Masking and Unmasking Maternal mRNA

THE ROLE OF POLYADENYLATION, TRANSCRIPTION, SPLICING, AND NUCLEAR HISTORY

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Funda Merci, Anjanette M. Searfoss, Michael Wormington, and Alan P. Wolfe

From the Laboratory of Molecular Embryology, NICHD, National Institutes of Health, Bethesda, Maryland 20892-5431 and the Department of Biology, University of Virginia, Charlottesville, Virginia 22903-2477

We establish that masked mRNAs synthesized from exogenous plasmid templates microinjected into the nuclei of Xenopus oocytes are translationally activated (unmasked) on oocyte maturation concomitant with polyadenylation. Synthetic mRNA injected into the cytoplasm of the oocyte is translated over an order of magnitude more efficiently than is the cognate mRNA synthesized in vivo. Both mRNA synthesized in vivo and mRNA microinjected into the oocyte cytoplasm require a cytoplasmic polyadenylation element in the 3’-untranslated region to activate translation on maturation. Although polyadenylation upon oocyte maturation can relieve the translational repression of mRNA synthesized in vivo, the excision of an intron within the nucleus does not relieve repression. We suggest that the translational repression coupled to the transcription process will more effectively repress inappropriate gene expression in the oocyte and offer the potential to achieve a wider range of gene regulation.

The translational control of maternal mRNA is a major regulatory mechanism for gene expression during Xenopus oogenesis, through oocyte maturation to the egg and during the first cleavage divisions of early embryogenesis (Standart, 1992; Wickens, 1992; Wormington, 1993). Two principal mechanisms have been proposed to contribute to translational control during early Xenopus development. Some maternal mRNAs that are translationally incompetent in the oocyte (masked maternal mRNA) have short poly(A) tails that are lengthened during oocyte maturation concomitant with transcriptional activation (unmasking) (McGrew et al., 1989; Sheets et al., 1995). Conversely a second class of mRNAs is polyadenylated and translated through oogenesis, but is deadenylated and translationally silenced at maturation (Fox and Wickens, 1990; Varnum and Wormington, 1990). Thus regulated polyadenylation contributes to translational control (Jackson and Standart, 1990; Richter, 1991; Bachvarova, 1992). A second mechanism involves the regulated assembly and disassembly of mRNA in ribonucleoprotein particles (mRNP) (Standart and Jackson, 1994; Wormington, 1994). All maternal mRNAs examined are assembled into translationally repressed mRNPs; however, specific mRNAs are recruited from the mRNP fraction to ribosomes for translation (Tafuri and Wolffe, 1993). The assembly of translationally repressed mRNPs depends on transcription and association of the mRNA with Y-box proteins such as Xenopus FRGY2 (Bouvet and Wolffe, 1994). The relative roles of polyadenylation and mRNP assembly in the control of translation have not yet been determined.

The class of maternal mRNAs possessing short poly(A) tracts that are translationally masked in the oocyte, require two distinct 3’-untranslated regions (3’-UTR) signals to direct polyadenylation and unmasking after oocyte maturation. These are a ubiquitous nuclear polyadenylation signal (AU AA) and a cytoplasmic polyadenylation element (UpAU) (Fox et al., 1989; McGrew et al., 1989). Proteins that recognize the cytoplasmic polyadenylation element and that are required for regulated polyadenylation have been defined (Hake and Richter, 1994; Gebauer and Richter, 1995; Ballantyne et al., 1995).

The assembly of maternal mRNA into masked mRNPs involves association of mRNA with members of a ubiquitous family of Y-box proteins (Wolffe et al., 1992; Wolffe, 1994) and repression of translation (Richter and Smith, 1984; Ranjan et al., 1993; Bouvet and Wolffe, 1994; Evdokimova et al., 1995). The stability of Xenopus FRGY2 binding to mRNA depends on phosphorylation (Murray et al., 1991) as does the efficiency with which FRGY2 inhibits translation (Kick et al., 1987). It is clear that the nuclear history of mRNA influences translational fate in the Xenopus oocyte (Wolffe and Merci, 1996). Microinjection of mRNA into the oocyte nucleus has been suggested to lead to a block in translation (Braddock et al., 1989, 1990). Covalent modification of mRNA (Braddock et al., 1991) or the excision of an intron (Braddock et al., 1994) might allow the mRNA injected into the nucleus to escape translational masking. Recent work has implicated specific promoter elements in directing the translational repression of mRNA (Gunkel et al., 1995). Consistent with the importance of nuclear history, Bouvet and Wolffe (1994) found that transcription of histone H1 mRNA in the oocyte nucleus was required for translational repression. However in contrast to earlier work (Braddock et al., 1989, 1990, 1991, 1994) microinjection of H1 mRNA into the nucleus did not lead to translational repression (Bouvet and Wolffe, 1994). Nevertheless a consensus exists that the FRGY2 protein is important in establishing the transcriptionally masked state (Bouvet and Wolffe, 1994; Braddock et al., 1994).

In this work we directly examine the relative roles of transcription-coupled translational masking and regulated polyadenylation in translational control. We also directly examine the influence of the splicing process on translational fate.

MATERIALS AND METHODS

Plasmid Constructions—The CMVCAT, pSPCAT and pH 1.10 and pH 1.11 constructs have been described elsewhere (Almouzni and Wolffe, 1995; Bouvet and Wolffe, 1994). The first intron of the Xenopus intestinal fatty acid-binding protein (IFABP) gene was amplified by

¶ To whom correspondence should be addressed. Tel.: 301-402-2722; Fax: 301-402-1323; E-mail: awlme@helix.nih.gov.
PCR from plasmid ID4g2X using the following primers: 5'-GGGCGCATGCTTCCACGACATCGTCTGCTTCCACTACGTATAAGGGTTG-3' (Shi and Hayes, 1994). This fragment was subcloned into the NcoI site immediately downstream from the coding region of H1 in the pH 1.10, to give CMV.H1+INT. Sp6.H1+INT (H1 plus intron) was constructed by subcloning the XbaI-SacI fragment of CMV.H1+INT into pSP64pa (Promega). To ensure efficient polyadenylation and appropriate cleavage (Wickens and Gurdon, 1983; Conaway and Wickens, 1985), the 220 nucleotide simian virus 40 late pre-mRNA polyadenylation sequence was amplified using the primers 5' SVLATE (5'-GGGGTCTAGAGTCGACCAGCTGGATCCAGACATGATAAGATA-3') and 3' SVLATE (5'-GGGGGGAATTCATTTGTAGAGGTTTTACTT-3') and subcloned into the XbaI and EcoRI fragment from CMVCAT to give CMV.SVLATE. CMV.luciferase was generated by subcloning the SacI/StuI fragment of the luciferase MCS construct (Wormington et al., 1996) into the SacI site of CMV.SVLATE. The 3'UTR of UBP3 was amplified from the LUCUBP3 construct (Wormington et al., 1996) and subcloned into BglII site of CMV.luciferase, to give CMV.lucUBP3. All constructions were confirmed by sequencing.

In Vitro Transcription—In vitro transcription reactions were performed using Sp6 and T7 polymerase, as described by Krieg and Melton (1984). Templates were digested down to their 3' end and subcloned into the XbaI and EcoRI sites of CMVCAT to give polyadenylated RNA. RNA synthesis was carried out in the presence of a 10:1 cap analog to rGTP ratio, to obtain 5'-capped transcripts (Bovet and Wolfe, 1994). Transcribed RNA was phenol/chloroform-extracted and ethanol-precipitated.

Xenopus Oocyte Microinjection—Xenopus oocytes were prepared as described previously (Drouin and Worch 1993). Stage V oocytes were sorted and used within 24 h of oocyte preparation. Oocytes were maintained at 18°C in modified Barth’s saline (Gurdon, 1977). To ensure accuracy in the nuclear versus cytoplasmic injection experiments, the injection volume was kept to 9.2 nl, and cytoplasmic injections were performed into the vegetal pole (Wormington, 1989). After injection, only oocytes with homogenous pigmentation and normal turgescence were collected. Collected oocytes were homogenized in 0.25 m Tris-HCl (pH 7.5) (10 μl/oocyte) and aliquoted for protein and RNA analysis. A minimum of 30–40 oocytes were injected per item of data. All results were reproduced in at least three separate experiments.

In vitro oocyte maturation was carried out in oocyte culture medium (Heasman et al., 1991) using 1 μg/ml of progesterone (Sigma). Oocyte maturation was determined by the appearance of a white spot at the animal pole indicative of germinal vesicle breakdown.

RNA Analysis—RNA was extracted from homogenate by the RNAzol method (TM Cinna Scientific). RNA was analyzed by primer extension as described by Toyoda and Wolfe (1992). Primer extension was performed using the primers 5'-CTAAAAAGGCCAAAGGCTGCTA-3' for H1 5'UTR and 5'-GGGGGAATTCATTTGTAGAGGTTTTACTT-3' for CAT RNA and 5'-GGTGGTATATCCAGTGATTTTTTTCTCCAT-3' for luciferase RNA. Capable of Polyadenylation-dependent Translational Activation—In earlier work we have described the translational masking of histone H1 mRNA by quantitating protein synthesis per mass of mRNA (Bovet and Wolfe, 1994). Histone H1 mRNA synthesized in vivo from a plasmid DNA molecule injected into the oocyte nucleus was translated 50–100-fold less efficiently than H1 mRNA microinjected into the nucleus (Bovet and Wolfe, 1994). We first wished to determine whether this result also represented the translational fate of other mRNAs in the Xenopus oocyte.

We find that chloramphenicol acetyltransferase (CAT) mRNA synthesized in vitro and microinjected into the oocyte cytoplasm (Fig. 1, A, lanes 2 and 3) is efficiently translated (Fig. 1B, lanes 2 and 3, and C). In contrast, CAT mRNA synthesized in vivo (Fig. 1, A, lanes 4 and 5) from a plasmid DNA molecule (CMVCAT) in which the cytomegalovirus promoter directs transcription is very inefficiently translated (Fig. 1, B, lanes 4 and 5, and C). We next examined the translational activities of polyadenylated reporter mRNAs containing luciferase coding sequences that either lacked a 3'-UTR (lucA) or were fused to 3'-UTR sequences containing a cytoplasmic polyadenylation element derived from UBP3 mRNA (Wormington et al., 1996). Alternatively, luciferase mRNA with or without the UBP3 3'-UTR was cloned into the oocyte cytoplasm (Fig. 2, A and B, RNA lanes). Quantitation of translational efficiency through assays for luciferase...
activity reveals a 10–50-fold reduction concomitant with mRNA synthesis in vivo compared with the microinjection of mRNA into the cytoplasm, suggesting masking is more efficient when coupled to transcription (Table I). We conclude that like H1 mRNA (Bouvet and Wolffe, 1994), a prokaryotic mRNA encoding luciferase are translationally repressed when transcribed polyadenylated luciferase mRNA with or without the UBP3 3′-UTR or with 5 ng of luciferase DNA with or without the UBP3 3′-UTR. After a 6-h incubation, oocytes were transferred into oocyte culture medium with or without 1 μg/ml of progesterone. Progesterone-treated, matured oocytes (+) and untreated (−) oocytes were collected after another 12 h and analyzed. A, primer extension analysis of oocytes injected with luciferase pA mRNA (lanes 1 and 2), luciferase pA DNA (lanes 3 and 4), luciferase UBP3 mRNA (lanes 5 and 6), luciferase UBP3 DNA (lanes 7 and 8). B, Western analysis of oocytes corresponding to the primer extension (A) was performed using anti-luciferase antibody. Noninjected oocytes were used as control (lane C). Luciferase protein is indicated with an arrow. The two lower molecular weight proteins seen in all lanes, including lane C, represent nonspecific proteins, these are indicated by the vertical bars labeled ns.

**TABLE I**

**Quantification of translational efficiency**

| Progesterone | Luciferase | Luc/mRNA: relative luciferase activity |
|--------------|------------|--------------------------------------|
| lucpA RNA    | 891,843    | 100.0                                |
| lucpA RNA    | 511,945    | 50.8                                 |
| lucpA DNA    | 2973       | 0.6                                  |
| lucpA DNA    | 3875       | 2.0                                  |
| lucUBP3 RNA  | 10,350     | 4.8                                  |
| lucUBP3 RNA  | 99,628     | 34.2                                 |
| lucUBP3 DNA  | 1409       | 0.3                                  |
| lucUBP3 DNA  | 9493       | 2.8                                  |

In contrast the lucUBP3 construct does contain the cytoplasmic polyadenylation element in the 3′-UTR, and on oocyte maturation the translational efficiency of lucUBP3 mRNA increases about 7-fold (Fig. 2B, lucUBP3, lanes 6 and 7, Table I). We next examined whether oocyte maturation would also activate translation of lucUBP3 mRNA synthesized in vivo. The translational efficiency of lucUBP3 mRNA synthesized in vivo is 15-fold less than mRNA injected directly into oocyte cytoplasm (Fig. 2B, lucUBP3, compare lanes 6 and 7 with lanes 8 and 9, Table I). However oocyte maturation up-regulates the translational efficiency of the mRNA synthesized in vivo. The translational efficiency of lucUBP3 mRNA synthesized in vivo increases 10-fold on maturation (Fig. 2B, lucUBP3 DNA, compare lanes 8 and 9, Table I). We assayed the increase in poly(A) tail length on the lucUBP3 mRNA synthesized in vivo that occurred on oocyte maturation. The average poly(A) tail length increased in length on maturation such that some poly(A) tails were as long as 180 nucleotides (Fig. 3). Thus the range of translational regulation remains unchanged whether lucUBP3 mRNA is synthesized in vivo or microinjected into oocyte cytoplasm (Fig. 4). However the silencing of translation prior to oocyte maturation is much more
efficient if the mRNA is synthesized in vivo.

Nuclear Injection and Splicing of mRNA Does Not Influence Translational Fate—Braddock et al. (1989, 1990, 1991, 1994) observed that microinjection of polyadenylated mRNA into the oocyte nucleus resulted in translational silencing. Bouvet and Wolffe (1994) found that polyadenylated mRNA microinjected into the oocyte nucleus was translationally competent. Braddock et al. (1994) suggested that the entry of a transcript into the splicing pathway allowed the mRNA to evade the masking process. We examined these issues using histone H1 mRNA that did or did not contain an intron from the intestinal fatty acid binding protein (Shi and Hayes, 1994). This mRNA was microinjected either into the oocyte nucleus or microinjected into the oocyte cytoplasm (Fig. 5). As a control we mixed the H1 mRNA with a CMV CAT construct that would be transcribed in the nucleus, but not the cytoplasm. We find that the mRNA injected into the nucleus is translated with comparable efficiency to that injected into the cytoplasm (Fig. 5, B and C).

We next examined the translational efficiency of mRNA transcribed in vivo or microinjected into the oocyte nucleus, with that of mRNA injected into the oocyte cytoplasm (Fig. 6).
As a control we mixed the H1 mRNA with the plasmid CMV CAT to confirm nuclear injection. We find that the translational efficiency of H1 mRNA injected into the oocyte nucleus or cytoplasm is equivalent (Fig. 6), whereas that of H1 mRNA synthesized in vivo is reduced. We then asked whether mRNA synthesized in vivo that contained an intron would be translated differently from mRNAs synthesized in vivo that lacked an intron (Fig. 7). We find that entry of a transcript into the splicing pathway does not influence translational fate (Fig. 7C). The synthesis of mRNA in vivo leads to translational repression independent of whether the mRNA has an intron excised or not. Control experiments indicated that the intron used in all of these experiments was excised in the majority of transcripts (Fig. 8).

**Fig. 6.** Nuclear versus cytoplasmic injection of in vitro transcribed mRNA does not alter its translational efficiency. Oocytes were injected with H1 mRNA either into the oocyte nucleus (N) or into the cytoplasm (C). For comparison, oocytes were injected into the nucleus with plasmid DNA encoding for H1 mRNA. All three groups were co-injected with CAT DNA as an injection control. After 16 h of incubation, oocytes were injected with [3H]arginine and [3H]lysine and collected after another 6 h. A, primer extension analysis was performed with H1 and CAT primers. Lanes 1 and 2 correspond to oocytes injected with 3 ng of H1 mRNA and 1 ng of CAT DNA into the nucleus or cytoplasm, respectively. Lane 3 corresponds to oocytes that have been co-injected with 3 ng of H1 DNA and 1 ng of CAT DNA. The primer extension products for the in vitro transcribed and injected H1 mRNA, and the H1 and CAT transcripts synthesized in the nucleus from microinjected DNA, are indicated with arrows. B, labeled proteins that were synthesized in oocytes injected with amino acids only (lane 2), injected with H1 mRNA and CAT DNA into the nucleus or cytoplasm (lanes 3 and 4, respectively), and in oocytes injected into the nucleus with H1 and CAT DNA (lane 5), were acid-purified as described under “Materials and Methods” and resolved on a 12% Tris-glycine gel. H1 protein is indicated with an arrow. C, Translational Efficiency (protein/mRNA, in arbitrary units) was calculated based on quantification of the autoradiograms in A and B with a Molecular Dynamics densitometer.

As a control we mixed the H1 mRNA with the plasmid CMV CAT to confirm nuclear injection. We find that the translational efficiency of H1 mRNA injected into the oocyte nucleus or cytoplasm is equivalent (Fig. 6C), whereas that of H1 mRNA synthesized in vivo is reduced. We then asked whether mRNA synthesized in vivo that contained an intron would be translated differently from mRNA synthesized in vivo that lacked an intron (Fig. 7). We find that entry of a transcript into the splicing pathway does not influence translational fate (Fig. 7C). The synthesis of mRNA in vivo leads to translational repression independent of whether the mRNA has an intron excised or not. Control experiments indicated that the intron used in all of these experiments was excised in the majority of transcripts (Fig. 8).
Our final experiment examined whether it was possible to titrate out the masking phenomenon that is coupled to transcription in _Xenopus_ oocytes (Fig. 9). We find that transcription as assayed by mRNA accumulation reaches maximum achievable levels without titrating the masking process. By comparison to the levels of injected CAT mRNA the oocyte has the capacity to mask at least 10 ng of mRNA synthesized _in vivo_ (Fig. 9A, compare lanes 2 and 4). Note that mRNA levels fall with increasing DNA, since high concentrations of DNA micro-injected into an oocyte nucleus exert a general inhibitory effect on the transcription process.

**DISCUSSION**

The major conclusion from this work is that the regulated polyadenylation process that occurs on oocyte maturation (Fox _et al._, 1989; McGrew _et al._, 1989) has the capacity to translationally activate mRNA that is masked through synthesis _in vivo_ (Figs. 1–4). Our results confirm the generality of the repression of translation coupled to mRNA synthesis in the oocyte nucleus (Bouvet and Wolffe, 1994). The main consequence of this repression is that mRNA is very efficiently masked prior to oocyte maturation (Fig. 2, Table I). However this masked mRNA remains accessible to the cytoplasmic polyadenylation components (Fig. 3), and the range of translational regulation achieved on this mRNA synthesized _in vivo_ is the same as that on mRNA injected into the cytoplasm (Fig. 4).

The advantages of repressing translation more effectively when mRNA is synthesized _in vivo_ are that precocious translation is avoided and that a wider range of gene regulation might be achieved. In this respect the transcription-coupled repression of translation (Bouvet and Wolffe, 1994) resembles the replication-coupled repression of basal transcription (Almouzni and Wolffe, 1993). In the latter case the packaging of nascent DNA with histones prevents the basal transcriptional machinery gaining access to promoters. In the phenomenon of transcription-coupled repression of translation, we have proposed that the packaging of mRNA with the Y-box proteins represents a default state that excludes the ribosomes from gaining access to RNA regulatory elements (Bouvet and Wolffe, 1994). However just as transcriptional activators can disrupt chromatin to allow the access of the transcriptional machinery to genes (Wolffe, 1995), so can regulated polyadenylation direct the recruitment of masked mRNA to the translational machinery (Figs. 2–4). We suggest that just as the repression of basal transcription by the histone proteins is an important contributory factor to transcriptional regulation (Han and Grunstein, 1988), so might the repression of translation by the Y-box proteins contribute to translational control.

Although changes in polyadenylation on oocyte maturation dependent on the cytoplasmic polyadenylation element (Wormington _et al._, 1996) can allow mRNAs to escape translational repression, the entry of a nuclear mRNA into the splicing pathway does not allow the unmasking of mRNA (Figs. 5–8). Our results are in contrast to those of Braddock _et al._ (1989, 1990, 1991, 1994). We have not been able to reconstitute the translational masking apparent in their experiments following the injection of mRNA into the oocyte nucleus (Figs. 5 and 6). Braddock _et al._ (1989, 1990, 1991, 1994) made use of a CAT mRNA fused to about 82 nucleotides of the human immunodeficiency virus long terminal repeat. Thus the mRNA used in our experiments differ. However a common theme in our results is the role of FRGY2 in translational repression (Braddock _et al._, 1994; Bouvet and Wolffe, 1994). Since Y-box proteins have the capacity to recognize mRNA with sequence selectivity (Bouvet _et al._, 1995), it is possible that the association of FRGY2 with the human immunodeficiency virus long terminal repeat mRNA might be favored. A preferred association with FRGY2 might also help explain how 30 ng of polyadenylated mRNA might be translationally repressed following nuclear injection (Braddock _et al._, 1994), when the oocyte normally only contains at most 7 ng of mRNA (Davidson, 1986). An alternate hypothesis to explain the discrepancy in data is to suggest that mechanisms exist to direct the dominant activation of histone H1 translation independent of the presence of an intron. This would appear unlikely since histone H1 mRNA is normally masked in the _Xenopus_ oocyte (Woodland _et al._, 1979; Tafuri and Wolffe, 1993).

Our demonstration of the translational regulation of mRNA
synthesized on exogenous plasmid DNA templates in response to oocyte maturation provides an opportunity to dissect the molecular mechanisms that maintain translational repression and that mediate the unmasking of mRNA. The capacity to synthesize as much as 10 ng of a particular masked mRNA and that mediate the unmasking of mRNA. The capacity to molecular mechanisms that maintain translational repression to oocyte maturation provides an opportunity to dissect the synthesized on exogenous plasmid DNA templates in response to oocytes will facilitate the further biochemical and structural characterization of the processes involved.

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