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Original Article

Expression of neurotrophin-4 mRNA during oogenesis in *Xenopus laevis*

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ABSTRACT **Neurotrophin-4 (NT-4), a recently discovered novel member of the family of neurotrophic factors structurally related to nerve growth factor (NGF), is abundantly expressed in the *Xenopus laevis* ovary. In this study we have localized NT-4 mRNA expressing cells in the *Xenopus* ovary by *in situ* hybridization and have used this technique together with Northern blot analyses to quantify NT-4 mRNA expression during oogenesis in *Xenopus*. *In situ* hybridization of sections through the *Xenopus* ovary using an α-[^{35}S]-dATP labeled *Xenopus* NT-4 mRNA specific probe showed an intense labeling over the cytoplasm of oocytes with a diameter of 50-200 μm corresponding to stage I according to Dumont (1972). Labeling was also seen over the cytoplasm of stages II to IV although with a lower intensity than over stage I oocytes. No labeling was seen over more mature oocytes of stages V and VI. NT-4 mRNA could not be detected in the early embryo from the onset of cleavage division to the neurula stage suggesting that the NT-4 gene is not expressed during *Xenopus* early embryogenesis. The confinement of NT-4 mRNA in the *Xenopus* ovary to immature oocytes suggests that NT-4 mRNA expression is strictly regulated during oogenesis and that the NT-4 protein could play a role as a maturation factor for immature oocytes.**

KEY WORDS: *in situ hybridization, Northern blot, oocyte*

Introduction

The nerve growth factor (NGF) family, collectively known as the neurotrophins, includes four structurally related proteins: NGF (Levi-Montalcini, 1987), brain-derived neurotrophic factor (BDNF) (Barde *et al.*, 1982; Leibrock *et al.*, 1989), neurotrophin-3 (also known as hippocampus-derived neurotrophic factor) (Ernfors *et al.*, 1990a; Hohn *et al.*, 1990; Jones and Reichardt, 1990; Kaisho *et al.*, 1990; Maisonpierre *et al.*, 1990b; Rosenthal *et al.*, 1990) and neurotrophin-4 (NT-4) (Hallböök *et al.*, 1991). These factors are required for the development and maintenance of the vertebrate nervous system (Barde, 1989). NGF is known to support *in vivo* the survival of sympathetic and neural crest-derived sensory neurons in the peripheral nervous system (Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980) as well as basal forebrain cholinergic neurons (Thoenen *et al.*, 1987; Whittemore and Seiger, 1987; Ebendal, 1989). BDNF increases the survival of peripheral sensory neurons *in vivo* (Hofer and Barde, 1988) as well as retinal ganglion cells (Johnson *et al.*, 1986), basal forebrain cholinergic cells (Alderson *et al.*, 1990) and mesencephalic dopaminergic cells (Hyman *et al.*, 1991; Knusel *et al.*, 1991) in cell cultures. Cells responding to NT-3 and NT-4 *in vivo* have not yet been identified but all four known neurotrophins have a partially overlapping spectrum of neurotrophic activities on peripheral ganglia in culture (Hallböök *et al.*, 1991). The neurotrophic specificities *in vitro* of NGF and BDNF have been shown to be acquired by specific combinations of amino acid motifs that are characteristic for each protein (Ibañez *et al.*, 1991).

NGF, BDNF and NT-3 are expressed in the brain with the highest levels of mRNAs for all three factors in the hippocampus where they are expressed in an overlapping but distinct set of hippocampal neurons (Ernfors *et al.*, 1990b; Phillips *et al.*, 1990). In the rat brain, expression of the neurotrophins is regulated during development (Ernfors *et al.*, 1990a; Maisonpierre *et al.*, 1990a) with a high but transient expression, during the first two postnatal weeks after birth, of NT-3 (Friedman *et al.*, 1991a) and BDNF (Friedman *et al.*, 1991b) mRNAs in parts of the neocortex and brain stem, respectively. This suggests that the dependencies of neurons for the neurotrophins change during development and that the spectrum of neurons supported by these factors is partially different in the developing and mature brain.

In peripheral tissues the levels of NGF mRNA and protein correlate with the degree of innervation by NGF-sensitive nerve

Abbreviations used in this paper: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3; NT-4, neurotrophin-4.

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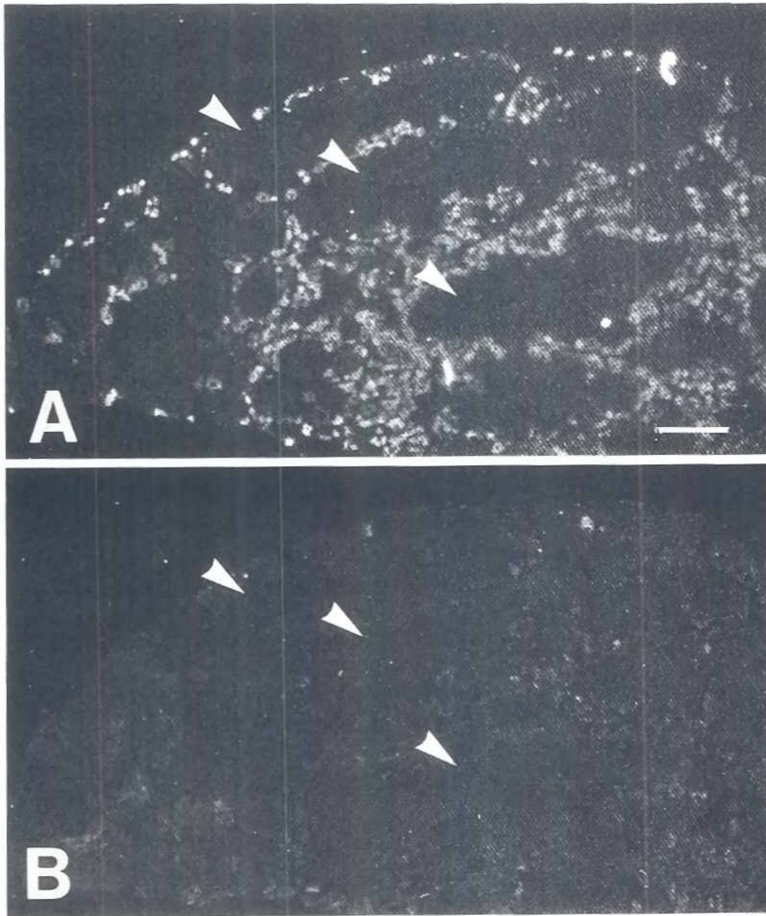

Fig. 1. NT-4 mRNA expression in the *Xenopus laevis* ovary. *Ovary from adult* Xenopus laevis *was sectioned in a cryostat (14 μm thick sections) and the sections were then hybridized to the indicated 53-mer oligonucleotides labeled with ^{35}S-dATP using terminal deoxynucleotidyl transferase.* **(A)** *Hybridization using a* Xenopus *NT-4 mRNA specific probe.* **(B)** *Hybridization using a control chicken BDNF mRNA oligonucleotide of similar G+C content. After hybridization, sections were washed in 1xSSC at 55°C followed by exposure to X-ray film for 10 days. Shown in the figure are photographs of the developed X-ray films. Note the intense labeling over many small cells with the NT-4 probe and the absence of labeling with the control probe. Arrows point at large (stage VI) oocytes which are not labeled with either of the two probes. Scale bar, 2 mm.*

fibers (Heumann *et al.*, 1984; Shelton and Reichardt, 1986). However, high levels of NGF mRNA and protein have also been found in the mouse submaxillary gland, snake venom gland (Levi-Montalcini and Angeletti, 1968), guinea pig and rabbit prostate (Harper *et al.*, 1979) and in bull seminal vesicle (Harper *et al.*, 1982). These findings open up the possibility of a non-neuronal function of NGF in these tissues. Further evidence for this comes from studies of NGF and its receptor in the male reproductive system. NGF mRNA and protein have been demonstrated in spermatocytes and early spermatids of rat and mouse and NGF protein has also been detected in mature spermatozoa in the lumen of the epididymis (Ayer-LeLièvre *et al.*, 1988). The 75 kilodalton, low-affinity NGF receptor (p75^{NGFR}) is expressed in Sertoli cells under negative control of testosterone (Persson *et al.*, 1990; Parvinen *et al.*, 1991) suggesting that NGF produced by spermatogenic cells mediates an interaction between germ and Sertoli cells. *In situ* hybridization (Persson *et al.*, 1990) and Northern blot analysis (Parvinen *et al.*, 1991) have shown that the mRNA for p75^{NGFR} is only expressed between stages VII-VIII of the cycle of the seminiferous epithelium (Leblond and Clermont, 1952) providing evidence for a function of NGF during spermatogenesis at the time of meiosis and spermiation. Using a microdissection technique to isolate defined stages of the seminiferous epithelium in the rat, Parvinen *et al.* (1991) recently showed that NGF stimulates DNA synthesis of seminiferous tubule segments with preleptotene spermatocytes cultured *in vitro*. The effect of NGF was restricted to stages VII-VIII of the seminiferous epithelium when meioses occurs and the possibility that NGF functions as a meiotic growth factor that acts through Sertoli cells was postulated. NT-3 has also been shown to be locally synthesized within the reproductive system, where its mRNA has been localized by *in situ* hybridization to epithelial cells surrounding secondary and tertiary follicles in the rat ovary (Ernfors *et al.*, 1990b).

We recently reported on the cloning of NT-4 from *Xenopus laevis* and *Vipera lebetina* and showed that NT-4 mRNA is abundantly expressed in the *Xenopus* ovary (Hallböök *et al.*, 1991). In this study we have used *in situ* hybridization and Northern blots to demonstrate high levels of NT-4 mRNA in immature *Xenopus* oocytes. The level of NT-4 mRNA in the oocytes decreased below the detection limit in fully grown oocytes suggesting that a putative role of NT-4 in the ovary is restricted to the early stages of oogenesis.

Results

Identification of cells expressing NT-4 mRNA in the Xenopus laevis *ovary by* in situ *hybridization*

Tissue sections through the adult *Xenopus laevis* ovary were hybridized to a ^{35}S-dATP labeled oligonucleotide probe specific for *Xenopus* NT-4 mRNA. As a control for the specificity of the hybridi-

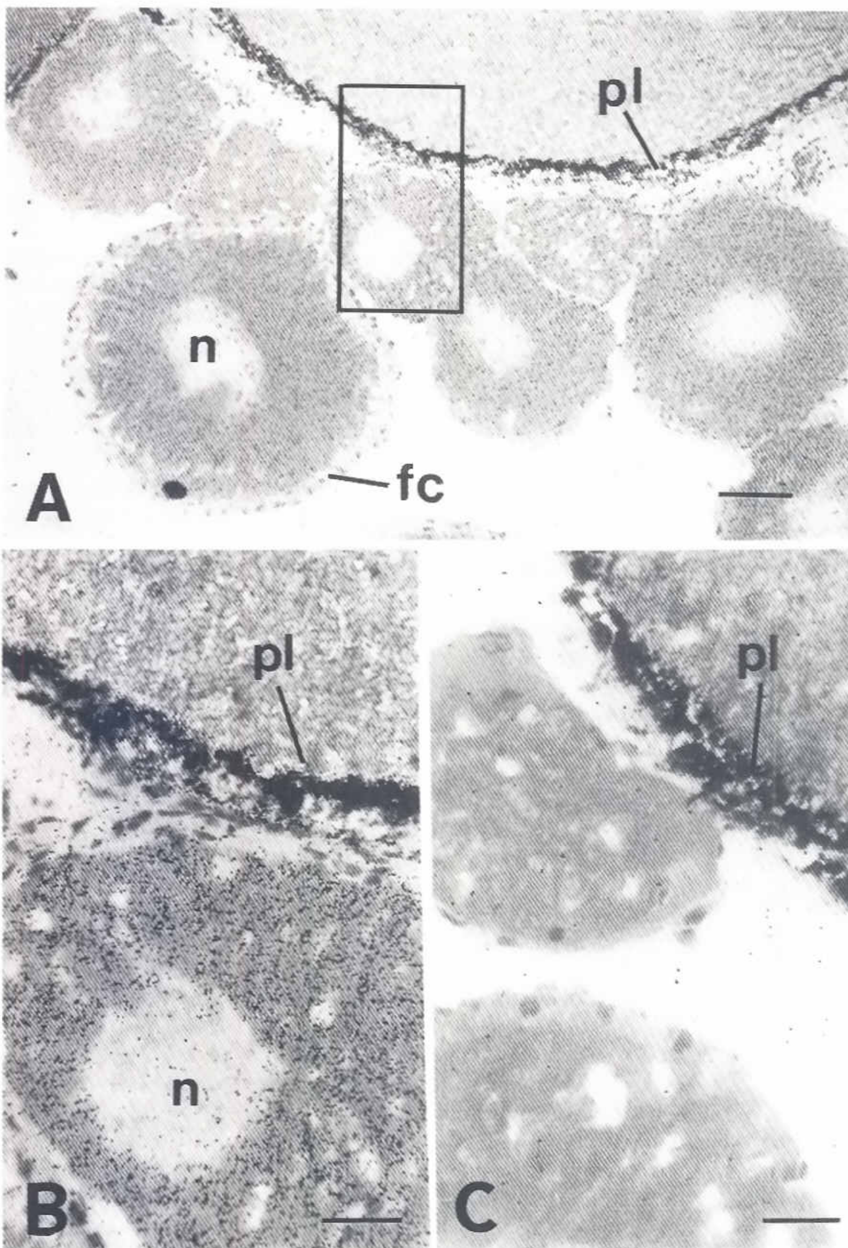

Fig. 2. Bright-field illumination of emulsion autoradiographs showing NT-4 mRNA expressing oocytes in the *Xenopus* ovary. *Sections hybridized to the* Xenopus *NT-4 mRNA specific* **(A, B)** *or control BDNF* **(C)** *probe were coated with Kodak NTB3 emulsion, exposed for 5 weeks, developed and lightly counterstained with cresyl violet. Shown in the figure are bright-field photomicrographs of the developed sections. Note in (A) the intense NT-4 mRNA labeling over small size oocytes (stages I and II) and the absence of labeling over large size (stages V and VI) oocytes. (B) shows a higher magnification of the boxed-in area in (A). Note the intense labeling over the cytoplasm of the stage II oocytes shown in the picture. (C) No labeling can be seen using the control chicken BDNF probe. Abbreviations: n, nucleus; fc, follicle cells; pl, pigmented layer. Scale bar in A, 50 μm; in B and C, 15 μm.*

zation, adjacent sections were hybridized to an oligonucleotide probe of the same length and GC-content complementary to mRNA for chicken brain-derived neurotrophic factor (BDNF). The NT-4 mRNA specific probe showed an intense labeling over many cells scattered throughout the ovary with a size (50-400 μm in diameter) corresponding to oocytes in early stages of oogenesis (Fig. 1A). No NT-4 mRNA could be detected over mature, post-vitellogenic stage VI oocytes (arrows in Fig. 1A). The chicken BDNF mRNA specific control probe did not label any cells in the *Xenopus* ovary.

Analysis of emulsion autoradiographs from the hybridized sections revealed an intense labeling over the cytoplasm of oocytes with a diameter of 50-200 μm (Fig. 2A and B) corresponding to stage I oocytes according to Dumont (1972). The NT-4 mRNA specific probe also labeled oocytes with a larger diameter corresponding to stages II to IV, though the intensity of labeling over these cells was lower than that seen over stage I oocytes. In agreement with the analysis of low magnification dark-field illuminations (Fig. 1), the emulsion autoradiographs did not show any labeling over more mature oocytes of stages V and VI. No labeling was seen over any cells after hybridization with the control BDNF probe (Fig. 2C).

To allow a more detailed determination of the level of NT-4 mRNA in oocytes during oogenesis, the number of grains per an arbitrarily chosen area unit was counted. The area unit chosen corresponded to approximately one hundredth of the cross section area of a stage

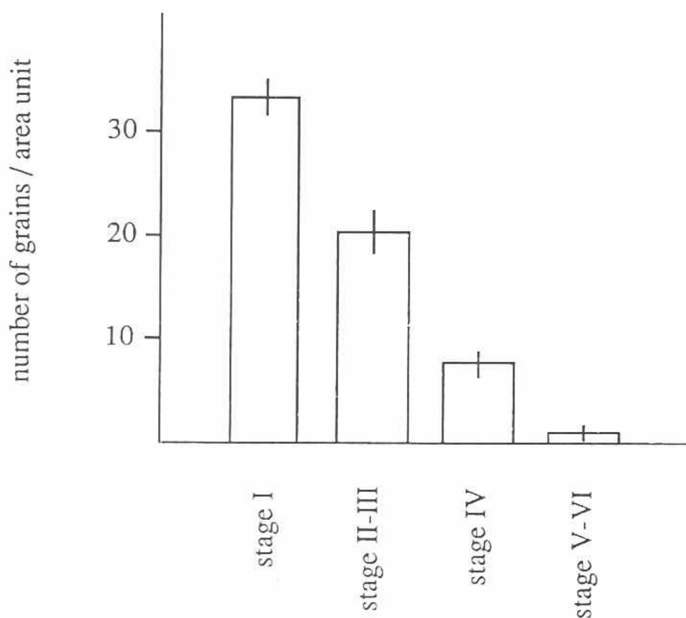

Fig. 3. Levels of NT-4 mRNA in oocytes at different stages of oogenesis. *Emulsion autoradiographs (shown in Fig. 2) of sections hybridized with the* Xenopus *NT-4 mRNA specific probe were used to count the number of grains over an area unit. The area unit chosen was about one hundredth of a stage I oocyte. Fifteen area units were analyzed in 10 different oocytes of the indicated stages. Error bars show ± S.D.*

I oocyte. The result of this analysis showed that the intensity of labeling over stage I oocytes was 1.7 and 4.3 times higher than over stage II/III and IV oocytes, respectively (Fig. 3). The number of grains per area unit over stage V and VI oocytes was not significantly above the background labeling.

Northern blot analysis of NT-4 mRNA expression during* Xenopus *oogenesis and early development

A fixed amount of total cellular RNA (40 µg) prepared from different stages of oocytes as well as from a fraction enriched for follicle cells was analyzed by Northern blots using a *Xenopus* NT-4 mRNA specific probe (Hallböök *et al.*, 1991). In agreement with the results of the *in situ* hybridization, the highest level of NT-4 transcripts, with sizes of 2.3 kb and 6.0 kb, was present in the smallest oocytes (stages I and II) (Fig. 4). A low level of a large NT-4 transcript (>10 kb), which may represent a precursor RNA, was also present in stage I and II oocytes. NT-4 mRNA was also abundant in stage II/IV oocytes but declined abruptly in more mature stage V and VI oocytes. A weak hybridization signal was seen in the follicle cell preparation, which was probably due to contamination with a small number of stage I and II oocytes. The same result was obtained when a fixed amount (5 µg) of polyadenylated RNA was analyzed (data not shown).

The analysis of NT-4 mRNA expression in the ovary showed that NT-4 mRNA is restricted to immature oocytes. To test the possibility that expression of NT-4 mRNA is induced after fertilization, the level of NT-4 mRNA was also assessed in developing *Xenopus* embryos by Northern blots of polyadenylated RNA. A low level of NT-4 mRNA was found in *Xenopus* somatic A_6 cultured kidney cells which were also included in the analysis. However, no NT-4 mRNA could be detected in early embryos from the onset of cleavage divisions to the neurula stage (data not shown).

Discussion

The abundant expression of NT-4 mRNA in the *Xenopus* ovary (Hallböök *et al.*, 1991) opens up the possibility that this member of the NGF family could play a role in oogenesis and/or early embryogenesis. The present study was performed in order to localize cells in the ovary expressing NT-4 mRNA as a first step towards an understanding of the putative function of the NT-4 protein in the ovary. In amphibians, as in all other vertebrates, fertilization of the egg triggers a period of rapid cell cleavage and this event is under the control of a class of soluble maternal mRNAs expressed during oogenesis and stored in the unfertilized egg for subsequent development (Davidson, 1986). This class of maternal mRNAs includes both basic fibroblast growth factor (Kimelman and Kirschner, 1987) and transforming growth factor-ß (Weeks and Melton, 1987), as well as several proto-oncogenes such as c-myc (Godeau *et al.*, 1986; Vriz *et al.*, 1989), c-fos (Mohun *et al.*, 1989), ras (Andéol *et al.*, 1990), ets-2 (Chen *et al.*, 1990) and c-mos (Sagata *et al.*, 1988). Immature stage VI *Xenopus* oocytes are arrested in prophase of meiosis I and both c-mos (Sagata *et al.*, 1988) and ets-2 (Chen *et al.*, 1990) have been shown to function during reinitiation of meiotic division. The finding of high levels of NT-4 mRNA in stage I and II oocytes with marked decrease in stage V and VI oocytes suggests that the NT-4 mRNA does not belong to the class of maternal mRNAs. This result also argues against a role of the NT-4 protein in the reinitiation of meiotic division or in early embryogenesis. In agreement with this, addition of recombinant NT-4 protein to immature stage VI oocytes failed to induce germinal vesicle breakdown *in vitro* and NT-4 mRNA was not detected in *Xenopus* early embryos (data not shown). Instead, the function of the NT-4 protein in the ovary appears to be coupled to events occurring in the pre-vitellogenic and early/mid vitellogenic oocyte.

NGF mRNA and protein have been detected in the rat ovary where the NGF content appears to be regulated by the degree of sympathetic innervation (Lara *et al.*, 1990b). Sympathectomy caused by neonatal administration of antibodies against NGF reduces follicle cell growth and the levels of androgens and estradiol suggesting that the trophic effect of NGF on the innervating sympathetic neurons in the ovary is important for the function of the mature ovary (Lara *et al.*, 1990a). A low level of NGF mRNA has also been detected in the *Xenopus* ovary (Carriero *et al.*, 1991; Hallböök *et al.*, 1991) where it is expressed in oocytes from stage I to VI (Carriero *et al.*, 1991). Similar results were also obtained in this study after hybridization of mRNA from different stages of oogenesis with a *Xenopus* NGF probe (data not shown). NGF mRNA belongs to a low abundance class of oocyte mRNAs and has been estimated to represent 0.01% to 0.001% of the total ovary mRNA population (Carriero *et al.*, 1991). Hybridization of the same Northern blots to *Xenopus* NGF and NT-4 probes showed that the level of NT-4 mRNA in immature oocytes is in the order of 100 times higher than the level of NGF mRNA. This demonstrates that NT-4 mRNA belongs to a class of highly abundant oocyte mRNAs with levels reaching 0.1% of the total mRNA.

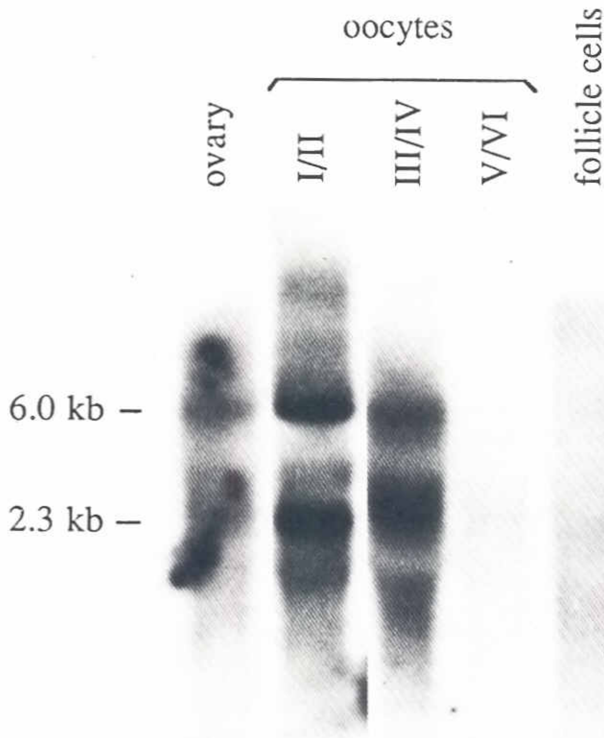

Fig. 4. Northern blot analysis of NT-4 mRNA expression during oogenesis in *Xenopus laevis*. *Ovaries from two adult* Xenopus laevis *were dissected out and treated with collagenase to remove follicle cells and release the oocytes. The oocytes were then sorted into the indicated groups following the stages described by (Dumont, 1972). Total ovary and the released follicle cells were also included in the analysis. Total cellular RNA was prepared and 40 mg/well of RNA was electrophoresed in a formaldehyde-containing 1% agarose gel. The gel was blotted onto a nitrocellulose filter and hybridized to a 350bp Hinc II fragment from the 3' exon of the* Xenopus *NT-4 gene. The filter was washed at high stringency and exposed for five days to an X-ray film. Note the marked decrease in the level of NT-4 mRNA in stage V and VI oocytes.*

Another striking feature of NT-4 mRNA expression in *Xenopus* oocytes is the marked decrease of NT-4 mRNA in more mature stage V and VI oocytes. This suggests that NT-4 mRNA expression is strictly regulated during oogenesis and that the high amount of NT-4 mRNA in immature oocytes gets degraded as the oocytes progress into more mature stages. Oocyte maturation is independent on new transcription (Maller, 1985) suggesting that the sequential synthesis of proteins required for maturation is regulated at the translational level. However, the transient appearance of NT-4 mRNA during oogenesis suggests that it represents a novel class of oocyte mRNAs that is highly expressed in immature oocytes (before the first meiotic division) but degraded in more mature oocytes. Regulation of expression of this class of oocyte mRNAs therefore appears to involve both a transcriptional down-regulation as well as a specific removal of sequences by mRNA degradation. The temporal appearance of such mRNAs suggests that the proteins translated from them, including the NT-4 protein, play a role in early oogenesis. Validation of this hypothesis should be obtained by the use of specific antibodies against the NT-4 protein to study its role during oogenesis in *Xenopus*.

Materials and Methods

Isolation, handling and culture of Xenopus *oocytes, embryos and cells*

Male and female *Xenopus laevis* frogs were maintained in the laboratory at 19°C. After immersion-anesthesia of the animals in 0.25% tricaine methane sulfonate (Sandoz, Switzerland), ovarian lobes were surgically removed, washed with modified Barth's saline Hepes (MBSH) (Gurdon and Wickens, 1983) and dissociated by overnight incubation at 20°C in calcium-free MBSH containing 2 mg/ml collagenase. Crude separation of pre-vitellogenic and vitellogenic oocytes was obtained by differential sedimentation, and oocytes were further sorted manually under a dissecting microscope into the developmental classes described by Dumont (1972). Synchronously cleaving embryos were obtained by *in vitro* fertilization essentially as described by (Newport and Kirschner, 1982). A_6 *Xenopus* kidney cells were cultured in Leibowitz L_{15} medium diluted with distilled water 60:40 (v/v) and supplemented with 10 mM Hepes pH 7.35, 10 µM hypoxanthine (Sigma), 4 mM glutamine and 10% fetal bovine serum (Gibco) at 20°C. Cultures were equilibrated with air and kept in the dark.

In situ *hybridization*

Fresh-frozen ovaries from adult *Xenopus laevis* frogs were sectioned (14 µm) in a cryostat (Leitz, Germany) and the sections were thawed onto poly-L-lysine (50 µg/ml) pretreated slides after which they were fixed in 10% formalin for 30 min and rinsed twice in PBS. Dehydration was carried out in a graded series of ethanol including a 5 min incubation in chloroform after which the slides were air-dried. Two 53-mer oligonucleotides, one specific for *Xenopus* NT-4 mRNA (5'CCCACAAGCTTGTTGGCATCTATGGTCAGAGCCCT CACATAAGACTGTTTTGC3') and another, as a control, specific for chicken BDNF mRNA (corresponding to amino acids 61 to 77 of the mature chicken BDNF protein (Hallböök *et al.*, 1991), were synthesized in an Applied Biosystems A381 DNA synthesizer. The oligonucleotides were labeled at their 3' end with α ^{35}S-dATP using terminal deoxyribonucleotidyl transferase (IBI, New Haven) to a specific activity of approximately 1×10^9 cpm/µg. Hybridization was performed at 42°C for 16 h in 50% formamide, 4x SSC, 1x Denhardts solution, 1% Sarcosyl, 0.02M $NaPO_4$ (pH 7.0), 10 % dextransulphate, 0.5 mg/ml yeast tRNA, 0.06M DTT, 0.1 mg/ml sheared salmon sperm DNA and 1×10^7 cpm/ml of ^{35}S-dATP labeled oligonucleotide probe. Sections were subsequently rinsed, washed 4 times (15 min each) at 55°C in 1 x SSC, rinsed in water, dehydrated in a graded series of ethanol and air-dried. The sections were exposed to X-ray film followed by coating in Kodak NTB-3 photo emulsion (diluted 1:1 in water), exposed for 5-6 weeks at -20°C, developed, fixed and counterstained with cresyl violet.

RNA blot analysis

The indicated samples were homogenized in 4M guanidine isothiocyanate, 0.1M ß-mercaptoethanol, 0.025M sodium citrate pH 7.0 and homogenized 3 times for 15 seconds with a Polytrone. Each homogenate was layered over a 4ml cushion of 5.7M CsCl in 0.025M sodium citrate pH 5.5 and centrifuged at 15°C in a Beckman SW41 rotor at 35 000 rpm for 16 h (Chirgwin *et al.*, 1979). Polyadenylated RNA (Poly(A)$^+$ RNA) was purified by oligo (dT) cellulose chromatography (Aviv and Leder, 1972) and the recovery of RNA was quantified spectrophotometrically before use in RNA blot analysis. Total cellular RNA (40 µg) or where indicated poly(A)$^+$ RNA (5 µg) from each sample was electrophoresed in a 1% agarose gel containing 0.7% formaldehyde. Uv-transillumination of the stained gel was used to confirm that all samples contained similar amounts of intact RNA. The gel was then transferred to a nitrocellulose filter. The filter was hybridized to a 350bp Hinc II fragment from the 3' exon of the *Xenopus* NT-4 gene (Hallböök *et al.*, 1991). The fragment was labeled with α-(^{32}P)-dCTP by nick-translation to a specific activity of around 5×10^8 cpm/µg and the hybridization was carried out as described (Ernfors *et al.*, 1988). Filters were washed at high stringency (0.1xSSC, 0.1% SDS, 54°C) and exposed to Kodak XAR-5 films at -70°C.

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