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Original Article

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

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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 cystein-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.

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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 *Wnt* mRNAs 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 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 60 % 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 *Awnt* mRNAs was then determined by RT-PCR using co-amplification of the 18S rRNA as an internal standard (Fig. 2C). This

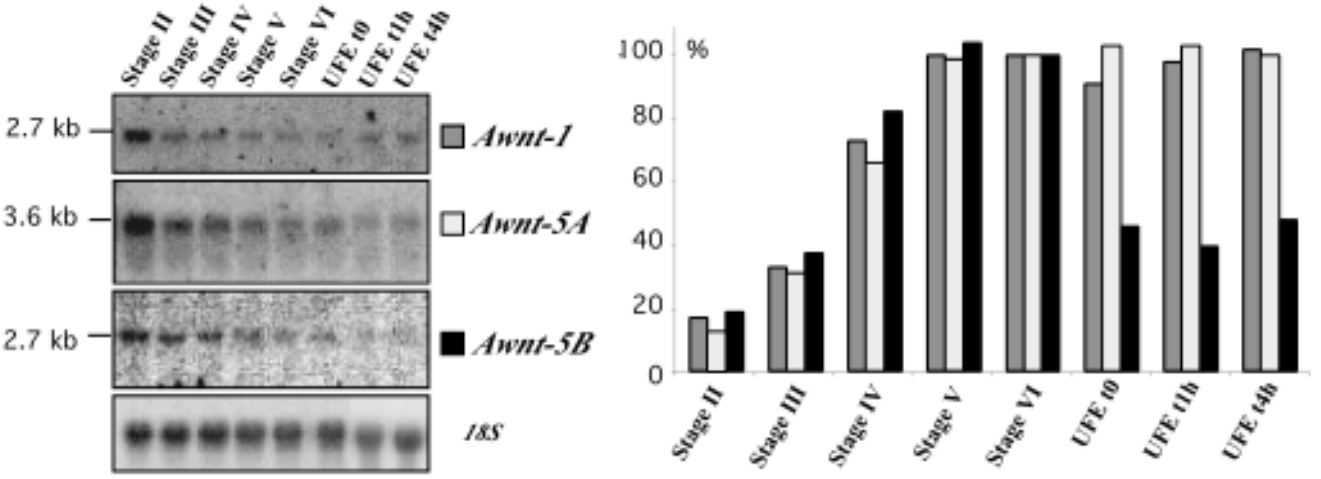

Fig. 1. Expression of *Awnt-1, Awnt-5A* and *Awnt-5B* mRNAs during axolotl oogenesis. *Northern blots were done with 10 µg of total RNA extracted from stage II to VI oocytes and from natural unfertilized eggs collected immediately (UFE t0), 1 h (UFE t1h) or 4 h (UFE t4h) after laying. The maternal 2.7 kilobases (kb)* Awnt-1, *3.6 kb* Awnt-5A *and 2.7 kb* Awnt-5B *mRNAs were revealed by specific ^{32}P labeled cDNA probes. The histogram displays relative levels of* Awnt *transcripts per oocyte.* Awnt *signals were quantified with a phosphoImager and the ImageQuant software, normalized with the axolotl 18S rRNA signal and adjusted for the RNA amount present in one oocyte. Stage VI oocytes were taken as 100 %.*

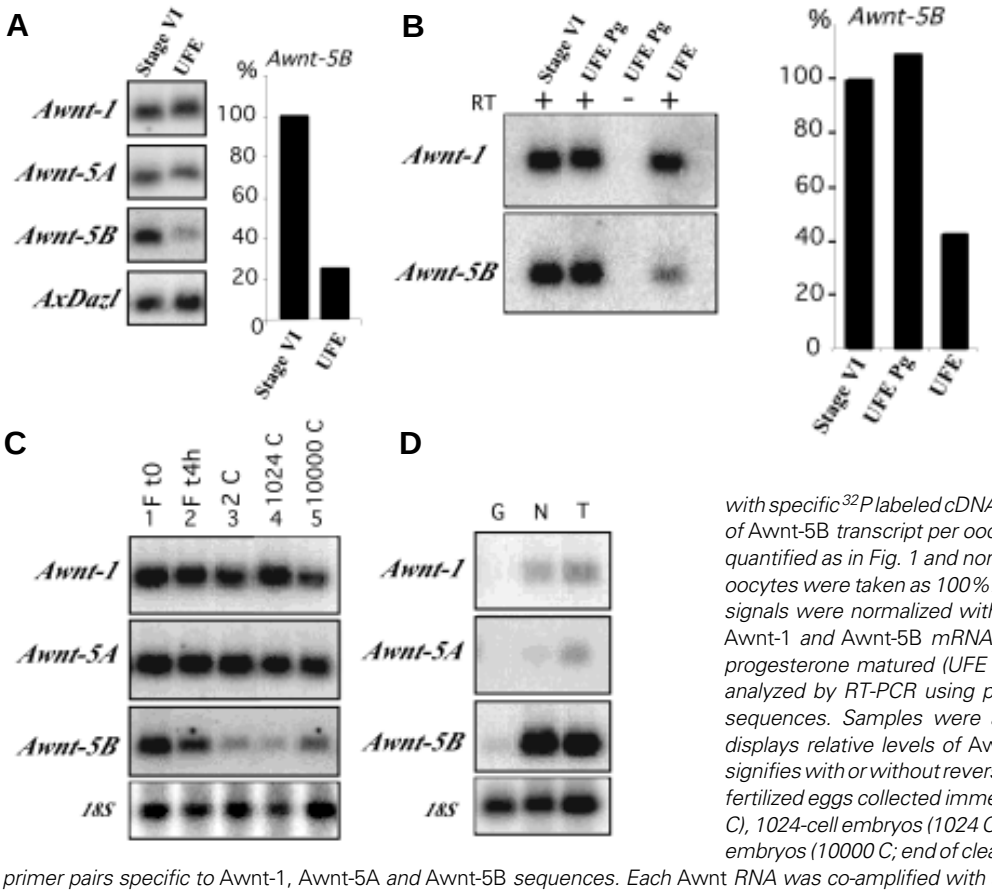

Fig. 2. Temporal changes in *Awnt* mRNA levels during meiotic maturation and after fertilization. (A) 2 μg *of total RNA from stage VI oocytes and natural unfertilized eggs (UFE) were analyzed by RT-PCR using primer pairs specific to* Awnt-1, Awnt-5A, Awnt-5B *and* AxDazl *sequences. After 15 cycles of PCR, samples were separated by gel electrophoresis, transferred to Nylon Hybond N+ membrane and hybridized with specific* ^{32}P *labeled cDNA probes. The histogram displays relative levels of* Awnt-5B *transcript per oocyte during maturation.* Awnt-5B *signals were quantified as in Fig. 1 and normalized with the axolotl* Dazl *mRNA. Stage VI oocytes were taken as 100%. Similar results were obtained when* Awnt-5B *signals were normalized with* Awnt-1 *or* Awnt-5A. **(B)** *Co-amplification of* Awnt-1 *and* Awnt-5B *mRNAs. 2 μg of total RNA from stage VI oocytes, progesterone matured (UFE Pg) and natural (UFE) unfertilized eggs were analyzed by RT-PCR using primer pairs specific to* Awnt-1 *and* Awnt-5B *sequences. Samples were analyzed as described in A. The histogram displays relative levels of* Awnt-5B *transcripts in the three stages. + or - signifies with or without reverse transcriptase (RT).* **(C)** *2 μg of total RNA from fertilized eggs collected immediately (F t0) or 4 h (F t4h) after laying, 2-cell (2 C), 1024-cell embryos (1024 C; MBT, Mid Blastula Transition) and 10000-cell embryos (10000 C; end of cleavage stages), were analyzed by RT-PCR using primer pairs specific to* Awnt-1, Awnt-5A *and* Awnt-5B *sequences. Each* Awnt *RNA was co-amplified with axolotl 18S rRNA (see Materials & Methods). Samples were separated by gel electrophoresis, transferred and hybridized as described in A.* **(D)** *2μg of total RNA from Gastrula (G), Neurula (N) and Tailbud (T) stages were analyzed by RT-PCR in the same conditions as described in C.*

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 polyadenylation 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 Pg, 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.

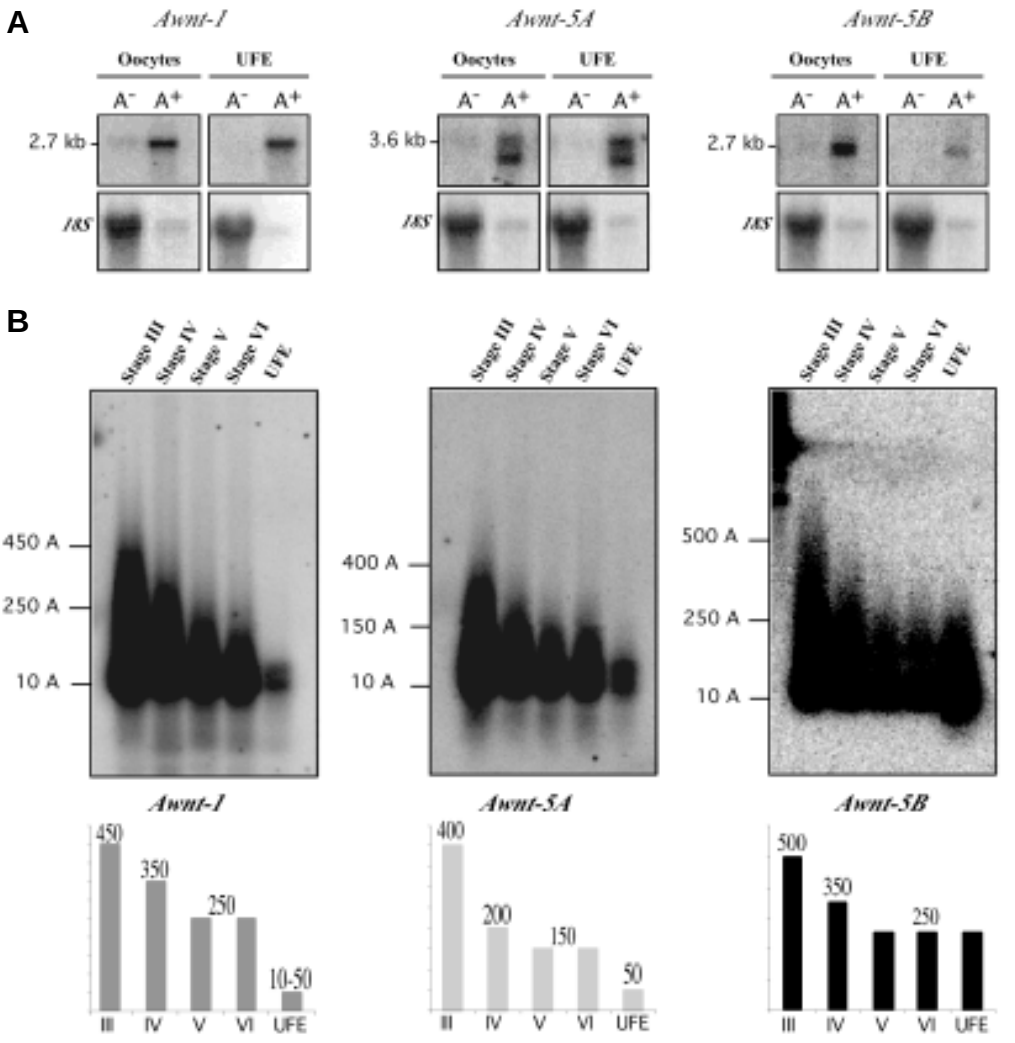

Fig. 3 *Awnt* poly(A) tail length variation during oogenesis and meiotic maturation. (A) *Total RNA was extracted from a pool of stages III to VI oocytes and from unfertilized eggs (UFE) and separated in poly(A)⁻ and poly(A)⁺ samples. 10 μg of poly(A)⁻ and 1 μg of poly(A)⁺ were analyzed by Northern blot 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 ^{32}P 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.*

Awnt-1 *and* Awnt-5A *Polyadenylation 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

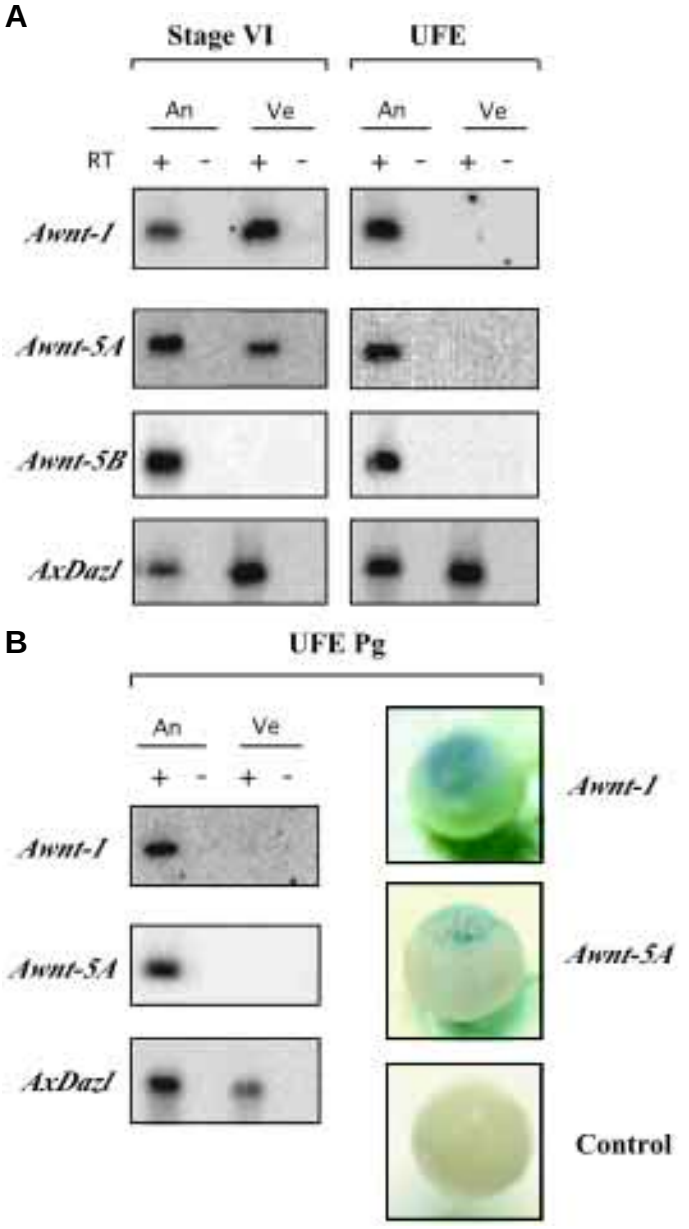

Fig. 4. Changes in *Awnt* transcripts localization during *in vivo* and *in vitro* oocyte maturation. (A) *Stage VI oocytes and natural unfertilized eggs (UFE) were sectioned in two parts corresponding to animal (An) and vegetal (Ve) hemispheres. Total RNA from each moiety was extracted and 2 μg were 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 ^{32}P 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 t*ranscripts was detected at the animal pole of* in vitro *matured oocytes (UFE Pg). No signal was observed at the vegetal pole.*

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 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*

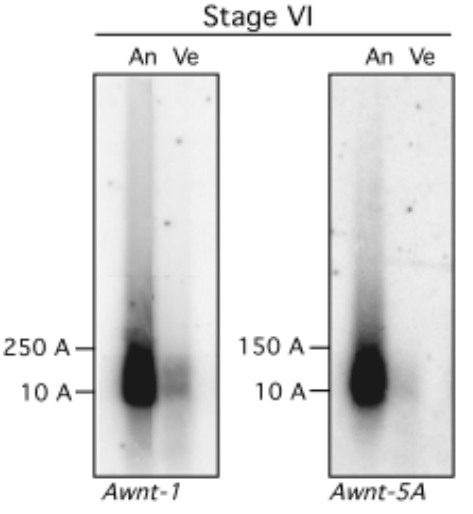

Fig. 5. Spatially regulated polyadenylation of *Awnt-1* and *Awnt-5A* along the animal-vegetal axis. *PAT experiments were performed as described in Fig. 3B with RNA extracted from animal (An) and vegetal (Ve) poles of stage VI oocytes. Sizes of adenylated products were evaluated using a DNA 1 kb ladder.*

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*/Ca^{2+} pathway (Kühl *et al.*, 2000). The *Wnt*/Ca^{2+} plays an essential role in mediating ventral signals in *Xenopus* embryos through suppression of the canonical *Wnt* pathway (Saneyoshi *et al.*, 2002). Particularly, it has been shown that *Xwnt-5A* can be regulated by calcium dependent processes (Slusarski *et al.*, 1997). During oocyte maturation, eggs acquire the ability to generate specialized Ca^{2+} signals in response to sperm entry. Such Ca^{2+} 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 ions and could be important for these early embryonic processes.

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 α*1 gap j*unction (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 *cyclin 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 *Awnt-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

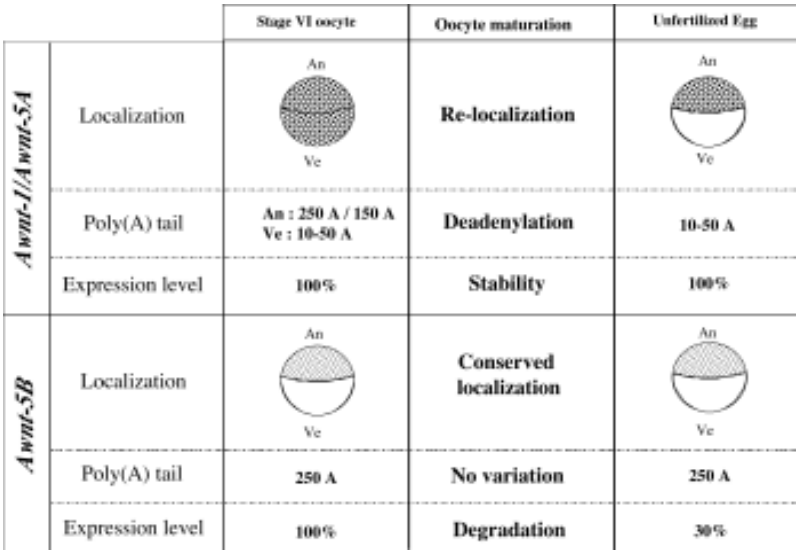

Fig. 6. Post-transcriptionnal regulation of *Awnt-1, Awnt-5A* and *Awnt-5B* during oocyte maturation. *Localization, poly(A) tail status and expression level of* Awnt-1, Awnt-5A *and* Awnt-5B *mRNAs were analyzed in stage VI oocytes and unfertilized eggs. An, animal hemisphere; Ve, vegetal hemisphere; A, adenosine.*

oocyte. 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.

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 spatial 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. 3B). Following germinal vesicle breakdown (GVBD), such deadenylation has been reported for many mRNAs (Varnum *et al*., 1992). This poly(A) shortening is referred to as the default pathway of deadenylation because no specific deadenylation sequences are required (Varnum and Wormington, 1990). During oogenesis and early development, poly(A) tail length is correlated with translational regulation: mRNAs that are polyadenylated are subsequently translated, while others remain dormant (Richter, 1999). The direct role of this tightly regulated polyadenylation as well as the relationships with mRNA localization processes will be further investigated with regard to a potential function of these *Awnt* genes before and after meiotic maturation.

Materials and Methods

Manipulation of Oocytes and Embryos

Stage I to VI oocytes were removed from axolotl adult female and manually defolliculated with forceps under microscope. They were staged according to Beetschen and Gautier (Beetschen *et al*., 1989) and then kept at 18° to 20°C in MBSH (Modified Barth High-Salt solution) (Gurdon and Wickens, 1983). Natural unfertilized eggs (UFE) were obtained 24 hours (h) after injection of human chorionic gonadotropin hormone (hCG 500 UI) (Chorulon, Intervet, France). For *in vitro* maturation, stage VI oocytes (1600-2200 µm in diameter) were incubated in 10^{-6} M progesterone (Sigma) in MBSH medium for 12 h. After treatment, only oocytes showing a white area around the first polar body at the animal pole were collected as *in vitro* mature UFE (UFE Pg). Embryos were obtained by natural fertilization and staged according to Signoret and Lefresne for the cleavage period (Signoret and Lefresne, 1971) and Schreckenberger and Jacobson for other developmental stages (Schreckenberger and Jacobson, 1975). For RNA localization, stage VI oocyte and UFE sections were performed manually under microscope by freezing them on dry-ice and cutting along the equatorial line in order to separate animal from vegetal hemispheres.

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

Total RNA was extracted from different oocyte stages, UFE and embryos by LiCl/Urea method (Auffray 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 ^{32}P labeled cDNA, washed at 65°C in 0.2X SSC, 0.1% SDS. Signals were quantified with a PhosphoImager and the ImageQuant software and normalized with axolotl 18S rRNA (Andéol *et al*., 1995a). cDNA probes were gel-purified fragments labeled with [α ^{32}P] 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.), GTCGATAGAATCCTGCA ; *Awnt-1* Reverse (Rev.), AGTCTTGCCATTGTACG, 50°C (Busse and Séguin, 1993); *Awnt-5A* For., GTGACTGGAACTTGTTC ; *Awnt-5A* Rev., CACTAGTGCTAAAGGTC, 50°C ; *Awnt-5B* For., CTGTAAGTGAATGAGCG, *Awnt-5B* Rev., GTCAGCGACCTATCTGCA, 50°C (Busse and Séguin, 1992); *AxDazl* For., GGAACTCTTTTGTATCTCAGGAGG, *AxDazl* Rev., 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 (pb), 489 pb and 419 pb, respectively. RT-PCR experiments, using axolotl 18S rRNA as internal control, were done with QuantumRNA^{TM} 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 (Sallès *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'-GCGAGCTCCGCGGCCGCGTTTTTTTTTTTT-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 d(T)-anchor and 50 pmol of specific sense primer (*Awnt-1*, GCT TTG TAA GTA GTC CCT TCC ; *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 ^{32}P 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 60°C in

40 mM sodium bicarbonate and 60 mM sodium carbonate for 20 min (*Awnt-1* and *Awnt-5B*) or 30 min (*Awnt-5A*). Whole mount *in situ* hybridization was performed as previously described (Kloc and Etkin, 1999) with anti-DIG antibody (1/2000) and 1% blocking reagent (Boehringer Mannheim). Hybridization was detected with an alkaline-phosphatase coupled antiDIG antibody and visualized using NBT (4.5 µL/mL) and BCIP (3.5 µL/mL) as a substrate (Boehringer Mannheim).

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