Differential post-transcriptional regulations of Wnt mRNAs upon axolotl meiotic maturation

SABINE VAUR1,2, NICOLE MONTREAU1,2, FRANÇOIS DAUTRY1 AND YANNICK ANDÉOL*,1,2

1Laboratoire des régulations post-transcriptionnelles, Institut André Lwoff, CNRS, UPR 1983, Villejuif, France and
2Laboratoire de Biologie du Développement, CNRS, UMR 7622, Université P. et M. Curie, Paris, France

ABSTRACT The products of the Wnt gene family play an essential role in several aspects of embryo patterning. We have investigated the post-transcriptional regulation of three of these genes: Awnt-1, Awnt-5A and Awnt-5B during axolotl (Ambystoma mexicanum) oogenesis, oocyte maturation and early development. We show that Awnt-1, Awnt-5A and Awnt-5B mRNAs are maternally expressed. The three transcripts are tightly regulated at specific times and display differential mRNA stability, poly(A) tail length and localization. In contrast to Awnt-5B which is restricted to the animal hemisphere, Awnt-1 and Awnt-5A have no particular localization in stage VI oocytes. Interestingly, these two mRNAs exhibit a polyadenylation gradient along the animal-vegetal axis. Moreover, after meiotic maturation, Awnt-1 and 5A mRNAs become exclusively localized to the animal pole. This is the first evidence of a complete mRNA re-localization to the animal hemisphere during oocyte maturation.

KEY WORDS: Wnt genes, meiotic maturation, mRNA degradation, polyadenylation, mRNA localization

Introduction

Post-transcriptional control of maternal mRNAs is a crucial aspect of gene regulation during early development. In amphibian, these maternal transcripts are synthesized and stockpiled in the growing oocyte for several months (Vassali and Stutz, 1995). At the end of oogenesis, transcription is silenced and mRNA synthesis from zygotic genome only starts at the midblastula transition (MBT) (Signoret et al., 1989). Thus, during the period encompassing the oocyte maturation and the first cleavages, the developing embryo is controlled and patterned by the maternally inherited mRNAs (Davidson, 1986). Expression of maternal mRNAs can be modulated at multiple steps. Changes in the stability of specific mRNAs lead to the removal of transcripts for subsequent embryonic development (Brown and Harland, 1990). Alternatively, protein synthesis can be regulated by acting on poly(A) tail length (Bachvarova, 1992), since deadenylation accompanies mRNA release from polysomes and cytoplasmic polyadenylation triggers translational activation. Finally, mRNA expression can also be spatially controlled by mechanisms leading to restricted mRNA localization (Bashirullah et al., 1998).

After fertilization, the rapid cleavages of the egg give rise to a multi-cellular blastula in which cell-cell communications are crucial for embryo patterning. Wnt genes encode a large family of cysteine-rich proteins which are essential intercellular signaling molecules during embryonic development (Nüsslein-Volhard and Wieschaus, 1980; McMahon and Moon, 1989; Wodarz and Nusse, 1998). However, little is known about the function and regulation of maternally expressed Wnt genes. In Xenopus, Xwnt-11 is the unique example of a maternal Wnt gene whose post-transcriptional regulation has been studied in detail. Xwnt-11 transcripts are tightly controlled by mechanisms leading to a restricted dorsal mRNA localization and a spatially regulated polyadenylation (Shroeder et al., 1999). These two regulatory events take place during oogenesis and fertilization, respectively and restrict Xwnt-11 translation to the future dorsal side of the embryo.

We were interested in investigating the maternal origin and the post-transcriptional regulation of Wnt mRNAs during axolotl (Ambystoma mexicanum) early development. Compared to Xenopus, axolotl provides an interesting alternative model since its long transcriptional quiescence can facilitate the study of maternal mRNA post-transcriptional regulation (Signoret and Lefresne, 1971; Andéol et al., 1998). Previous studies have characterized three Wnt genes in axolotl: Awnt-1, Awnt-5A and Awnt-5B (Busse and Séguin, 1992; Busse and Séguin, 1993). The AWNT-5 proteins share 83% amino acid identity but only 42% with AWNT-1. Moreover, Awnt mRNAs have quite divergent non-coding sequences suggesting they display different post-transcriptional regulations. Their temporal and spatial expression have been analyzed from MBT to hatched larvae. The

Abbreviations used in this paper: MBT, mid blastula transition; PAT, poly(A) test; UFE, unfertilized egg.
three Awnt mRNAs are zygotically expressed from the neurula until late embryogenesis. Much earlier, between MBT and early gastrula stages, Awnt-5B mRNA is not detected whereas Awnt-1 and Awnt-5A are expressed and located in ventral region and dorso-ventral vegetal region, respectively. To analyze whether these Wnt mRNAs were already localized in the blastula during early cleavages and before fertilization, we investigated their temporal and spatial expression during early development before MBT and much earlier during oogenesis and meiotic maturation. 

In the present study, we show that Awnt-1, Awnt-5A and Awnt-5B mRNAs are maternally expressed with similar patterns of accumulation during oogenesis. In stage VI oocytes, while Awnt-5B mRNA is localized to the animal hemisphere, Awnt-1 and Awnt-5A are uniformly distributed and differentially polyadenylated along the animal-vegetal axis. Upon meiotic maturation Awnt transcripts acquire different stability, poly(A) tail length and localization. Importantly, Awnt-1 and 5A mRNAs exhibit a novel pattern of localization and become exclusively recovered at the animal hemisphere. This re-localization is also observed after progesterone-induced maturation. 

Results 

Awnt-1, Awnt-5A and Awnt-5B mRNAs are Differentially Regulated during Oocyte Maturation and after Fertilization 

The temporal expression of the three Awnt mRNAs was analyzed from early to late axolotl oogenesis and during oocyte maturation (Fig. 1). The three WntmRNAs were detected throughout oogenesis. In Northern blot, where equal RNA amounts were loaded, Awnt signals decreased progressively. When results were expressed per oocyte, as in the histogram, they indicated progressive accumulation of these maternal transcripts from stages II to V and stabilization between stages V to VI. Awnt-1 and Awnt-5A transcript levels remained stable between stage VI oocytes and unfertilized eggs (UFE) whereas Awnt-5B was partially degraded (60%) in UFE. 

These results showed that the Awnt transcripts were of low abundance, especially for Awnt-5B mRNA at the end of oogenesis. For this reason, we performed semi-quantitative RT-PCR analyses. The number of PCR cycles needed for quantification was determined for each Awnt mRNA. The intensity of the three Awnt signals increased exponentially up to 20 cycles of PCR and reached a plateau, as previously described (Busse and Séguin, 1992). Accordingly, 15 cycles of PCR amplification were chosen for the semi-quantitative analysis. This approach was used to further analyze the differential behaviors of Awnt mRNAs before and after meiotic maturation. 

Figure 2A shows that Awnt-1 and Awnt-5A mRNAs were stable during oocyte maturation whereas Awnt-5B was degraded. Awnt-5B signals were quantified and normalized according to AxDazl mRNA expression (Johnson et al., 2001). As shown in the histogram, only 30% of the Awnt-5B transcripts, present in stage VI, were still detected after meiotic maturation. Similar variations were obtained when Awnt-5B signals were normalized according to Awnt-1 or Awnt-5A expression. These semi-quantitative RT-PCR confirmed Awnt-1/ Awnt-5A stability and Awnt-5B specific degradation during oocyte maturation, as observed by Northern analysis. 

Since oocyte maturation can be reproduced in vitro by progesterone treatment, the stage-specific degradation of Awnt-5B was then analyzed in progesterone-matured oocytes (UFE Pg) and compared to in vivo matured oocytes (UFE). Awnt-1 and Awnt-5B were co-amplified in stage VI oocytes, UFE Pg and UFE (Fig. 2B). No degradation was observed for Awnt-1 mRNA. A significant decrease of Awnt-5B level was observed in UFE Pg as described above, but surprisingly this stage-specific degradation was not reproduced by in vitro progesterone stimulation (UFE Pg). As shown in the histogram, a 80% degradation of Awnt-5B mRNA occurred in UFE whereas no decrease was detected in UFE Pg. Thus, in vivo oocyte maturation results in differential effects on Awnt mRNA levels and targets Awnt-5B for specific degradation. However, progesterone treatment is insufficient to trigger this decay although germinal vesicle breakdown (GVBD) is induced. 

To investigate the behavior of the three Awnt mRNAs during early development, total RNA was extracted from undivided fertilized eggs, 2-cell, 1024-cell (MBT) and 10000-cell embryos (late blastula). The level of WntmRNAs was then determined by RT-PCR using co-amplification of the 18S rRNA as an internal standard (Fig. 2C). This
Post-Transcriptional Regulation of Wnt mRNAs

strategy required the determination of an appropriate ratio of 18S primers and competimers to modulate 18S rRNA amplification (see Materials & Methods) and to obtain comparable 18S and Awnt signals. The three Awnt mRNAs were detected from the time of fertilization up to 30 h (10000-cell embryo). Awnt-1 and Awnt-5A mRNAs were stable until MBT. In contrast, Awnt-5B mRNA level continued to decrease after fertilization and throughout cleavage stages. In early gastrula, Awnt-1 and 5A transcripts were no longer present and only a residual signal persisted for Awnt-5B mRNA (Fig. 2D). In neurula and tailbud stages, the three Awnt mRNA were detected as zygotic transcripts. The zygotic expression of Awnt-1 and 5A mRNAs was weaker than that of Awnt-5B as previously described (Busse and Séguin, 1993).

Our results show that Awnt-1, Awnt-5A and Awnt-5B mRNAs are maternally expressed and that their levels are differentially modulated during meiotic maturation. Awnt-1 and Awnt-5A mRNAs remained stable whereas Awnt-5B decay was initiated during this period and continued after fertilization until gastrulation. Progesterone-induced maturation did not lead to the expected Awnt-5B mRNA decay suggesting that hormonal stimulation incompletely mimics the in vivo process.

The Poly(A) Tail is Differentially Modulated upon Meiotic Maturation

We characterized the poly(A) tail of these Awnt mRNAs. First, total mRNAs were separated into poly(A)+ and poly(A)− fractions and analyzed by Northern blot (Fig. 3A). The three Awnt mRNAs were detected in the poly(A)+ fractions of oocytes and UFE. A single transcript was detected for Awnt-1 and Awnt-5B mRNAs and two Awnt-5A transcripts were observed before and after oocyte maturation. The existence of several isoforms has been already observed for other Wnt genes (Shroeder et al., 1999).

The variation of Awnt poly(A) tail length was investigated by using a polyaadenylation test (PAT) (Fig. 3B). Awnt-1 poly(A) tail extended to approximately 450 adenosines (A) in stage III oocytes, were reduced to 350 A in stage IV and finally measured 250 A in stages V and VI. A similar deadenylation profile was observed for Awnt-5A poly(A) tail which comprised 400 A in stage III oocytes and measured 150 A in the post-vitellogenic V and VI oocytes. After oocyte maturation (UFE), Awnt-1 and Awnt-5A mRNAs were further deadenylated with poly(A) tails reduced to 10-50 A. PAT analysis indicated that the poly(A) tail of Awnt-5B mRNA extended to approximatively 500 A in stage III oocytes. It was significantly longer than in stages IV, V and VI where poly(A) tails were respectively 350 and 250 A long. Poly (A) tail length was also estimated to 250 A in UFE, suggesting that no variation occurred during meiotic maturation. Thus, in contrast to the further deadenylation of Awnt-1 and Awnt-5A, the poly(A) tail of Awnt-5B mRNA was unchanged during maturation process.

Asymmetric Distribution of Awnt mRNAs in the Animal Hemisphere after Oocyte Maturation

The spatial distribution of Awnt mRNAs was determined in stage VI oocytes and in UFE. These cells were cut in two parts correspond-
ing to animal and vegetal halves, RNA was extracted from each moiety and analyzed by RT-PCR (Fig. 4A). *AxDazl* was used as control and detected in animal as well as in vegetal hemispheres from stage VI oocytes and UFE (Johnson *et al.*, 2001).

In stage VI oocytes, *Awnt*-1 and *Awnt*-5A mRNAs were both found in the two hemispheres, suggesting that they were distributed along the animal-vegetal axis. In contrast, *Awnt*-5B mRNAs were only concentrated in the animal half. For this transcript, no signal was detected in the vegetal half even after 30 PCR cycles, indicating that it was clearly restricted to the animal hemisphere. RT-PCR was also performed on naturally matured oocytes (UFE). Since *Awnt*-5B mRNA was partially degraded at this stage, 30 PCR cycles were used to allow a better detection in each fraction. Whereas *Awnt*-5B mRNA conserved its restricted localization in the animal pole, *Awnt*-1 and 5A transcript distribution was modified compared to stage VI oocytes. These two mRNAs were exclusively detected in the animal hemisphere of UFE. This implies that the change in *Awnt*-1 and 5A mRNA distribution occurred during the meiotic process.

To further attest this time specific re-distribution, *Awnt*-1 and 5A mRNA localization was analyzed after progesterone stimulation (Fig. 4B). RT-PCR experiments indicated that *Awnt*-1 and 5A transcripts were only detected at the animal hemisphere in UFE, as previously observed in UFE. Similar results were obtained for *Awnt*-5B mRNA (data not shown). Thus, *Awnt* mRNAs exhibited identical localization during *in vivo* and *in vitro* oocyte maturation, suggesting that progesterone is sufficient to induce localization changes of *Awnt*-1 and 5A transcripts. To confirm the animal pole targeting of *Awnt*-1 and 5A induced by progesterone treatment (UFE Pg) we used whole mount *in situ* hybridization (Fig. 4B). A strong signal was observed over the animal pole with *Awnt*-1 and *Awnt*-5A antisense probes. No specific signal was detected in control with digoxigenin sense probe. Similar *in situ* experiments were performed in stage VI oocytes but staining could not be distinguished from background (data not shown). A diffuse localization of *Awnt* mRNAs leading to low signal intensity could account for this result.

In summary, *Awnt*-5B mRNA was localized to the animal pole of stage VI oocytes and conserved its localization in UFE. In contrast, *Awnt*-1 and *Awnt*-5A, distributed throughout the oocytes, were recovered only at the animal pole in UFE. Moreover, *Awnt*-1 and 5A localization changes during *in vivo* maturation can be reproduced by hormonal stimulation. This asymmetric distribution in UFE and UFE Pg could be either the consequence of a migration from vegetal to animal hemisphere or the result of a selective degradation in the vegetal pole.

**Awnt-1 and Awnt-5A Polyaadenylation is Spatially Regulated along the Animal-Vegetal Axis**

In stage VI oocytes, by contrast to *Awnt*-5B, *Awnt*-1 and *Awnt*-5A mRNAs were found to be uniformly expressed along the animal-vegetal axis. To investigate whether the equally distributed maternal *Awnt* mRNAs were differentially adenylated along this axis, PAT was performed on animal and vegetal halves (Fig. 5). In the animal pole, *Awnt*-1 mRNAs

---

**Fig. 3** *Awnt* poly(A) tail length variation during oogenesis and meiotic maturation. (A) Total RNA was extracted from a pool of stages III to VI oocytes and from unfertilized eggs (UFE) and separated in poly(A)− and poly(A)+ samples. 10 µg of poly(A)− and 1 µg of poly(A)+ were analyzed by Northern blot with *Awnt*-1, *Awnt*-5A and *Awnt*-5B probes. 18S: axolotl rRNA used to control the purity of the poly(A)− and poly(A)+ fractions. (B) Total RNA extracted from stages III to VI oocytes and UFEs was reverse-transcribed with an oligo (dT)-anchor primer followed by PCR with the anchor primer and a specific *Awnt*-1, *Awnt*-5A and *Awnt*-5B primer to estimate poly(A) tail length. Samples were separated by gel electrophoresis, transferred to Nylon Hybond N+ membrane and hybridized with specific 32P labeled cDNA probes. PAT results were analyzed as described in Materials & Methods. Sizes of adenylated products were evaluated using a DNA 1 kb ladder. Histograms represent poly(A) tail length variations observed on the Southern blot. A, Adenosine.
Post-Transcriptional Regulation of Wnt mRNAs

Fig. 4. Changes in Awnt transcripts localization during in vivo and in vitro oocyte maturation. (A) Stage VI oocytes and natural unfertilized eggs (UFE) were sectioned in two parts corresponding to animal (An) and vegetal (Ve) hemispheres. Total RNA from each moiety was extracted and 2 µg were analyzed by RT-PCR, with (+) or without (-) reverse transcriptase (RT), using primer pairs specific to Awnt-1, Awnt-5A, Awnt-5B and AxDazl sequences. Samples were separated by gel electrophoresis after 15 PCR cycles for stage VI oocytes and 30 PCR cycles for UFE, transferred to Nylon Hybond N+ membrane and hybridized with specific 32P labeled cDNA probes. (B) In vitro matured oocytes (UFE Pg) were sectioned into animal (An) and vegetal (Ve) halves. RNA extractions, RT-PCR, electrophoreses and hybridizations were performed as in A. In situ hybridizations were carried out with digoxigenin Awnt-1 and Awnt-5A antisense probes. Control was performed using digoxigenin Awnt-5A sense probe. A specific localization of Awnt-1 and Awnt-5A transcripts was detected at the animal pole of in vitro matured oocytes (UFE Pg). No signal was observed at the vegetal pole.

Fig. 5. Spatially regulated polyadenylation of Awnt-1 and Awnt-5A mRNAs

expressed, share a common pattern of accumulation during oogenesis but display different patterns of decay, polyadenylation and localization upon meiotic maturation. We provide evidence for the existence of a RNA localization process, which is activated during maturation and targets Awnt-1 and 5A mRNAs to the animal pole in UFE. In addition, this process has been reproduced in progesterone-matured oocytes. This in vitro maturation will be a useful system to further explore the molecular mechanisms involved in Awnt-1 and 5A mRNA localization pathways.

Temporal Expression Profile of Awnt-1 and Awnt-5A mRNAs

Awnt-1 and 5A mRNAs remain stable from oocyte maturation until MBT and completely disappear at gastrula stage (Fig. 2C). Numerous maternal mRNAs described in amphibian development behave similarly and are only degraded after MBT (Duval et al., 1990). This implies that factors necessary for their degradation are absent or inactive in the pre-MBT embryos and are only synthesized or activated after MBT. The presence of Awnt-1 and 5A displayed a long poly(A) tail of 250 A. In the opposite hemisphere, Awnt-1 poly(A) tail was significantly shorter and only measured 10-50 A. Awnt-5A mRNAs displayed the same differences between the two poles. Awnt-5A poly(A) tail extended to approximatively 150 A in animal pole. In the vegetal moiety, PAT signal was hardly detectable although Awnt-5A transcripts were equally distributed in each pole (Fig. 4A). This indicates that the vegetal Awnt-5A mRNAs either had very short poly(A) tails or no tail at all. Thus, our results demonstrate that Awnt-1 and Awnt-5A poly(A) tails are spatially regulated along the animal-vegetal axis of stage VI oocytes.

Discussion

We have investigated the expression of Awnt-1, Awnt-5A and Awnt-5B mRNAs during axolotl oogenesis, oocyte maturation and early development (Fig. 6). These three mRNAs are maternally
mRNAs raises the possibility that these genes could play a role during axolotl early development. In *Xenopus*, *Xwnt-1* has no maternal expression and is only activated during neurulation whereas *Xwnt-5A* is maternally expressed and detected during development (Noordermeer et al., 1989; Christian et al., 1991). This maternal *Xwnt-5A* mRNA acts as a modulator of morphogenetic movement (Moon et al., 1993). The function of *Awnt* maternal mRNAs remains to be elucidated. It has been shown that the secreted WNT protein family comprises two functional groups, the canonical Wnt and Wnt/Ca²⁺ pathway (Kühler et al., 2000). The Wnt/Ca²⁺ plays an essential role in mediating ventral signals in generating specialized Ca²⁺ signals in response to sperm entry. Such Ca²⁺ signals are crucial for egg activation and the initiation of embryonic development (Leclerc et al., 1999; Leclerc et al., 2000; Machaca and Haun, 2001). Axolotl Wnt genes may also be regulated by calcium dependent processes (Slusarski et al., 1997). During oocyte maturation, eggs acquire the ability to generate specialized Ca²⁺ signals in response to sperm entry. Such Ca²⁺ signals are crucial for egg activation and the initiation of embryonic development (Leclerc et al., 1999; Leclerc et al., 2000; Machaca and Haun, 2001).

**Awnt-5B mRNA Decay is Initiated at Oocyte Maturation**

One interesting feature of *Awnt-5B* mRNA expression profile concerns its partial degradation (60 to 70%) during meiotic maturation (Fig. 2B). Surprisingly, this stage specific decay is not reproduced in progesterone-matured oocytes (UFE Pg), suggesting that hormonal stimulation only partially mimics the in vivo process. Differences between naturally and hormonally progesterone-matured oocytes have been previously reported (Gautier and Tencer, 1986; Charbonneau et al., 1990). Thus, after in vivo maturation, only a small part of the *Awnt-5B* maternal stockpile persists in unfertilized eggs (UFE). Such time specific decay is particular to a small class of maternal mRNAs which includes *c-myc* in axolotl (Andéol et al., 1998), and α1 gap junction (Gimlich et al., 1990) or *Xfin* (Altaba et al., 1987) mRNAs in *Xenopus*. Studies on axolotl *c-myc* mRNA regulation have demonstrated the existence of a nuclear degradation factor released at GVBD (Andéol et al., 1995b). This raises the possibility that such a nuclear factor could also be responsible for *Awnt-5B* mRNA decay upon *in vivo* meiotic maturation. During this process, *Awnt-5B* mRNAs are partially degraded whereas they have a long poly(A) tail of 250 A. In contrast to *Awnt-1* and *5A* mRNAs, they do not undergo the default deadenylation pathway. After fertilization, this mRNA keeps its 250 adenosines but its level continues to decrease (unpublished results). These results suggest that *Awnt-5B* degradation is independent of its poly(A) tail length and therefore could involve a deadenylation-independent decay process (Brown and Harland, 1990; Beelman and Parker, 1995).

**Localization of Maternal Awnt mRNAs during Oocyte Maturation**

The distribution of the three *Awnt* mRNAs along the animal-vegetal axis was investigated before and after oocyte maturation (Fig. 4A). *Awnt-5B* transcripts are exclusively recovered in the animal moiety of stage VI oocytes and UFE. This mRNA is probably already localized before stage VI and conserves its restricted expression after meiotic maturation. Similar localization patterns have been reported for *Oct-60* in *Xenopus* (Mowry and Cote, 1999) and for *cycarin B* mRNA in zebrafish (Howley and Ho, 2000). By contrast, the localization of *Awnt-1* and *5A* mRNAs changes during oocyte maturation. In stage VI oocytes, these two mRNAs are distributed along the animal-vegetal axis while in UFE, they are no longer detected in the vegetal half and are exclusively present in the animal hemisphere. This asymmetric distribution is also reproduced *in vitro* in progesterone-matured oocytes (UFE Pg), indicating that hormonal treatment is sufficient to mimic the *in vivo* localization changes (Fig. 4B).

Polarized localization of maternal mRNAs, which is an early step in embryonic patterning, can occur during oogenesis (King et al., 1999). Processes leading to vegetal mRNA localization have been extensively studied and two distinct mechanisms have been characterized, an early and a late pathway (Bashirullah et al., 1998). The early pathway, which takes place during stages I-II of oogenesis, is independent of cytoskeletal network. The late pathway appears efficient between stages III-IV in oocytes and is microtubule-dependent. By contrast, very little is known about animal pole localization of mRNAs, as well as the timing and the mechanisms involved. Our results show the existence of a new mRNA localization process which triggers a complete *Awnt-1* and *5A* re-distribution to the animal pole during oocyte maturation. Such asymmetry could be explained by selective degradation occurring in the vegetal hemisphere or by transport from vegetal to animal pole. Since no variation in the *Awnt-1* and *5A* mRNA levels is observed during meiotic maturation, our results argue in favor of an active re-localization process. However, we cannot totally exclude the possibility of a selective degradation in the vegetal hemisphere, especially for *Awnt-5A* mRNA. Indeed, since *Awnt-5A* level is lower in vegetal than in animal half, it remains possible that its degradation in the vegetal hemisphere would not significantly influence the overall mRNA level in the whole egg.
Spatio-Temporal Regulation of Awnt-1 and Awnt-5A Poly(A) Tail Length

In stage VI oocytes, Awnt-1 and Awnt-5A mRNAs are uniformly distributed but display different polyadenylation states along the animal-vegetal axis. In the animal pole, Awnt-1 and Awnt-5A poly(A) tails extend to 250 and 150 A respectively, while they exhibit only few adenosine residues in the opposite pole (Fig. 5A). Such spatial regulation of poly(A) tail length along the animal-vegetal axis has never yet been described for maternal Wnt mRNAs. Spatially regulated polyadenylation of uniformly localized transcripts is known to occur during embryo axis formation. In Xenopus egg, the maternal Xwnt-11 mRNA is evenly distributed but differentially polyadenylated along the dorso-ventral axis (Shroeder et al., 1999). This asymmetric polyadenylation leads to a spatially regulated translation of Xwnt-11 protein on the dorsal side of the egg. Thus, these data strongly argue in favor of an asymmetric translation of Awnt-1 and AWnt-5A in the animal pole of stage VI oocytes. Moreover, after meiotic maturation, while Awnt-1 and Awnt-5A mRNAs are targeted to the animal pole, their poly(A) tails are reduced from 250/150 to 10-50 A (Fig. 5B).

Total RNA Extraction, Poly (A)+ Isolation and Northern Analysis

Total RNA was extracted from different oocyte stages. UFE and embryos by LiCl/Urea method (Aufray and Rougeon, 1980). Poly (A)+ RNAs were isolated from 1 mg of total RNA with the PolyATtract mRNA Isolation System (Promega). The flow through was kept as the poly(A)+ fraction. For Northern analysis (Sambrook et al., 1989), RNAs were electrophoresed in 1.1% formaldehyde agarose gels and transferred in 20X SSC to Nylon Hybond N+ membrane (Amersham). Prehybridization and hybridization were performed at 65°C in Church buffer (Church and Gilbert, 1984). Each blot was hybridized with specific 32P labeled cDNA, washed at 65°C in 0.2X SSC, 0.1% SDS. Signals were quantified with a Phospholmager and the ImageQuant software and normalized with axolotl 18S rRNA (Andéol et al., 1995a). cDNA probes were gel-purified fragments labeled with [α-32P] dCTP by random priming.

RT-PCR

RNA samples were treated by RQ1 DNase (Promega) for 20 min at 37°C, purified by phenol/chloroform extraction, ethanol precipitated and dissolved in water. Each RT-PCR was done with the Access RT-PCR System (Promega). 2 µg of RNA were reverse transcribed at 48°C for 45 min. In expression profile experiments, 15 cycles of PCR were performed as previously described (Busse and Séguin, 1992; Busse and Séguin, 1993). For RNA localization experiments, 15 and 30 PCR cycles were performed. Annealing, melting (94°C), and extension (68°C) were for 1 min each. The primers and annealing temperatures used were: Awnt-1 Forward (For.), GTG CAT GAA TAC CGT; Awnt-1 Reverse (Rev.), AGT CTG GCA TGT AGC, 50°C (Busse and Séguin, 1993); Awnt-5A Forward, GTGACTGGAACCTGT; Awnt-5A Reverse, GTCAGCAGCATTCTCGA, 50°C (Busse and Séguin, 1992); AxDaZl Forward, GGA ACT CTT TTT GAT TCT CGG AG, 50°C; Awnt-5B Forward, CTG TAAGTGAATGAGCG, 50°C; Awnt-5B Reverse, GTCAGGCCATCTCTCGA, 50°C (Busse and Séguin, 1992); AxDaZl Reverse, GGAGACAGGCACATACAATCA ACC, 54°C (Johnson et al., 2001). Sizes of the PCR products for Awnt-1, Awnt-5A and Awnt-5B were 477 base pairs (bp), 489 bp and 419 bp, respectively. RT-PCR experiments, using axolotl 18S rRNA as internal control, were done with QuantumRNA™ 18S Internal Standard kit (Ambion). 18S rRNA primers and competimers were mixed in a 1/4 ratio.

PAT Analysis (Poly(A) Test)

The PAT procedure was used to measure poly(A) tail length (Salles et al., 1999). 2 µg of total RNA were first annealed with 100 ng of phosphorylated oligo(dT) at 42°C in the presence of T4 DNA ligase (Gibco-BRL). After 30 min at 42°C, 500 ng of oligo (dT)-anchor (5'-GGCAGGCTCGCCGGCCGCGTTTTTTTTTTTTTTT-3') were added and the mix was transferred at 12°C for 2 h. cDNAs were synthesized with 200 U of superscript Reverse Transcriptase (Gibco-BRL) at 42°C for 1 h. PCR was carried out in a final volume of 50 µL containing 2.5 µL of reverse-transcription reaction, 1 µL of 10 mM dNTPs, 50 pmol of oligo (dT)-anchor and 50 pmol of specific sense primer (Awnt-1, GCT TTG TAA GTA GTG CTC TCT ; Awnt-5A, GAT GCA ACA GCA ACC CTG GC ; Awnt-5B, CTG CGA TGG GTG GTA GGG G), 2.5 units Taq DNA polymerase and 5 µL of supplied buffer. Amplifications were carried out for 40 cycles at 94°C (1 min), 58°C (1 min), 72°C (1 min). PCR products were electrophoresed in 1.5% agarose Nusieve gel, blotted and probed with specific 32P labeled cDNA. In the distribution of amplified fragments, the superior limit in size was taken as the poly(A) tail length. To determine these limits, signal intensities were quantified in each lane along the migration axis with the ImageQuant software and data were plotted on a graph. In each sample, the superior limit of the smear was defined as the point where the signal arose above background.

Whole Mount In Situ Hybridization

Axolotl albino stage VI oocytes and UFE were fixed in MEMFA (100 mM MOPS pH 7.4, 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde) for 3-4 h, and transferred into 100% methanol. Sense and anti-sense RNA probes labeled with digoxigenin were prepared from Awnt cDNAs subcloned into pBluescript KS+ (Busse and Séguin, 1992; Busse and Séguin, 1993). The transcript size was reduced by hydrolysis at 80°C in the in vitro progesterone system will be useful for the molecular characterization of this time specific localization as well as the investigation of its function during meiotic maturation.
Acknowledgements

The authors are very grateful to M. Benard, L. Dandolo and C. Maric for helpful comments. They thank C. Séguin for providing the Awnt-1, Awnt-5A and Awnt-5B cDNA clones. This work was supported by grants from the ‘Ligue Régionale de Normandie contre le cancer’ and from the ‘Ligue Régionale Ile de France contre le cancer’. S.V. is supported by a fellowship from the Ministère de l’Education Nationale, de la Recherche et de la Technologie. Y.A. is a member of University Paris VI, P. et M. Curie.

References


Received: April 2002
Reviewed by Referees: May 2002
Modified by Authors and Accepted for Publication: July 2002
Edited by Anne Marie Duprat