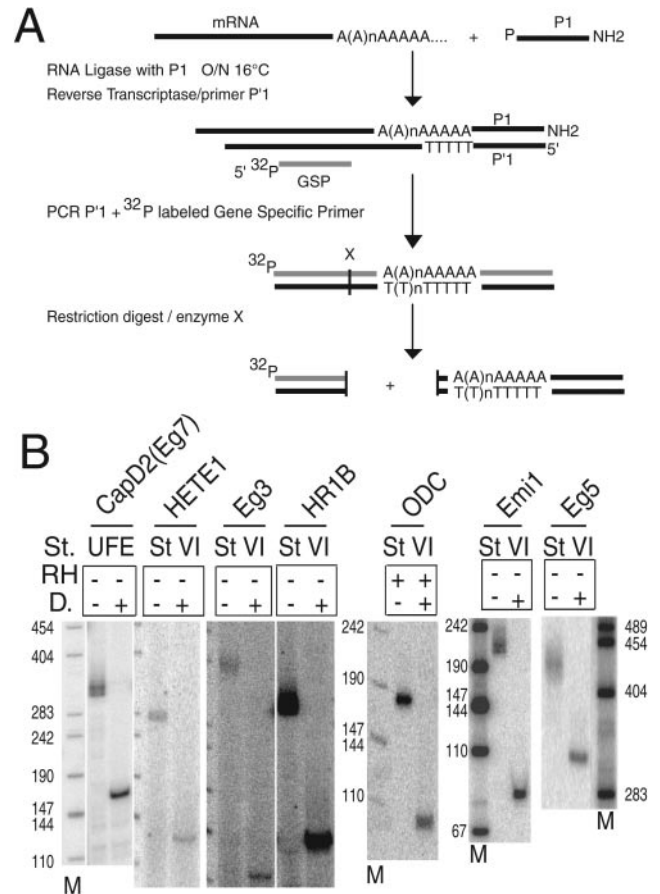

Figure 3. RL-PAT specificity. (**A**) Schematic of the RNA ligation mediated poly(A) test, as described by Rassa *et al.* (35). P1 denotes the primer ligated to the 3′-UTR of the RNA, the 3′ end of P1 is blocked by an amino group. cDNA synthesis is achieved from primer P′1 and PCR products are radiolabeled by the ^{32}P phosphorylated gene-specific primer (GSP). X indicates a restriction site, radiolabeled fragments are in gray. (**B**) Specificity of RL-PAT was tested by restriction digest (D) of PCR products obtained from StVI oocyte or UFE RNA as indicated (St., stage). The identity of the tested mRNAs is indicated above each lane. When indicated, RNase H (RH) digestion in the presence of oligo(dT) was performed prior to the RL-PAT. M, radiolabeled DNA size markers as indicated (base pairs).

probably does not produce a sufficiently stable oligo(dT)-RNA hybrid to allow degradation by RNase H. During maturation however, Eg3 behave differently from Eg5 and CAPD2(Eg7) mRNAs. Eg3 mRNA contains a poly(A) tail of about 50 As in stage VI oocytes and does not appear to be further polyadenylated during oocyte maturation (Figure 4A); western analysis has shown that Eg3 protein is present in StVI oocyte and only increases modestly during oocyte maturation (16). In contrast, RL-PAT indicated that Eg5 and CAPD2(Eg7) contain a poly(A) tail in StVI and are further polyadenylated during maturation, in agreement with the increase in protein level observed for both Eg5 and CAPD2(Eg7) during oocyte maturation (31,42).

To determine whether the microarray analysis is a good prediction tool, we analyzed by RL-PAT several mRNAs with unknown adenylation behaviors. We identified Emi1 (Early mitotic inhibitor 1) mRNA as being deadenylated during oocyte maturation (see Table 5). RL-PAT (Figure 4B) showed that Emi1 maternal mRNA is clearly deadenylated
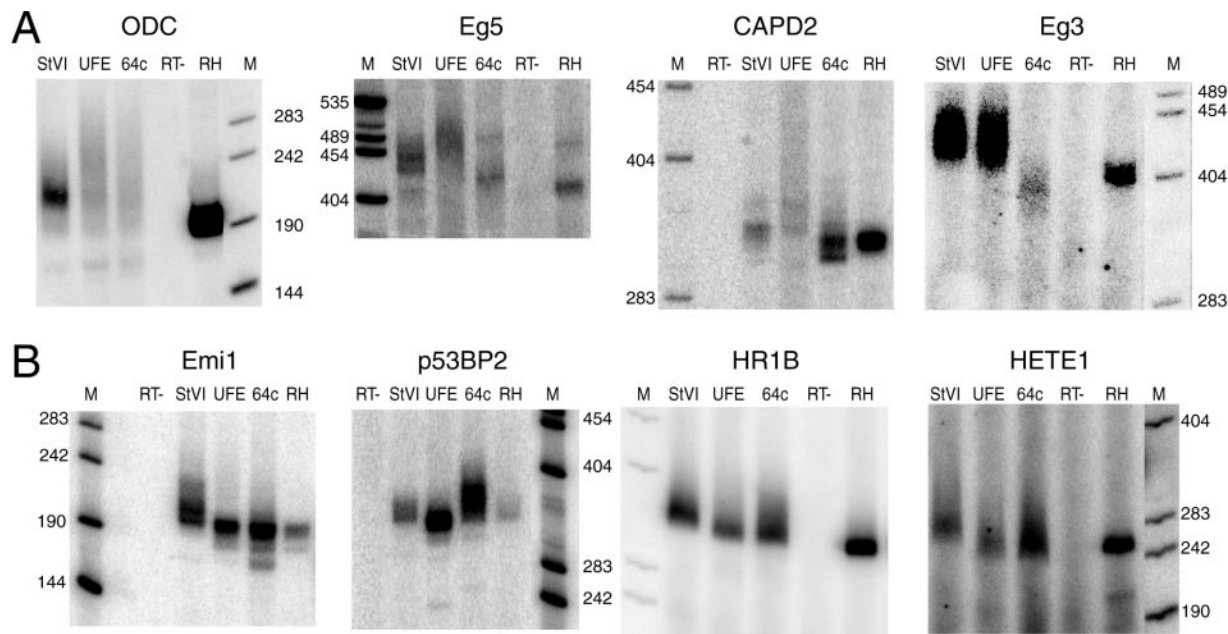
Figure 4. Adenylation behaviors of selected *X.tropicalis* endogenous mRNAs. Total RNAs from StVI oocytes, UFE and 64C embryos were analyzed by RL-PAT. The identity of the tested mRNAs is indicated above each panel. Oligo(dT)/RNase H digestion (RH) was performed on StVI oocyte total RNA. M, DNA size markers. In RT- control lanes, ligated RNAs were not reverse transcribed before PCR. (A) RL-PAT results for mRNAs with known adenylation behaviors in *X.laevis*. (B) RL-PAT results for mRNAs with unknown behaviors.

from stage VI to UFE, with a poly(A) tail shortened from about 50–60 As in St VI oocytes to the poly(A)⁻ form in UFE. Emi1 mRNA remains deadenylated at least up to the 64-cell embryos. Emi1 can therefore be placed in category 1 both by microarray analysis and RL-PAT.

The p53 binding protein 2 (p53BP2) that binds to both p53 and Bcl2 (43,44), enhances the trans-activation function of p53 and may induce the cdk inhibitor p21 (45). RL-PAT of p53BP2 maternal mRNA revealed that its poly(A) tail is shortened during maturation and lengthened after fertilization (Figure 4B) as predicted by the microarray analysis (Table 4).

Finally, as shown in Figure 4B, both HETE1 and HR1B are deadenylated during maturation and polyadenylated after fertilization as predicted by microarray analysis and qRT–PCR (Table 5 and Figure 1). These RL-PAT assays clearly show that adenylation changes may range from dramatic (p53BP2, Emi1 or Eg mRNAs) to more discrete changes in the length of the poly(A) tail (HR1B).

DISCUSSION

Work on *X.laevis* has led to the conclusion that the adenylation and translational status of a mRNA are correlated; deadenylated mRNAs are poorly translated while poly(A)⁺ mRNAs are better translated. The work presented here identified differentially adenylated mRNAs during *X.tropicalis* oogenesis and early development and classified them according to their adenylation behaviors (see Table 4, complete results are available upon request).

In addition to the global analysis into categories that is discussed below, the adenylation behavior of a number of specific *X.tropicalis* mRNAs was studied. In all these cases, where a behavior had been described in *X.laevis* it

was reproduced in *X.tropicalis*, indicating a strong conservation of controlled polyadenylation between these two species. This is coherent with the wider conservation already described by Verrotti *et al*. (46).

About two-thirds of the genes analyzed by the microarray were expressed maternally. This may seem a rather high estimate, but gene-probes spotted on the arrays were mainly chosen for their involvement in embryonic development and it is therefore conceivable that maternally expressed genes are over-represented.

Comparing microarray and qRT–PCR results we found that about 75% of the changes observed by microarray were qualitatively reproduced by qRT–PCR. This result is in agreement with a large-scale comparative study realized by Dallas *et al*. (47) who found that statistically significant correlations ($P < 0.05$) were observed between qRT–PCR microarray results for about 70% of the human genes they studied. It should be realized that both of these methods are based on the recognition of relatively short sequence elements in the mRNA. Therefore, if several differentially spliced isoforms of an mRNA exist, the two methods may detect different populations which could lead to the observed apparent discrepancy for about 25–30% of the mRNAs.

By microarray analysis, we found that about 24% of the expressed genes presented changes in adenylation status during the period studied. These mRNAs were grouped, according to their adenylation changes into nine categories. Because transcription is silent from stage VI of oogenesis to the MBT, when zygotic transcription is massively activated, and as mRNA degradation pathways that rely on prior deadenylation are not efficient before the MBT (4,5), changes in poly(A)⁺ mRNA abundance are mainly due to changes in the presence or absence of a poly(A) tail. However, two mRNAs, Xlhbox2B and Xoo1, (48,49), are known to

be specifically cleaved during oocyte maturation in *X.laevis*. Therefore, mRNA specific degradation cannot be excluded and could apply to mRNAs in categories 4, 5 or 8.

Among the nine conceivable categories, that containing the most mRNAs was category 9; these are mRNAs for which no adenylation changes were detected. However, the proportion of mRNAs in this category may be over-estimated. First, only the adenylation changes between three specific stages of oogenesis and early development (St VI, UFE, 64 cells embryos) were studied. Therefore, we cannot exclude the possibility of more discrete patterns of adenylation/deadenylation such as a polyadenylation followed by a deadenylation between StVI and UFE.

Second, as poly(A)⁻ mRNAs are not detected in our microarray screen, the category 9 mRNAs must correspond to poly(A)⁺ transcripts. Therefore, these mRNAs should contain CPEs to resist default deadenylation during maturation. However, when we assessed the number of CPEs in this category it was similar to the proportion observed in the global population of UTRs. Therefore, the mRNAs in this category may have adenylation changes below the threshold chosen for the statistical analysis of the microarray data and which, consequently, would not be detected. This, for instance, was observed for CAPD2(Eg7) mRNA during oocyte maturation. However, when the analysis was repeated with a larger allowed false discovery rate, very few gene products were excluded from category 9, indicating that the chosen criteria were not producing major artefacts. Third, we determined that the oligo(dT)-T7 promoter-primer reverse transcription conditions were such that mRNAs bearing a poly(A) tail as short as 10 As were amplified while mRNAs with 5 As were not. Therefore, the technique used probably does not efficiently detect the elongation of a pre-existing poly(A)tail. Alternatively as *X.tropicalis* oocyte maturation is about two times faster than *X.laevis* maturation (50,51) it is possible that some category 9 mRNAs devoid of CPE are not deadenylated by default fast enough to achieve a poly(A) tail size below 10 As in the time-course of the experiment. A last parameter that needs consideration is the microarray signal quality. If this were too noisy (large variations between samples) then any changes would be classified as insignificant by the SAM analysis.

Among mRNAs whose adenylation does change, the most numerous are those deadenylated during oocyte maturation and that then remain deadenylated (category 1). In maturing oocytes, mRNAs devoid of any specific polyadenylation element are the substrate for a default deadenylation activity (26,27) and, consistently, such polyadenylation elements were under-represented in category 1 mRNAs (see Figure 2, compare cat1 with cat UTR). At present, no sequence elements are known to trigger specific deadenylation in the maturing Xenopus oocyte. However, the 3′-UTR of the category 1 mRNA Emi1 (from GenBank accession no. CX483179) contains both a consensus CPE (UUUUAUU) downstream of a NPS (AAUAAA) and an ARE (UAUUUAUU). Therefore, putative elements for both polyadenylation and deadenylation are present in this mRNAs. The AREs studied to date do not trigger deadenylation during oocyte maturation (5), so one would predict that the deadenylation of Emi1 mRNA during maturation is either due to the presence of novel sequence specific deadenylation

elements in the 3′-UTR, or that in the context of this particular 3′-UTR, the CPE is inactive allowing default deadenylation or, alternatively, that the ARE is active during oocyte maturation. Inactivation of CPEs or eCPEs can be achieved by silencer elements (24,36).

It is interesting to note that the adenylation changes observed for Emi 1 mRNA are consistent with the reported expression for the protein. The amount of Emi1 protein, an F-box protein that inhibits Anaphase Promoting Complex activity in Xenopus (52), increases from stage V to stage VI of oogenesis. During oocyte maturation, Emi 1 protein level remains constant and then, after fertilization, drops in a cell cycle-dependent manner (53). As overexpression of Emi1 blocks mitotic division (53) we can hypothesize that the deadenylation of the mRNA is required to arrest translation and thereby to maintain a low level of the protein after fertilization.

Category 6 mRNAs are also deadenylated during oocyte maturation but, in contrast to category 1 and 8 mRNAs, they are re-adenylated after fertilization. Three examples of these mRNAs are illustrative: p53BP2, HR1B and HETE1. The 3′-UTR of p53BP2 3′-UTR (from GenBank accession no. CR848117) contains CPEs (UUUUUUAUU and UUUU-AUU), a NPS (AAUAAA) and an ARE (UAUUUAUU) but is devoid of an eCPE. We have previously shown that maturation types CPEs are active in embryos (4,28). Therefore, it is conceivable that the CPEs present in p53BP2 mRNAs trigger the embryonic polyadenylation. The deadenylation of this mRNA during oocyte maturation could either be directed by the ARE, by novel deadenylation elements or by a silencing of the CPE as evoked above for the Emi1 category 1 mRNA.

HR1B and HETE1 mRNAs are even more interesting. Both are deadenylated during maturation and polyadenylated after fertilization. HR1B mRNA (from CR760960) contains a NPS (AAUAAA) and an ARE (UAUUUAUU) and HETE1 3′-UTR (from BC059759) contains only a NPS (AAUAAA) but neither contains a CPE, atypical CPEs or eCPE, normally considered as a prerequisite for polyadenylation during maturation or after fertilization (54). However, in a screen performed to identify polyadenylation-element-containing mouse neuronal mRNAs, only about 60% of the 66 mRNAs that were polyadenylated in maturing *X.laevis* oocytes contained CPE sequences (55). Also, a precedent for CPE/eCPE independent polyadenylation is BMP-7 mRNA that is polyadenylated after fertilization in *X.laevis* (56) in the absence of CPEs or eCPEs sequences.

In our analysis, only about 50% of the mRNAs of categories 4, 5 and 30% of category 2, that are poly(A)⁺ after oocyte maturation contain a CPE and even less (17%) an eCPE. For category 3 and 6, mRNAs that are polyadenylated after fertilization respectively 39 and 33% contain a CPE and only a very small proportion (about 6%) of category 3 and 6 mRNAs contain an eCPE. It is therefore becoming clear that for many mRNAs cytoplasmic polyadenylation occurs in the absence of eCPEs or CPEs in their 3′-UTR. Accordingly, we propose that polyadenylation can be targeted by novel sequence elements that remain to be characterized.

The conceptual category 7, corresponding to mRNAs polyadenylated both at maturation and after fertilization, is devoid of candidates. This is consistent with (i) the absence

of transcription during the period studied and (ii) the methodology used for priming the reverse transcription [oligo(dT)-T7 primer anchored to the 3′-UTR] that is not optimal for discriminating between an existing poly(A) tail and an elongated poly(A) tail. Therefore, mRNAs bearing even short poly(A) tail (10 A's) would be considered poly(A)$^+$.

It is conceivable, therefore, that mRNAs activated (polyadenylated) during oocyte maturation are under-represented which is exemplified by Eg5 and CAPD2(Eg7) mRNAs that are not considered to be polyadenylated during maturation by the microarray analysis while RL-PATs clearly showed the presence of a poly(A) tail in St VI oocytes that is further elongated during maturation (Figure 4A).

Taken together, these results suggest that as yet unidentified regulatory elements exist in mRNAs that either trigger translational activation and polyadenylation or repress it.

We consider that the results presented here will accomplish two main goals. First, they should facilitate the characterization of regulatory sequences by identifying suitable candidate gene product. This characterization will include both refining the information known about identified regulatory elements such as the EDEN, ARE or CPE and defining new regulatory elements and associated RNA binding proteins. In somatic cells, sequences controlling the polyadenylation status of an mRNA have a direct effect on the stability of the mRNAs. In post-MBT embryos and somatic cells, a regulation of mRNA stability is important for a proper control of development (57,58) and cell proliferation (59). Therefore, the changes in polyadenylation behavior reported here are probably important not only for oogenesis or early development but also for the control of protein level in older embryos or adult cells. This is clearly demonstrated by the role of CPE-dependent polyadenylation in the translational activation of localized mRNAs in neurons (60) as well as the role of EDEN-BP-dependent deadenylation in regulating somitogenesis (57).

Second, these results should advance our understanding of the functional significance of the observed regulations. This could be achieved through a combined approach of overexpressing downregulated mRNAs and by the specific knockdown of activated mRNAs that have been identified in this study.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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