The nuclear–cytoplasmic distribution of the *Xenopus* nuclear factor, xnf7, coincides with its state of phosphorylation during early development

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Summary

We describe the characterization in *Xenopus laevis* of a nuclear protein, xnf7, which is first detected in the oocyte GV and is eventually enriched in nuclei of cells of the adult brain. Previous studies have shown that this protein contains zinc-finger-like structures and acidic domains typical of transcriptional activators, and is phosphorylated *in vitro* by p34cdc2 protein kinase. The protein also binds to double-stranded DNA. These data suggest that xnf7 may function as a transcription factor. During oocyte maturation, xnf7 is released into the cytoplasm and is not detectable in nuclei until the mid-blastula-gastrula stage of development. Western blot analysis of xnf7 isolated from oocytes and eggs showed the existence of multiple bands or isoforms of the protein. Unique isoforms that are generated during oocyte maturation are the result of phosphorylation. The phosphorylated isoforms remain in the cytoplasm until the mid-blastula stage. The re-accumulation of protein in the embryonic nuclei at this time correlates with the increase in abundance of the less phosphorylated isoforms. The xnf7 protein possesses a nuclear localization signal (NLS) similar to the bipartite signal found in nucleoplasmin. Newly synthesized xnf7 accumulated in the oocyte GV to detectable levels within a few hours following synthesis suggesting that retention of the protein in the cytoplasm during early cleavage may be due to a process that interferes with the function of the NLS. These data suggest that compartmentalization and/or post-translational modification of the nuclear protein xnf7 may be involved in regulating its function during early development.

Key words: *Xenopus*, DNA-binding protein, isoforms, nuclear localization, development.

Introduction

An important question concerns the mechanism by which the embryo regulates the function of maternal gene products that may not be required until specific developmental stages such as the MBT (Newport and Kirschner, 1982). In *Xenopus*, there are a number of maternal gene products that exhibit interesting patterns of behavior during early development that may indicate a role of compartmentalization and post-translational modification in regulating their function. Dreyer (1987, 1989) described a number of antigens that originate in the oocyte nucleus (GV), become cytoplasmic during oocyte maturation, and re-enter the embryonic nuclei at specific times during development. Some re-enter immediately following fertilization while others re-enter either at the blastula or neurula stages of development.

Recently, we have been studying in *Xenopus* the gene xnf7 whose protein product originates in the oocyte GV, becomes cytoplasmic at maturation and is retained in the cytoplasm until it re-enters the embryonic nuclei by the mid-blastula stage (Dreyer et al. 1983; Miller et al. 1989; Kloc et al. 1989; Reddy et al. 1991). It ultimately becomes enriched in nuclei of the central nervous system at larval stages of development. Xnf7 also binds to double-stranded DNA and is detected associated with lampbrush chromosomes (Miller et al. 1989; D. Wedlich, unpublished observations). We have also shown that the conceptual xnf7 protein possesses several zinc-finger-like regions, two acidic regions that are similar to transcriptional activating domains, and a p34cdc2 protein kinase phosphorylation site (Reddy et al. 1991). In addition, it is an *in vitro* substrate for p34cdc2 protein kinase. Recently, we have shown that one of the acidic domains can transactivate a reporter gene in a transfection assay (B. Reddy and L. Etkin, unpublished observations). These results suggest that
the xnf7 protein may function as a transcription factor during development.

In the present study, we were interested in determining several of the characteristics of the xnf7 protein that may help us to define further its behavior and understand its function during development. We report here that (1) the xnf7 protein is phosphorylated at maturation resulting in the formation of slowly migrating isoforms, (2) the phosphorylated slow isoform is in the cytoplasm of maturing oocytes, (3) the phosphorylated slow isoform predominates during early cleavage when the protein is cytoplasmic while the fast migrating isoform becomes predominant again by the mid-blastula stage when the protein is accumulating in nuclei, and (4) the xnf7 protein possesses a putative nuclear localization signal (NLS) similar to that of nucleoplasmin and can efficiently enter the oocyte GV within a short time following synthesis. These results suggest that both phosphorylation and cytoplasmic sequestration are involved in regulating the function of xnf7 during early development. They also suggest that the xnf7 protein may be retained in the cytoplasm during early development by interfering with the nuclear localization mechanism.

Materials and methods

Animals

Xenopus laevis were obtained from Nasco (Wisconsin). Frogs and tadpoles were maintained according to Gurdon (1967). Ovulation was induced by injection of 800 i.u. human chorionic gonadotropin (hCG) (Sigma) into the dorsal lymph sacs of females. Fertilization was performed either by spawnings where males were injected with 300 i.u. hCG or by in vitro techniques (Etkin et al. 1984; Tompkins, 1978).

Tissue extract preparations, labelling of oocyte phosphoproteins, and of bacterially expressed protein with MPF

GVs were manually isolated in 1× Modified Barth’s Saline (MBS). Oocytes were either manually defolliculated in MBS or defolliculated by incubation in 50 μg ml⁻¹ collagenase (type IV; Sigma) in calcium- and magnesium-free OR-2 (Wallace et al. 1973) at 25–30°C for 45–90 min. Maturation of defolliculated oocytes was induced by culturing in 1× MBS with 10 μg ml⁻¹ progesterone (Sigma). Maturation (GVBD) was determined by the presence of a white spot in the animal pole or by dissection of oocytes. To study phosphorylation, oocytes were incubated in 0.5 MCl⁻¹ inorganic 32p-phosphate (carrier-free; Amersham) for 4 h in 1× MBS. The free label was removed by extensive washing with 1× MBS and oocytes were matured with progesterone. Ovulated eggs were dejellied with 2.5 % cysteine in 50 mM Tris-HCl (pH 7.8) and extensively washed with 1× MBS. Protein extracts were prepared by homogenization of GV, oocytes, and eggs in a buffer consisting of 10 mM Tris-HCl (pH 8.0), 10 mM DDT, 0.1% Triton X-100 and 5 mM EDTA. Protease inhibitors included 0.1–1 mM PMSF, 0.5 μg ml⁻¹ leupeptin and 0.7 μg ml⁻¹ pepstatin. Phosphatase activity was inhibited by the addition of 50 mM β-glycerophosphate (Sigma). Protein concentration determinations of extracts were performed using the Bio-Rad Protein determination kit with BSA as a standard.

Immunoprecipitation, electrophoresis and immunoblotting

Immunoprecipitation was performed according to Anderson and Blobel (1983) using 1:50 diluted antisera and protein A–sepharose beads (Pharmacia) as a solid phase immunoadsorbant. Samples were separated electrophoretically under denaturing conditions (Dreyfuss et al. 1984; Laemmli, 1970). Western blots were performed either as in Miller et al. (1989) or using a semidry Sartoblot apparatus (Sartorius, Hayward, CA) as recommended by the manufacturer. Two antibodies were used, one was the monoclonal antibody, 37-1A9, prepared from Xenopus GV proteins (Dreyer et al. 1981) and a second antibody, the polyclonal antibody (L24), produced from the bacterially expressed xnf7 protein, produced identical results (Reddy et al. 1991). The preparation of monoclonal and polyclonal antibodies and the developmental fates of their antigens is described elsewhere (Dreyer et al. 1981, 1982, 1983, 1985; Reddy et al. 1991).

Phosphatase treatments

Samples were combined with an equal volume of 0.2 mM Tris-HCl (pH 9.5), 0.2 mM NaCl and 10 mM MgCl₂ and incubated at 37°C for 0–18 h with 0.01–2 mg ml⁻¹ bacterial alkaline phosphatase (BAP) (Sigma). Effective concentrations of BAP were determined experimentally.

Immunohistochemistry

Embryos, tadpoles and portions of adult tissues were fixed in 100 % methanol, overnight, at 4°C, dehydrated with 100 % ethanol, cleared in xylene, embedded in paraplast and cut into 5 μm sections. Sections were treated with 3% H₂O₂ in paraplast. For immunofluorescent staining, the non-specific protein-binding sites were blocked by incubation in blocking solution (3 % BSA, 10 % goat serum in PBS) for 30 min–1 h (Wedlich and Dreyer, 1988). Subsequently, sections were incubated for 1 h with the 37-1A9 monoclonal antibody (1:500 in blocking solution) and washed two times (10 min each) in PBS. Fluorescein–isothiocyanate (FITC)–conjugated goat anti-rabbit IgG (BMB) (1:20 to 1:2000 dilution in PBS) was used as a secondary antibody. After 1 h incubation sections were washed in PBS (two times, 15 min each) and mounted in 50 % glycerol in PBS.

Nuclear localization of xnf7 in oocytes

Stage 6 oocytes (Dumont, 1972) were labeled with [35S]methionine (specific activity 1000 Ci mm⁻¹; Amersham) in MBS during which samples were taken at various time points. At each time point, 25 oocytes were manually dissected into cytoplasms and GV. The protein was extracted, and xnf7 was immunoprecipitated using the L24 polyclonal antibody. The immunoprecipitated protein was analyzed by PAGE and fluorography.

Results

Xnf7 is phosphorylated at maturation resulting in the formation of new isoforms

We previously reported the presence of a fast-migrating isoform of xnf7 in oocytes and a slow-migrating form present in eggs (Miller et al. 1989). The presence of a fast isoform in oocytes and a slow isoform in eggs suggested that the slow isoform was generated during oocyte maturation and may be the result of phosphorylation. This was tested by maturing oocytes in vitro with
progesterone and analyzing samples taken during maturation by western blot.

Fig. 1 shows that there is a dramatic shift in the isoforms from the faster oocyte form to the more slowly migrating egg forms in oocytes that were matured in vitro. No shift to the egg isoforms was observed in oocytes cultured in the absence of progesterone. The peak shift to the egg isoforms occurred at 0.6–0.8 GVBD when the immature oocytes matured. The slowest egg isoform is the one addition and 1.0 GVBD is the time of progesterone addition. The arrows point to the positions of the different isoforms.

In the first set of experiments, defolliculated oocytes were incubated in 1×MBS in the presence or absence of progesterone. Proteins from progesterone-treated oocytes were extracted at 30 min intervals and blotted. Lane 1, protein samples (2 oocyte equivalents) at time 0; lane 2, 30 min; lane 3, 60 min; lane 4, 90 min; lane 5, 120 min; lane 6, 150 min; lane 7, 180 min; lane 8, 210 min; lane 9, 240 min; lane 10, 270 min following progesterone addition. Lane 11, control oocytes not treated with progesterone but cultured for 270 min. The arrows point to the positions of the different isoforms.

In the second set of experiments, extracts prepared from oocytes and eggs were treated with BAP to substantiate further the role of phosphorylation in the production of the xnf7 isoforms. Fig. 2B shows an immunoblot of oocyte and egg extracts treated with BAP. The BAP treatment did not affect the mobility of the fast oocyte isoform; however, it did produce a shift in migration of the slow egg isoforms to the fast oocyte isoforms (lanes 2 and 4). This confirms that phosphorylation of the oocyte isoforms results in their reduced mobilities on gels and clearly demonstrates that the isoforms of xnf7 produced during oocyte maturation are due to phosphorylation.

**Phosphorylated xnf7 is located in the cytoplasm during oocyte maturation**

We were interested in determining whether the new phosphorylated isoforms of xnf7 produced during maturation were located in the nucleus or the cytoplasm. Therefore, we treated defolliculated oocytes with progesterone and at subsequent times prepared extracts from the GVs, enucleated cytoplasms and intact oocytes.

Phosphorylated xnf7 is located in the cytoplasm during oocyte maturation.
Fig. 3. Egg-specific isoforms are found only in the cytoplasm of maturing oocytes before GVBD. Oocytes were induced to mature with progesterone (time 0'). At 30 min intervals following progesterone addition, 10 oocytes were manually enucleated and extracts prepared from the GVs (GV) and the enucleated cytoplasms (c). Total oocytes were also extracted for comparison (t). Each lane received 2 oocyte equivalents (e.g. 2 GVs or 2 enucleated cytoplasms). At 210 min after progesterone addition 50% of all oocytes had undergone GVBD. 100% GVBD was at 4 h.

Fig. 3 shows that the majority of xnf7 was localized in the GV with a small amount in the enucleated oocyte cytoplasm from the time of progesterone addition until 150 min later. This is consistent with observations using immunocytochemistry (Dreyer et al. 1983). Interestingly, the small amount of xnf7 detected in the cytoplasm prior to 150 min was the fast oocyte isoform. The onset of the major shift to the slow egg isoforms began at 150 min after progesterone addition which was well before GVBD. Furthermore, after 150 min, it was the slow egg-specific isoform that was present in the cytoplasm while the antigen present in the intact GV was the fast oocyte isoform. By 210 min, roughly 50% of all oocytes had undergone GVBD and it was impractical and difficult to isolate intact GVs. These data show that during oocyte maturation a small amount of xnf7 is found in the cytoplasm before GVBD and that prior to 150 min it all remains in the hypophosphorylated fast oocyte form. After 150 min (but prior to GVBD), however, the phosphorylated cytoplasmic slow-migrating egg form of xnf7 appears.

The hypophosphorylated xnf7 isoforms become predominant during development concurrent with accumulation in the nuclear compartment

Previous studies have shown that xnf7 is retained in the cytoplasm until it reenters the nuclei by the mid-blastula to gastrula stages of development (Dreyer et al. 1983). We were interested in determining the distribution of the different isoforms during development. Fig. 4 shows a western blot of protein extracts prepared from embryos of different developmental stages. During cleavage until stage 8 the predominant form was the slow-migrating egg-specific isoform, though a small amount of the fast-migrating form appears at stage 3. By stage 8-9 the fast-migrating oocyte isoform becomes predominant. Small amounts of the slow egg isoforms were still detected as late as early tailbud stages although their abundance decreased 2- to 3-fold. We also examined the time at which xnf7 reenters the nuclei by performing immunofluorescence on histological sections of early blastula (stage 7) and early gastrula (stage 10) embryos. Fig. 5 shows that xnf7 was not detectable in stage 7 nuclei; however, at stage 10 almost all nuclei showed staining. More importantly, the accumulation of xnf7 protein in the nuclei between stage 7 and 10 correlates well with the predominance of the faster migrating oocyte-like isoform (Fig. 4). Since we have shown that these isoforms differ in their degree of phosphorylation, this result suggests that the state of phosphorylation of xnf7 changes during development coincident with its transport from the cytoplasm to the nucleus.

Xnf7 possesses a putative NLS and accumulates in oocyte GVs within a short time after synthesis

One possible explanation for why the protein does not accumulate in nuclei until the blastula stage is that the xnf7 protein possesses a NLS that functions poorly, resulting in a slow accumulation of the protein in the nuclei. The conceptual sequence of the xnf7 protein shows the presence of a putative NLS signal KRKIEE-PEPEPKKAK located between amino acids 106 and 120 (Reddy et al. 1991). This is very similar to the
Phosphorylated isoforms of xnf7

Fig. 5. Immunofluorescence of the accumulation of the xnf7 protein during development. (A and B) Histological sections through stage 7 embryos (early blastula) stained with DAPI (A) or FITC (B). (C and D) Histological sections through stage 10 embryos stained with DAPI (C) or FITC (D).

bipartite NLS, KRPAATKAGQAKKKK, found in nucleoplasm (Robbins et al. 1991). As a preliminary assessment of the ability of xnf7 to enter nuclei, we performed the following experiment.

Newly synthesized xnf7 protein was labeled by incubation of stage 6 oocytes in [35S]methionine during which samples of GVs and cytoplasms were taken. Protein was extracted and xnf7 was immunoprecipitated with the L24 polyclonal antibody (Reddy et al. 1991) and analyzed by PAGE and fluorography. We calculated the relative concentration of the protein in the GV and cytoplasm based on densitometry measurements of the bands on the fluorograph and the differential volume of the two compartments. Fig. 6 shows that the xnf7 protein was detected in the GV 5 h following initiation of labeling (Lane 1). Based on the 8-fold difference in volume of the GV and cytoplasm (Burglin and DeRobertis, 1987), we calculated that the xnf7 protein was 4-fold more concentrated in the GV than cytoplasm at this time point. At 15 h (Lanes 3 and 4) the protein was 8-fold more concentrated and at 30 h (lanes 5 and 6) 11 times more concentrated in the GV.

We calculated the lag time between the detection of xnf7 in the cytoplasm and its accumulation in the nuclei from this and two other experiments. The results indicated that the translocation time from the cytoplasm to the nucleus for xnf7 was 2 h. This translocation time is consistent with the immunofluorescent data (Fig. 5) showing that the xnf7 protein accumulated in nuclei during the several hour period between stages 7 and 10. These data show that the xnf7 protein possesses an efficient NLS and suggest that the maternal protein present at fertilization does not enter nuclei prior to the

Fig. 6. Entry of endogenous xnf7 protein in GVs. Stage 6 oocytes were pulse labeled with [35S]methionine (1000 Ci mmol-1) as described in Materials and methods. At 5, 15 h, and 30 h oocytes were dissected into cytoplasms and GVs, protein extracted and xnf7 protein was immunoprecipitated and analyzed by PAGE and fluorography. Lanes 1 and 2, GVs and cytoplasms, respectively, from oocytes cultured for 5 h; Lanes 3 and 4, GVs and cytoplasms from oocytes cultured for 15 h; Lanes 5 and 6, GVs and cytoplasms from oocytes cultured for 30 h. N, nucleus; C, cytoplasm
blastula stage due to a process that interferes with the function of the NLS.

**Discussion**

We report that a nuclear protein (xnf7) that is enriched in the adult central nervous system of *Xenopus* is phosphorylated during oocyte maturation following its release into the cytoplasm. The phosphorylation results in the formation of several isoforms of the protein. Xnf7 is retained in the cytoplasm until the mid-blastula stage when it accumulates in the nuclei at detectable levels. The reaccumulation in nuclei is accompanied by a concurrent dephosphorylation of the xnf7 protein. The xnf7 protein is efficiently accumulated in oocyte GVs suggesting that its retention in the cytoplasm during early development may be due to a mechanism that interferes with the function of the NLS.

The conceptual xnf7 protein contains several putative zinc fingers, acidic domains similar in structure to activation domains found in numerous transcription factors (one of which is capable of activating a reporter gene), and three potential p34^cdc2^ protein kinase phosphorylation sites (Reddy et al. 1991). Xnf7 binds to double-stranded DNA (Miller et al. 1989) but not to single-stranded DNA (C. Dreyer, unpublished observations). In addition, the bacterially produced protein from the xnf7 cDNA as well as the endogenous xnf7 from the oocyte reacts with a polyclonal antibody made from the Krüppel finger link region (both xnf7 and Krüppel have a common epitope recognized by the antibody, B. Reddy, C. Dreyer and L. Etkin, unpublished observations). These data, taken together, support the conclusion that xnf7 is a DNA-binding protein and may function as a transcription factor.

Our evidence for phosphorylation of xnf7 during oocyte maturation includes the shift in gel mobility of xnf7, incorporation of inorganic ^32^P into immunoprecipitable xnf7 and the removal of phosphate groups with bacterial alkaline phosphatase (BAP). Additionally, we have shown that bacterially produced xnf7 is phosphorylated by MPF (Reddy et al. 1991). The removal of phosphate groups with BAP converts the egg isoforms into species that co-migrate with the oocyte isoforms.

We have shown that xnf7 is an *in vitro* substrate for a cell-cycle-specific kinase, MPF and have mapped the site of phosphorylation to a putative p34^cd2^ protein kinase consensus site (Reddy et al. 1991; Gautier et al. 1988; Moreno and Nurse, 1990). Our data demonstrating the phosphorylation of xnf7 by MPF *in vitro* and the observation that xnf7 is phosphorylated during oocyte maturation suggest that it may also be an *in vivo* substrate (Reddy et al. 1991).

There is also a close correlation between the state of phosphorylation of xnf7 and its accumulation in nuclei during cleavage. The embryonic relocalization of xnf7 during development correlates with the increased accumulation of the faster moving (lower state of phosphorylation) oocyte isoform. The observation that both faster and slower migrating isoforms co-exist in embryos reflects that the shift from the cytoplasm to the nucleus is not an all-or-none phenomenon, but takes place over a period of embryonic development culminating in the highest levels of accumulation at the mid-blastula stage. This is supported by immunohistochemical data showing that oocyte isoforms of xnf7 are nuclear, while the egg forms are largely cytoplasmic during early development. It was found that the nuclear transport kinetics of the SV40 T antigen were effected by sequences outside of the nuclear transport signal that are sites of potential phosphorylation (Rihs and Peters, 1989). Interestingly, in xnf7, the major site for *in vitro* phosphorylation actually overlaps the putative NLS suggesting that phosphorylation of this site may affect the functioning of the NLS (Reddy et al. 1991). However, our understanding of the relationship between phosphorylation and nuclear/cytoplasmic partitioning for xnf7 will have to await the results of further studies.

There are several examples of temporally and spatially specific movements of proteins from the cytoplasm to the nucleus during development and cell differentiation at which time they are functional. An example is the *Drosophila dorsal* gene product which encodes a transcription factor required for the development of ventral structures (Hunt, 1990; Roth et al. 1989; Rushlow et al. 1989; Steward, 1989). *Dorsal* appears to function by activating the transcription of at least two genes, *twist* and *snail*, that are expressed in the ventral region of the embryo. Interestingly, during normal development the *dorsal* gene product is present in all regions of the embryo, but in the ventral region it is localized in the nuclei, and in the dorsal region in the cytoplasm. The difference in nucleocytoplasmic compartmentalization of the *dorsal* gene product may be due to its interaction with the *cactus* gene product resulting in anchoring of the *dorsal* protein in the cytoplasm. This interaction may be augmented by differential phosphorylation of the *dorsal* protein in different regions of the embryo, which may affect its binding to the *cactus* protein (Hunt, 1990). Another protein whose function may be regulated by its nuclear–cytoplasmic distribution is the transcription factor NF-kB, which functions in the activation of the kappa chain gene in B lymphocytes (Lenardo and Baltimore, 1989). In unstimulated cells, this protein is complexed with another protein I-Kb resulting in sequestration in the cytoplasm and inability to translocate into the nucleus. The interaction appears to be dependent on the phosphorylation of the I-Kb protein. In *Xenopus*, the c-myc protein is retained in the cytoplasm in the oocyte and becomes nuclear following fertilization suggesting a possible association with a cytoplasmic anchor protein in oocytes (Gusse et al. 1989).

The xnf7 protein becomes cytoplasmic at oocyte maturation, is retained in the cytoplasm as a phos-
phorylated form, and accumulates in nuclei by the mid-blastula to gastrula stages of development. We do not believe that the delay in accumulation of the xnf7 protein during development is due to an inefficient nuclear translocation signal since our data show that newly synthesized endogenous xnf7 protein accumulates in the GV efficiently with kinetics similar to proteins such as nucleoplasmin that are detected in embryonic nuclei immediately following fertilization. We also have preliminary data suggesting that the xnf7 protein associates with large protein complexes during cleavage stages when it is cytoplasmic (X. Li and L. Etkin, unpublished observations). These data suggest that the xnf7 protein may be actively sequestered in the cytoplasm during early cleavage stages, perhaps by interaction with other proteins until it is needed at or soon after the mid-blastula stage. We propose that the compartmentalization in the cytoplasm and post-translational modification of proteins such as xnf7 are important mechanisms by which Xenopus regulates the function of nuclear proteins during cleavage stages prior to the MBT.

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