

Cytoplasmic Retention of *Xenopus* Nuclear Factor 7 before the Mid Blastula Transition Uses a Unique Anchoring Mechanism Involving a Retention Domain and Several Phosphorylation Sites

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Abstract. *Xenopus* nuclear factor 7 (xnf7) is a maternally expressed protein that belongs to the B-box zinc finger gene family consisting of transcription factors, protooncogenes, and ribonucleoproteins. Its function is regulated by retention in the cytoplasm from oocyte maturation until the mid blastula transition (MBT) when it reenters the nucleus. We defined a 22-amino acid cytoplasmic retention domain (CRD) in xnf7 that functioned cooperatively with two phosphorylation sites within the xnf7 molecule to retain the protein in the cytoplasm until the MBT. Deletion of this region or mutations in the phosphorylation sites resulted in the early entry of xnf7 into the nucleus. A mutation changing one of the phosphorylation sites to a glutamic acid resulted in the prolonged retention of the

xnf7 protein in the cytoplasm until stages 9–10, well past the MBT. Additionally, a mutant form of xnf7 possessing a second nuclear localization signal at the COOH terminus was retained in the cytoplasm. This suggests that retention of xnf7 was not due to the masking of its NLS as is the case with NF κ B and dorsal but was due to a novel anchoring mechanism in which the CRD interacts with an anchor protein. The CRD sequence is also found in another B-box zinc finger protein that is also retained in the cytoplasm until the MBT in the newt. Therefore, we believe that this may be an important mechanism whereby the function of a number of nuclear proteins is regulated during development.

An important problem in development concerns the strategy used by the embryo to regulate nuclear processes during embryogenesis. One mechanism used in a variety of systems is selective transport of proteins into the nuclei at specific times or in specific regions of the embryo during development or cellular differentiation (Hunt, 1989; Nigg et al., 1991; Schmitz et al., 1991; Silver, 1991; Forbes, 1992; Whiteside and Goodbourn, 1993). Several notable examples include the transcription factor NF- κ B which functions in the activation of the κ chain gene during B lymphocyte differentiation (Lenardo and Baltimore, 1989; Ghosh and Baltimore, 1990; Kerr et al., 1991) and the nuclear/cytoplasmic distribution of the dorsal gene product during development in *Drosophila* which controls dorsal-ventral axis formation (Hunt, 1989; Roth et al., 1989; Rushlow et al., 1989; Schmitz et al., 1991; Steward, 1989; Govind and Steward, 1992).

Another example of selective nuclear transport of proteins during development is in the vertebrate *Xenopus laevis* (Dreyer et al., 1983; Dreyer, 1987). Using immunohistochemistry with antibodies against GV proteins, Dreyer

(1987) described the developmental fate of a number of maternal nuclear proteins which were first detected in the oocyte nucleus (GV). After oocyte maturation these proteins were released into the cytoplasm after which some immediately reentered the nuclei (early shifting) while others remained in the cytoplasm until specific stages during development (late shifting).

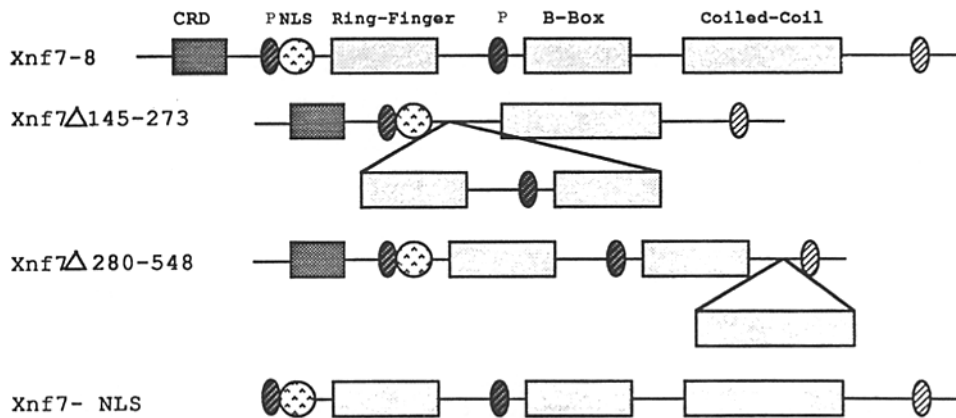
Several of the late shifting proteins reenter the nucleus at the mid blastula transition (MBT).¹ This is a crucial time during *Xenopus* development when the zygotic genome is activated and there is a dramatic change in the cell cycle (Newport and Kirschner, 1982; Etkin, 1988). Thus, it is likely that some of these proteins play important roles in regulating these processes.

We have analyzed one of the late shifting proteins, xnf7 (*Xenopus* nuclear factor 7), a maternally expressed putative transcription factor whose protein product is retained in the cytoplasm until it reenters the embryonic nuclei at the MBT (Dreyer et al., 1983; Miller et al., 1989, 1991; Reddy et al., 1991). Xnf7 is a member of a novel family of zinc finger proteins, the B-box family, consisting mainly of transcription

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1. *Abbreviations used in this paper:* CRD, cytoplasmic retention domain; MBT, mid blastula transition; NLS, nuclear localization signal; xnf7, *Xenopus* nuclear factor 7.

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factors and protooncogenes (Reddy and Etkin, 1991; Reddy et al., 1992; Freemont, 1993). When *xnf7* is released into the cytoplasm during oocyte maturation it is hyperphosphorylated, however, it is dephosphorylated coincident with nuclear reentry at the MBT. Previously, we have mapped one of the phosphorylation sites to threonine 103 which is adjacent to the NLS (Reddy et al., 1991; Li and Etkin, 1993). Additionally, there are several other putative phosphorylation sites located at threonines 209, 212, and 218 between the two zinc finger domains. It is likely that these sites are phosphorylated at maturation (Li, X., and L. D. Etkin, unpublished observations). Therefore, the potential nuclear function of *xnf7* is suppressed by its retention in the cytoplasm before the MBT and its nuclear/cytoplasmic distribution may be regulated by its state of phosphorylation.

The *xnf7* nuclear localization signal (NLS) is found between amino acids 106 and 120 and is very similar to the bipartite basic NLS of nucleoplasmin (Reddy et al., 1991). However, nucleoplasmin, unlike *xnf7* reenters the nucleus immediately following fertilization (Dreyer et al., 1983). Recently, we analyzed the function of the *xnf7* NLS in oocytes and developing embryos and showed that the bipartite basic NLS functions efficiently in nuclear transport suggesting that the retention of *xnf7* in the cytoplasm is due to a process that either interferes with the function of the NLS or anchors it to a subcellular structure or complex (Li and Etkin, 1993).

In the present study we have identified a *cis*-acting cytoplasmic retention domain (CRD) in *xnf7* and have shown that phosphorylation is intimately involved in its function. Also, we found that *xnf7* was retained in the cytoplasm even when a second NLS was added at the COOH terminus suggesting that its retention is due to intermolecular interactions with a cytoplasmic anchor involving the CRD. NFkB, on the other hand, entered the nucleus upon the addition of a second NLS at the COOH-terminal end of the protein indicating that it was retained through a mechanism that masked the normal NLS. Therefore, the cytoplasmic retention of *xnf7* may involve a novel mechanism different from that of NFkB or dorsal.

Figure 1. Accumulation of deletion mutants of *xnf7* in nuclei during development. (A) Diagram of deletion constructs used in these experiments. (B) In vitro-transcribed mRNA made from the above constructs was injected into fertilized eggs. Injected embryos at stage 7 and 9 were fixed, sectioned and treated with the T7 polyclonal antibody followed by immunoperoxidase staining. The panels represent, *xnf7*Δ145-273 injected embryos analyzed at stage 7 and 9; *xnf7*Δ280-548 injected embryos at stage 7 and 9; and *xnf7*-NLS injected embryos analyzed at stage 7 and 9. Arrows are pointing to nuclei. P, phosphorylation sites.

Materials and Methods

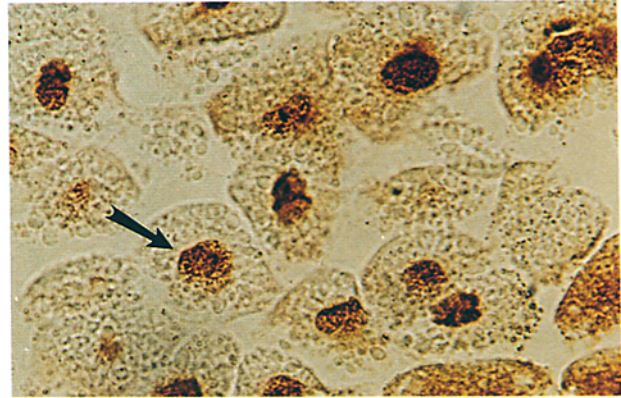
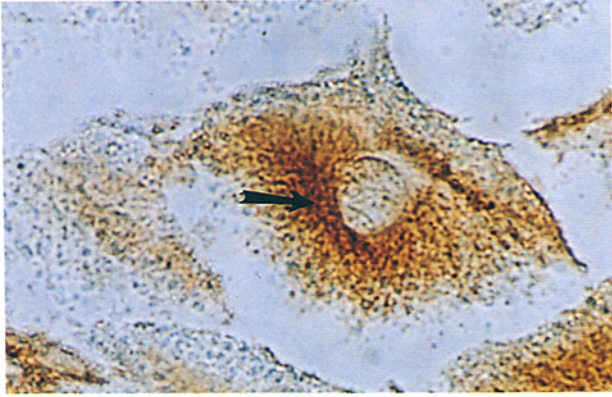
Expression Vectors

A vector was constructed for synthesis of *xnf7* mRNA. The vector consisted of pBS, into which a synthetic oligonucleotide containing the 5' untranslated sequences of the *Xenopus* β-globin gene and a 12-amino acid-long T7 viral coat protein tag was inserted at the XbaI and KpnI sites. The T7 viral coat peptide was used to distinguish the exogenous *xnf7* from the endogenous protein with polyclonal antibodies. The *xnf7*-8 cDNA (this cDNA lacks 30 NH₂-terminal amino acids of the full length cDNA to ensure use of the globin translational start site (Reddy et al., 1991) was cloned into the EcoRI site of the vector, fusing it in frame with the upstream T7 sequence. The *xnf7*-1 construct was made by deleting the NH₂-terminal 128 amino acids, including the NLS. *Xnf7*Δ145-273 and *xnf7*Δ280-548 were constructed by internal deletions of the *xnf7*-8 cDNA using convenient restriction sites. *Xnf7*NLS was constructed by inserting the NLS oligonucleotide (5' AAC TCCCCAGAAAGAGAAAGATTGAGGAACCTGAGCCTGAACCGAAGAAAAGCAAAG 3' coding for Lys Thr Pro Gln Lys Arg Lys Ile Glu Glu Pro Glu Pro Glu Pro Lys Lys Ala Lys) upstream of the NH₂-terminal truncated *xnf7*-1 cDNA. *Xnf7*-1-CRD was made by cloning an oligonucleotide containing the CRD sequence downstream (3') of the NLS in the *xnf7*NLS construct. This oligonucleotide consisted of 5'GAAGAGTAT-TACGCGCATTACGTGGGCTTGAACCGCCGCAAAAACGAATGG-GTGGACAAATCTCGT3' coding for Glu Glu Tyr Ala His Tyr Val Gly Leu Asn Arg Arg Gln Asn Glu Trp Val Asp Lys Ser Arg. *Xnf7*-8C-NLS and *xnf7*-1C-NLS were generated by inserting an NLS oligonucleotide at the C-terminus of the *xnf7*-8 and *xnf7*-1, respectively. For *xnf7*ΔCRD (Δ52 to 73), fragments upstream and downstream of the CRD were amplified by PCR, ligated, and cloned into the same expression vector as above. All the phosphorylation mutants were generated by the PCR soeing technique (Horton et al., 1990). The PCR soeing is performed as follows: four oligonucleotides are required for making each mutation, including the 5' and 3' end oligonucleotides from the expression vector flanking the insert and two complementary oligonucleotides containing the mutation (listed below). Three PCR reactions are performed for each mutation. The first PCR amplifies the upstream fragment using the 5' oligonucleotide and one of the mutant oligonucleotides as the primers. The second PCR is to amplify the downstream fragment using the 3' oligonucleotide and the complementary mutant oligonucleotide as primers. The last PCR reaction is to ligate the upstream and downstream fragments by using 5' and 3' end oligonucleotides as primers and the PCR products from reaction 1 and 2 as templates. Oligonucleotides containing the mutations for different mutants are listed as follows: oligonucleotides for making *Xnf7*P1 are 5' TCAAAGGCGCCCA-GAAGAGAAAG 3' (nt 336 to 360) and its complement 5' CTGGGGCGC-CCTTGAGTCTGGCTG 3'. Oligonucleotides for making *Xnf7*P2 are 5' GCCTGTGCGCCGGTTCGCACCTGTAGAGAAAAGGCACGG 3' (nt 654 to 693) and its complement 5' CCGTGCCTTTTCTCTACAGGTG-

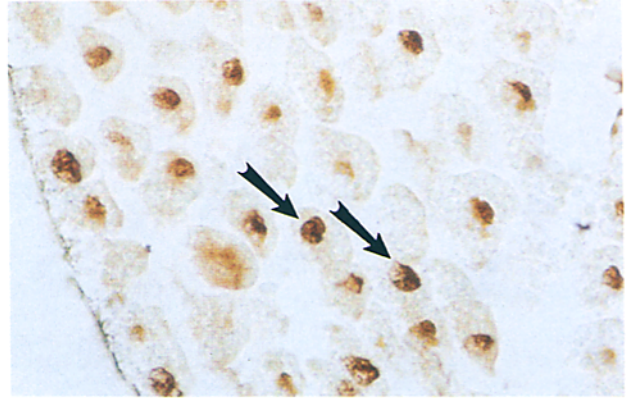
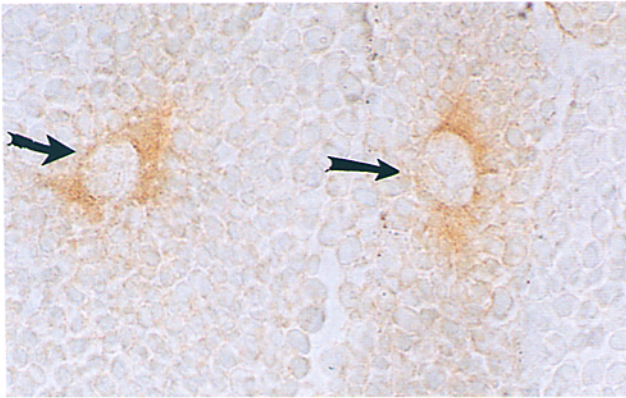
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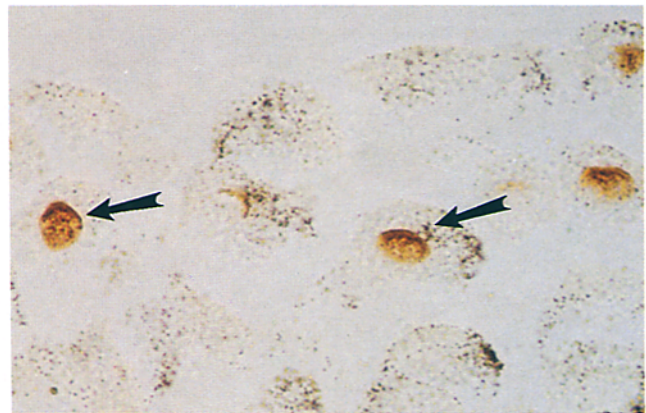
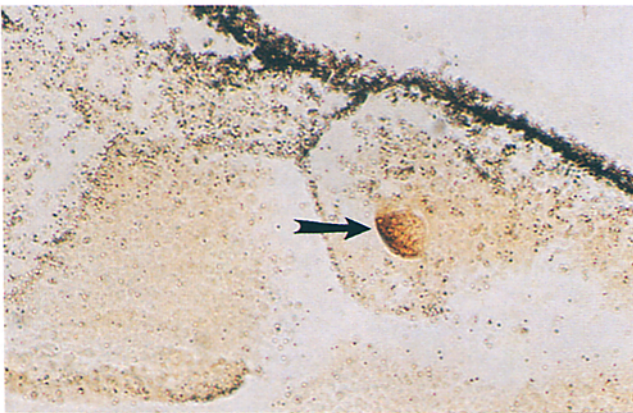
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xnf7- Δ 145-273



xnf7- Δ 280-548



xnf7-NLS

Figure 1.

CGACCGCGCACAGGC 3'. Oligonucleotides for making Xnf7/thr-Glu are 5' GGCCAAGAAAGCCGCTGTGAGCCGGTCAACCTGTAGAG-AAAAGGCACGGCC 3' (nt 644 to 696) and its complementary 5' CCA-GGGGCCGTTCTTTTCTCTACAGGTTGACCGGCTCACAGGC-GGC 3'. All constructs were sequenced and several independent clones were analyzed for each mutant to ensure against potential mutations generated during PCR.

Microinjection of Embryos and Oocytes

Eggs were fertilized in vitro. Eggs were transferred into 1× modified Barth saline (MBS) and microinjected as previously described (Etkin et al., 1984; Etkin and Pearman, 1987). 10 ng of RNA was injected into the equatorial region of the one-cell embryos. One hour after microinjection, embryos were transferred into 0.1× MBS and allowed to develop. Oocytes were injected in the equatorial region with 10 ng of RNA.

Immunoperoxidase Staining of Sections

Embryos were fixed in 100% methanol at 4°C overnight, embedded in Paraplast Plus (Oxford Labware, St. Louis, MO), and serially sectioned into 10-µm sections. Immunoperoxidase staining was carried out according to Cornish et al. (1992). Deparaffinized and hydrated sections were bleached in 6% hydrogen peroxide in methanol for 20–30 min. After two 10-min PBS washes, nonspecific staining was blocked with a 1-h incubation in a blocking buffer consisting of 10% goat serum and 3% BSA in TBS with 0.5% Tween 20 (TBST). Sections were incubated 1 h in a 1:50–1:200 dilution of the L24 polyclonal antibody (Reddy et al., 1992) in blocking buffer then washed two times for 10 min each in PBS. For control sections no primary antibody was used. The sections were treated with the secondary antibody (1:50 dilution of goat anti-rabbit conjugated with peroxidase) for 1 h and washed 2 × 10 min in PBS. The color reaction was initiated with 1 mg/ml DAB + 0.03% H₂O₂ in PBS for 5 min and was stopped by two 5-min washes with water. The sections were counterstained with either hematoxylin or Azur B and mounted with Permount (Fisher Scientific, Pittsburgh, PA) or a 1:1 dilution of PBS with glycerol.

Labeling of Exogenous xnf7 Mutant Phosphoproteins during Oocyte Maturation

Maturation of defolliculated injected oocytes was induced by culturing in 1× MBS with 10 µg ml⁻¹ progesterone (Sigma Chemical Co., St. Louis, MO). 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 mCi ml⁻¹ inorganic ³²P-phosphate (carrier-free; Amersham Corp., Arlington Heights, IL) for 4 h in 1×MBS. The free label was removed by extensive washing with 1×MBS and oocytes were matured with progesterone. Protein extracts were prepared by homogenization of oocytes 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 Chemical Co.). Protein concentrations of extracts were determined using the Bio Rad Protein kit (Bio Rad Laboratories, Richmond, CA) with BSA as a standard. Exogenous and endogenous xnf7 was immunoprecipitated according to Li and Etkin (1993) and analyzed by SDS-PAGE and autoradiography.

Results

Retention of xnf7 in the Cytoplasm Is Dependent on the Presence of a 22-residue CRD Located between Amino Acids 52 and 73

Previous studies (Li and Etkin, 1993) suggested that retention of xnf7 in the cytoplasm was due to either a mechanism that interfered with the function of its NLS or an anchor mechanism tethering the protein to a cytoplasmic complex or structure.

To map putative retention domains in xnf7 we created a series of deletion mutants and determined whether they were retained in the cytoplasm by injecting synthetic mRNA into

Table 1. Comparison of CRD Sequences between xnf7 and PwA33

Gene	Amino acids	Sequence
xnf7	52–73	EEYYAHYVGLNRRQNEWVDKSR
PwA33	79–100	EEFYVHYVGLNRRQNEWVDKSR

fertilized eggs. Injected embryos were analyzed at different stages during development using immunostaining with the T7 antibody to detect the epitope tagged protein. Deletion mutants included xnf7Δ145–273, xnf7Δ280–548, and xnf7-NLS, which lacks amino acids 1–128 but contains an oligonucleotide with the NLS at the NH₂ terminus (Fig. 1 A). Fig. 1 B shows that at stage 7 xnf7Δ145–273 and xnf7Δ280–548 appeared in the cytoplasm, while at stage 9 all were nuclear. This was identical to the time of nuclear entry of the xnf7–8 construct and endogenous xnf7 (Reddy et al., 1991; Li and Etkin, 1993). The xnf7NLS protein, on the other hand, was detected within the nucleus by stages 6–7, well before the MBT and the nuclear accumulation of the xnf7–8 and other mutant proteins. These data show that a signal located within the domain from amino acids 1–128 was necessary for the retention of xnf7 in the cytoplasm during early development.

Recently, the protein product of another B box zinc finger gene (PwA33), cloned from the newt *Pleurodeles waltii* was also shown to be retained in the cytoplasm until MBT (Belini et al., 1993). The NH₂ terminus of the gene was different from that of xnf7 except for a 22-amino acid domain, which was 97% identical (Table I). Therefore, we tested the function of this domain in the cytoplasmic retention of xnf7 by creating a mutant gene lacking this sequence (xnf7ΔCRD).

Synthetic mRNA from deletion mutant xnf7ΔCRD was injected into fertilized eggs and the fate of the protein followed by immunostaining. Fig. 2 A shows that while wild-type xnf7–8 was not detected at stage 7 the mutant was detected in the nucleus by stage 6–7 (Fig. 2 B). These data suggest that retention of xnf7 in the cytoplasm before MBT was dependent on the presence of the 22-amino acid *cis*-acting CRD within the NH₂ terminus.

The translational efficiency and the stability of the protein from each of the deletion mutants was analyzed by extraction of protein from injected oocytes and embryos and analysis by Western blotting. We also analyzed the newly synthesized exogenous xnf7 protein by labeling with [³⁵S]methionine, immunoprecipitation, and analysis by SDS-PAGE and fluorography. We found no differences between the amount of synthesis or the stability of the protein produced by the various constructs (data not shown). The apparent differences in intensity of staining in the cytoplasm surrounding the nucleus with some of the constructs were due to the association of the wild-type protein with structures located at the poles that may be centrosomes, while some of the deletion mutants were distributed evenly throughout the cytoplasm. In all of these cases the nucleus/cytoplasm ratio of the exogenous protein at stage 7 was similar. Also, when first detected in the nucleus the protein is localized in an asymmetric pattern (see Fig. 2 B) due to its association with the chromosomes which are located to one side of the nu-

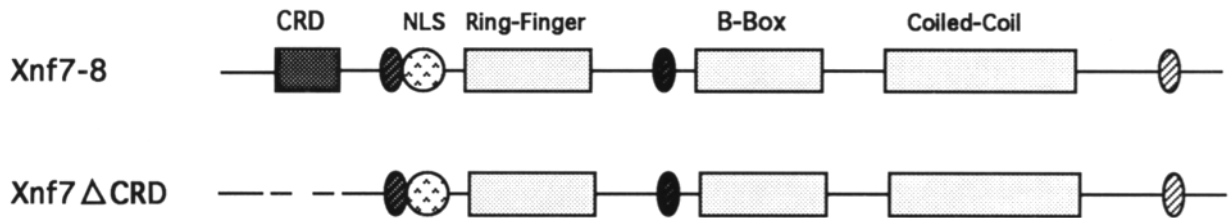
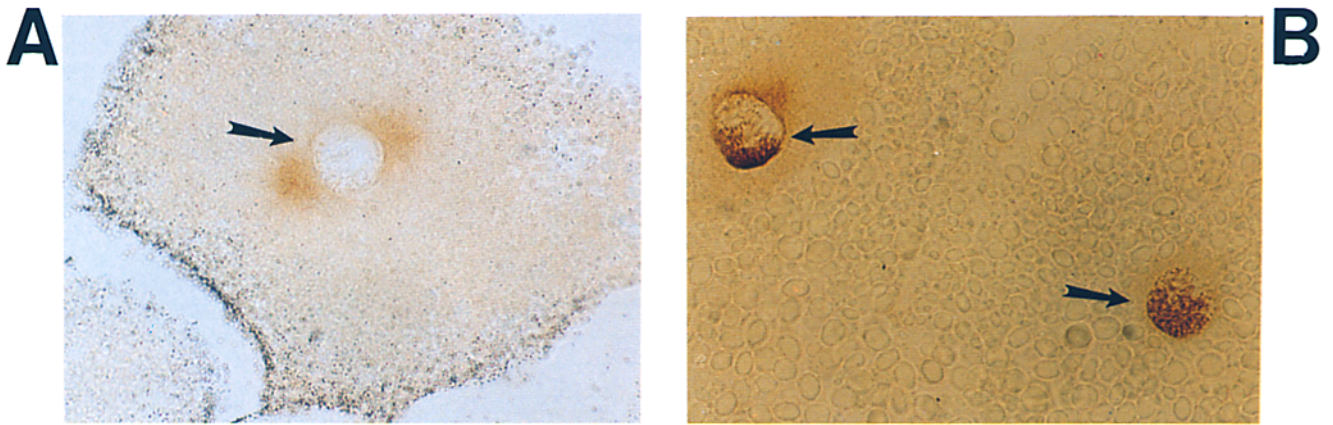
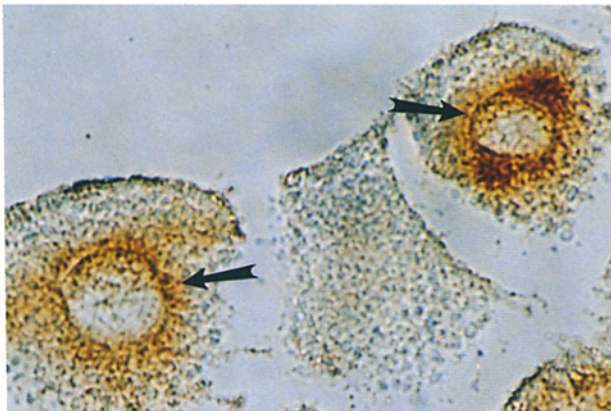


Figure 2. Role of the CRD in cytoplasmic retention. In vitro transcribed RNAs from the *xnf7-8* and *xnf7ΔCRD* constructs were injected into fertilized eggs. *A* shows the cytoplasmic localization of the *xnf7-8* protein at stage 8; *B* shows the nuclear localization of the *xnf7ΔCRD* protein by stage 7. Arrows point to nuclei. Diagram of constructs is shown below.

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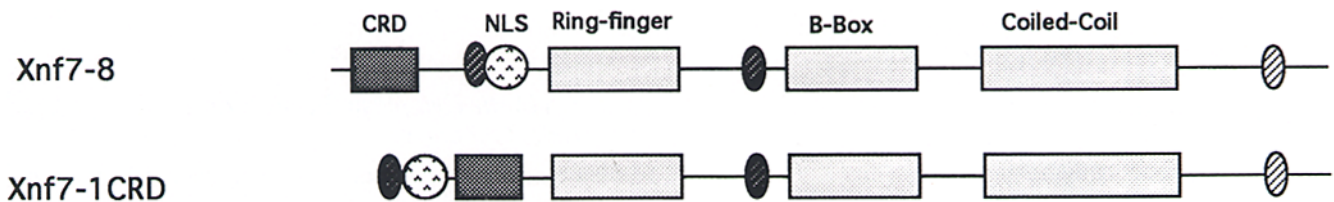
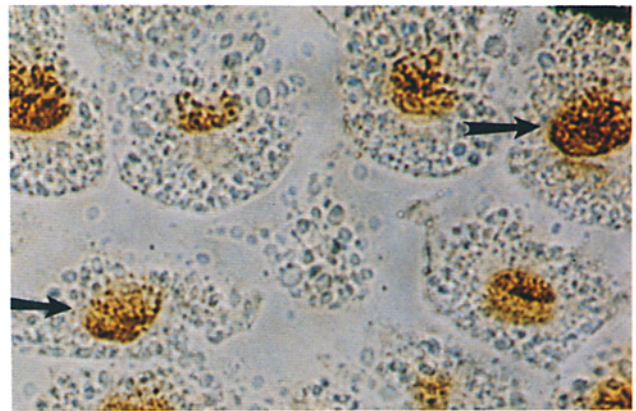
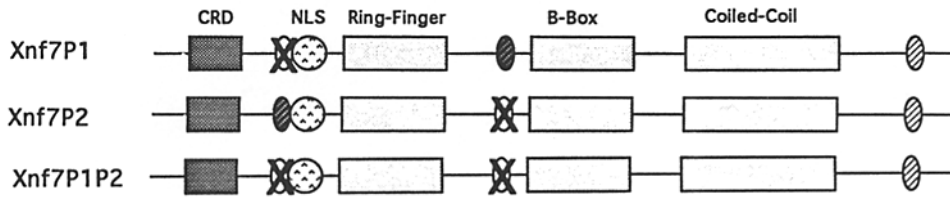


Figure 3. The CRD sequence functions at a different position in *xnf7*. In vitro-transcribed mRNA from the *xnf7-1* CRD construct was injected into the cytoplasm of fertilized eggs. Analysis for nuclear localization was performed at stage 7 and 9. The arrows point to the nuclei and the diagram of the constructs is shown below.

A



cleus as determined by DAPI staining. Xnf7 later acquires a more uniform staining pattern throughout the nucleus.

CRD Function Is Not Dependent upon Surrounding NH₂-terminal Sequences

We tested the ability of the 22-amino acid CRD to function autonomously by placing the CRD between the NLS and the first zinc finger domain in the xnf7-1NLS construct (xnf7-1CRD). This eliminated the possibility that sequences adjacent to the 22-amino acid CRD at the NH₂ terminus were involved in its function. Fig. 3 shows that the xnf7-1NLS-CRD protein was retained in the cytoplasm until the MBT. Thus, deleting other NH₂-terminal sequences surrounding the CRD did not effect its ability to function.

We also tested the ability of the CRD to function autonomously in a heterologous protein, pyruvate kinase (pk), by making a construct containing the pk xnf7 NLS and CRD with the pk cDNA. The protein from this construct was detected in nuclei prior to the MBT and was not retained in the cytoplasm (data not shown). This suggests that the CRD sequence may require the proper context within the xnf7 protein for proper functioning. Alternatively, it is possible that the positioning of the CRD in the pk protein may have interfered with its function. Further analysis will be required to fully explain this result.

CRD Function Is Dependent upon the Phosphorylation of One of Two Sites within xnf7

Previous studies showed a tight correspondence between the hyperphosphorylation of xnf7 which occurs at maturation and its retention in the cytoplasm (Miller et al., 1991). We identified a phosphorylation site at threonine 103 (referred to as site 1) and potential sites at threonines 209, 212, and 218 (referred to as site 2), based on homologies to consensus phosphorylation sequences and phosphoamino acid mapping analysis (Reddy et al., 1991). In the following experiments we use a functional assay to determine the role of phosphorylation in the cytoplasmic retention of xnf7 by creating a series of point mutations changing these threonines to alanines to inhibit phosphorylation of these sites (Fig. 4 A).

After injection of RNA from site 1 mutants (xnf7P1) or site 2 mutants (xnf7P2), mutant protein behaved like the wild-type xnf7 protein, entering the nucleus at MBT (Fig. 4 B). However, when both sites 1 and sites 2 were mutated in the

Figure 4. The mutation in phosphorylation sites effects the nuclear retention of xnf7. (A) Diagram of the constructs used to make the in vitro transcribed RNA; (B) examples of embryos at stage 7 and stage 9 injected with: the xnf7P1 mutant in which the thr of the first phosphorylation site was mutated to ala, the xnf7P2 mutant in which the three thr at positions 209, 212, and 218 were mutated to ala, and xnf7PIP2 in which both phosphorylation sites were mutated.

same molecule (xnf7PIP2) the protein entered the nucleus by stage 6-7 (Fig. 4 B). These data strongly suggest that the phosphorylation of either site 1 or site 2 was required for the proper function of the CRD and that mutation of both phosphorylation sites resulted in the loss of function of the CRD. They also indicate that the function of phosphorylation sites 1 and 2 is redundant in the function of the CRD. In addition, this experiment indicates that xnf7, which is normally phosphorylated during oocyte maturation, can be phosphorylated during early cleavage stages.

To further verify the importance of phosphorylation of sites 1 and 2 we created a mutant xnf7 protein by replacing the threonines at site 2 with glutamic acid residues (xnf7Thr-Glu). The rationale for this experiment was that the negatively charged glutamic acids would mimic the phosphorylation of site 2 and xnf7 would remain cytoplasmic. Fig. 5 shows that the protein produced by xnf7Thr-Glu mutant was indeed retained in the cytoplasm at stage 10, well past the MBT, demonstrating the importance of the phosphorylation of this site in the functioning of the CRD.

It is possible that removal of the CRD sequence effects the phosphorylation of xnf7 resulting in early entry of the protein into the nucleus. To determine this we tested the ability of mutant xnf7 lacking the CRD sequence (xnf7ΔCRD) to be hyperphosphorylated during oocyte maturation by injecting synthetic mRNA from this mutant into oocytes. Oocytes were incubated in ³²P and matured by adding progesterone. Phosphorylated xnf7 was analyzed by immunoprecipitation with the L24 polyclonal antibody against xnf7 and autoradiography according to Reddy et al. (1991). Fig. 6 shows that the endogenous wild-type xnf7 and the exogenous xnf7ΔCRD mutant were hyperphosphorylated during oocyte maturation. Although we did not analyze the precise sites phosphorylated during maturation these results demonstrate that the removal of the CRD sequence does not effect hyperphosphorylation of xnf7 which most likely includes sites 1 and 2.

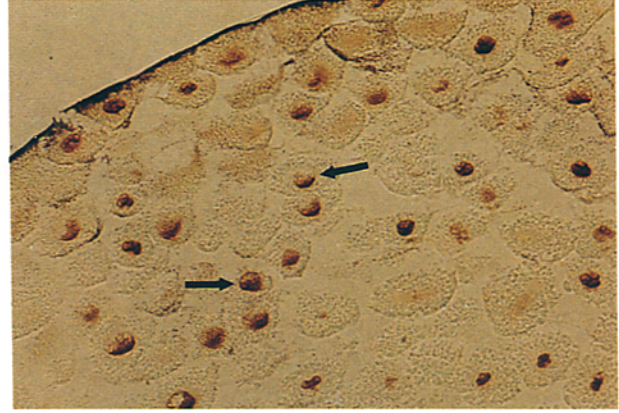
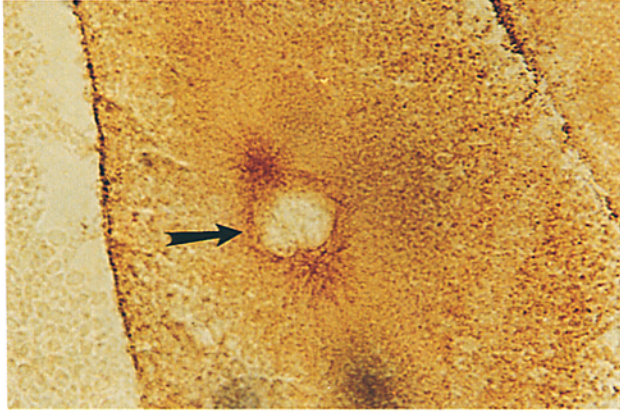
Retention in the Cytoplasm Involves an Anchoring Mechanism

There are several possibilities regarding the mechanism involved in the cytoplasmic retention of xnf7. These include an intramolecular mechanism in which the protein structure changes upon phosphorylation, placing the CRD over the NLS to mask its function, or a mechanism in which hyper-

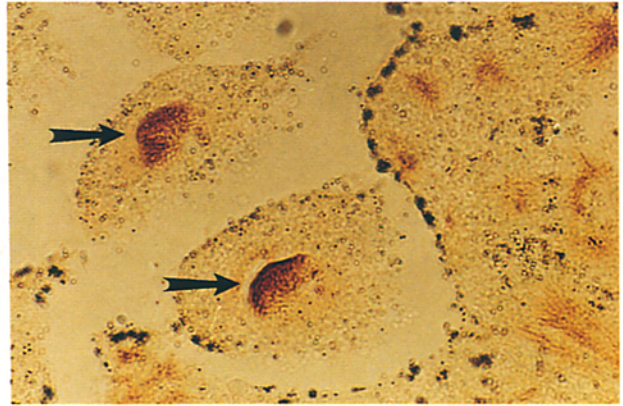
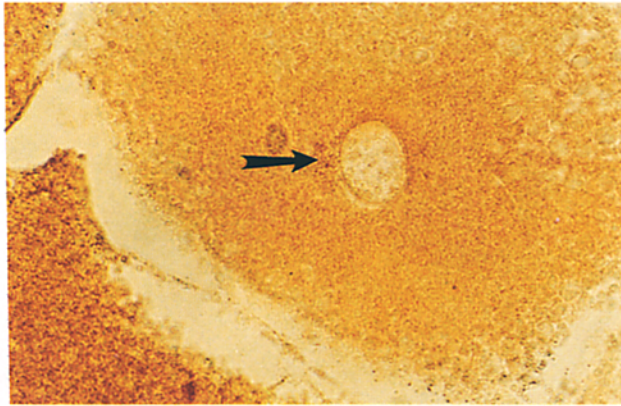
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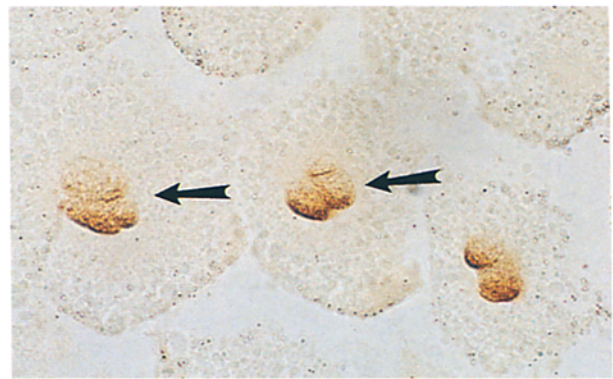
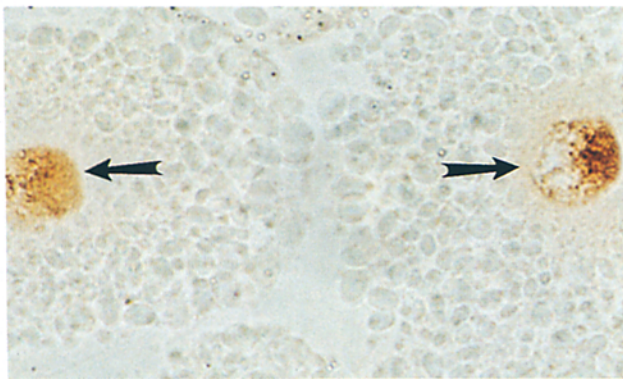
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Xnf7P1



Xnf7P2



Xnf7P1P2

Figure 4.

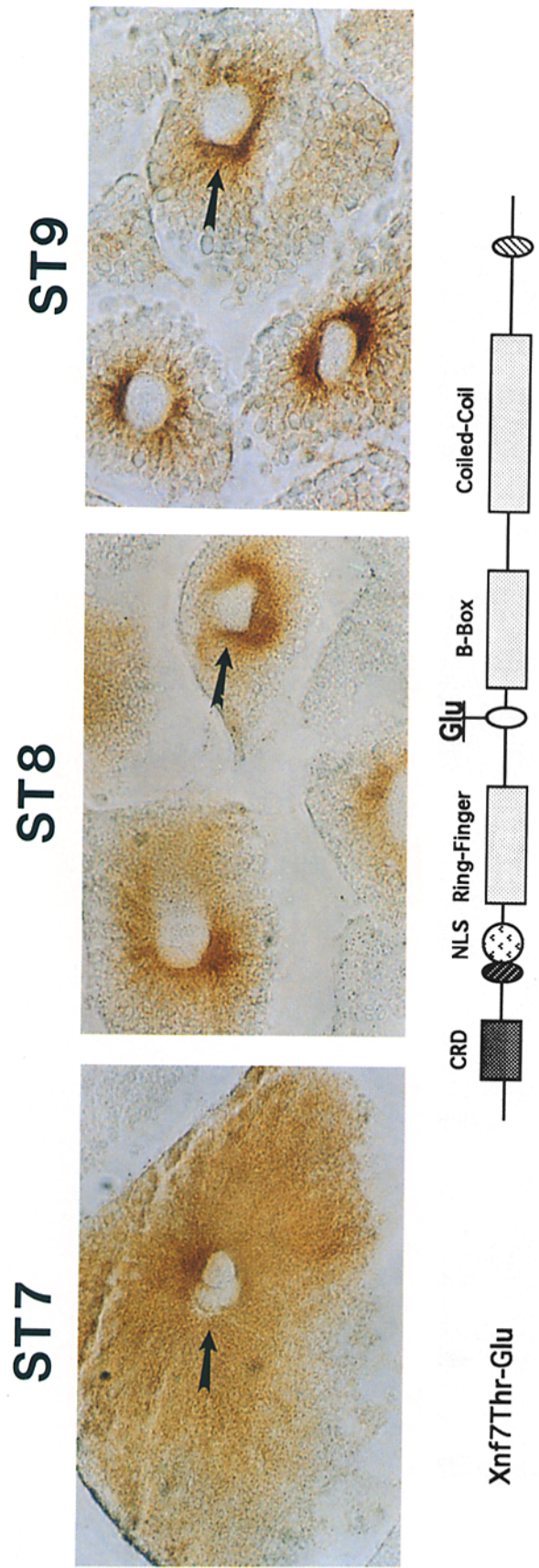


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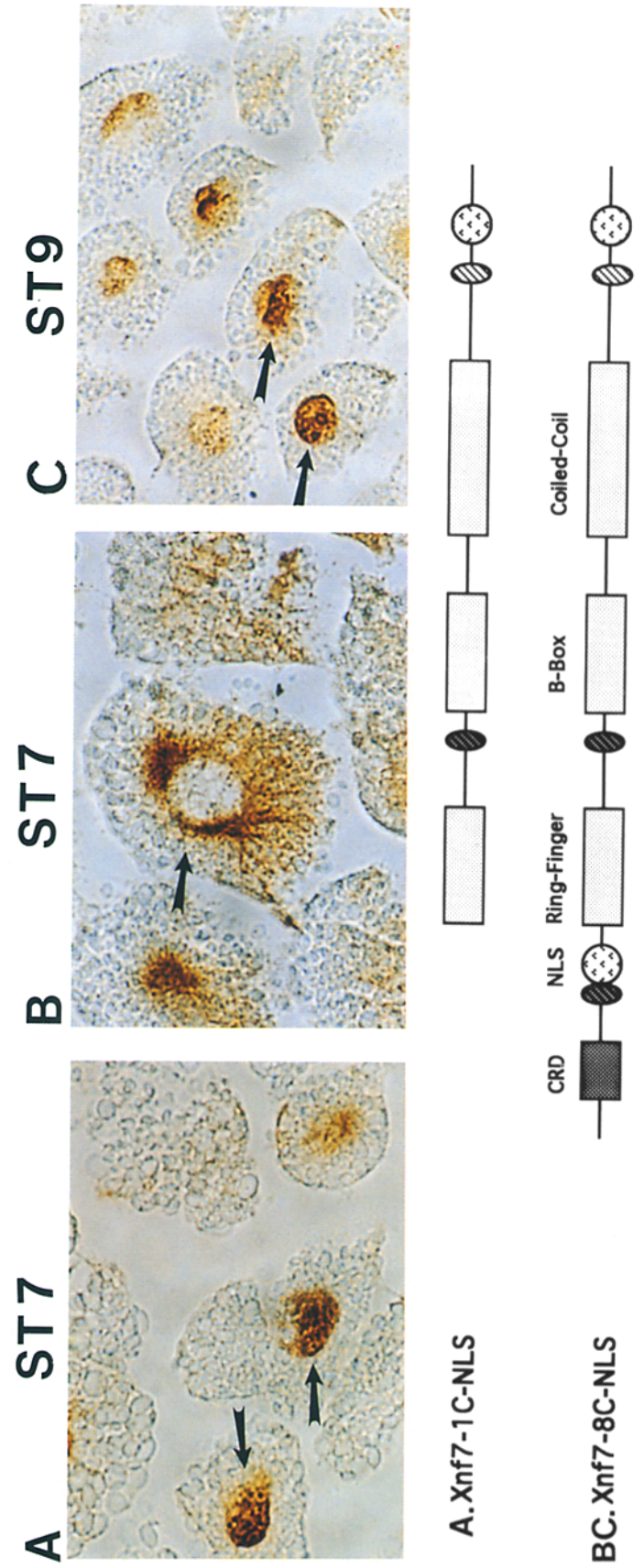


Figure 7.

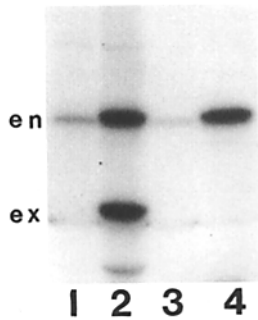


Figure 6. Xnf7 Δ CRD mutant is phosphorylated during oocyte maturation. Oocytes were injected with xnf7 Δ CRD mutant mRNA and incubated with 0.5 mCi/ml 32 P-carrier free inorganic phosphate for 4 h. Oocytes were matured in vitro with progesterone. After maturation 10 oocytes from each group were homogenized and endogenous and exogenous xnf7 was immunoprecipitated using the L24 polyclonal antibody that recognizes both forms

(Reddy et al., 1991). The endogenous and exogenous forms can be distinguished from one another on the basis of mobility on SDS-PAGE gels. The radioactive immunoprecipitates were visualized by autoradiography. (Lane 1) endogenous (*en*) xnf7 and exogenous mutant xnf7 Δ CRD (*ex*) protein from oocytes prior to maturation; (lane 2) protein from the same group of oocytes as in lane 1 after oocyte maturation; (lane 3) endogenous xnf7 protein from noninjected oocytes before maturation; (lane 4) protein from the same group of oocytes as in lane 3 after oocyte maturation.

phosphorylation anchors xnf7 to a cytoplasmic anchor protein through interaction with the CRD.

To distinguish between these two alternatives we created a construct in which an extra NLS was placed at the carboxy-terminus of the xnf7-8 protein (xnf7-8C-NLS). The expectation was that if cytoplasmic retention was due to an intramolecular mechanism the extra NLS would result in early entry into the nucleus; if on the other hand an anchoring mechanism was involved, the extra NLS would have no effect and the protein would enter the nucleus at the MBT. As a control for the proper function of the NLS at the COOH terminus we placed the NLS on the xnf7-1 construct, which lacked the CRD and NLS at the NH₂ terminus (xnf7-1C-NLS).

Fig. 7 A shows that the xnf7-1C-NLS protein lacking the CRD entered the nucleus by stage 6-7, indicating that the NLS placed at the COOH terminus functioned efficiently. However, the xnf7-8C-NLS protein product which possessed the CRD and the COOH-terminal NLS was retained in the cytoplasm until MBT, suggesting that an anchoring mechanism was responsible for cytoplasmic retention (Fig. 7, B and C).

Discussion

The retention of xnf7 in the cytoplasm before the MBT illustrates an important level of regulation of nuclear proteins and is one of the few reported examples of selective nuclear transport used in a developing system. We have identified a *cis*-acting domain, the CRD, located between amino acids 52

and 73 that is required for the retention of xnf7 in the cytoplasm. The CRD likely functions by interacting with an anchor protein in the cytoplasm, and its function is dependent upon the phosphorylation of one of two phosphorylation sites on the xnf7 protein.

Selective nuclear transport has been demonstrated in a number of instances of proteins involved in cell regulatory processes including the SW15 gene product whose nuclear or cytoplasmic localization is regulated during the cell cycle by the phosphorylation state of the protein (Moll et al., 1991); lamin B₂ in which the phosphorylation of protein kinase C sites inhibits its nuclear import (Hennekes et al., 1993); Simian Virus 40 T antigen in which phosphorylation effects its entry into nuclei (Jans et al., 1991); and p53, a tumor suppressor gene, whose function may be regulated by retention in the cytoplasm (Moll et al., 1992; Gannon and Lane, 1991). In the case of the glucocorticoid receptor it is known that binding to the hsp 90 protein in the cytoplasm interferes with the function of the NLS and that nuclear localization is dependent upon the presence of the hormone which causes dissociation of the hsp 90 from the receptor (Picard and Yamamoto, 1987; for review see Pratt, 1992).

During development the dorsal gene product in *Drosophila* is present in all regions of the embryo, but in the ventral region it is localized in the nucleus, while in the dorsal region it is in the cytoplasm. The variation in nucleocytoplasmic compartmentalization of the dorsal gene product in different regions of the embryo may be due to its interaction with the cactus gene product resulting in anchoring of the dorsal protein in the cytoplasm or the masking of the NLS. 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 (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989).

Interestingly, both NF κ B and dorsal are related in that they share homology with the c-rel protooncogene (Kieran et al., 1990; Ghosh et al., 1990). It is known that the I κ B partner of NF κ B contains a number of ankyrin repeats that are associated with proteins that interact with the cytoskeleton. Thus, one possible mechanism for cytoplasmic retention of NF κ B, and perhaps, dorsal is that their partners interact with the cytoskeleton to retain them in the cytoplasm. Release is triggered by changes in phosphorylation of either the anchor or nuclear protein. However, recent evidence shows that the cytoplasmic retention of NF κ B may be due to a mechanism in which binding to I κ B masks the function of the NLS. Therefore, cytoplasmic retention of NF κ B may not involve anchoring to a subcellular structure or the cytoskeleton (Blank et al., 1991; Begg et al., 1992; Henkel et al., 1992).

Xnf7 is phosphorylated at oocyte maturation and remains predominately in the hyperphosphorylated state until the

Figure 5. The mutation of phosphorylation site 2 to glutamic acid results in retention of xnf7 in the cytoplasm. Synthetic mRNA from the xnf7thr-glu mutant was injected into fertilized eggs. The developing embryos were analyzed at stage 7, 8, and 9. The arrows are pointing to the nuclei and the construct is diagrammed below.

Figure 7. The xnf7 protein is retained in the cytoplasm through an anchoring mechanism. Synthetic mRNAs produced from the xnf7-1C-NLS and the xnf7-8C-NLS construct were injected into the cytoplasm of fertilized eggs. (A) The accumulation of the mutant xnf7-1C-NLS protein in nuclei at stage 7; (B) the mutant xnf7-8C-NLS was not detected in nuclei at stage 7; however, (C) the protein is detected in the nuclei at stage 9. Arrows point to nuclei and the constructs are diagrammed below.

MBT, when it is dephosphorylated coincident with nuclear entry (Miller et al., 1991). This tight correlation between hyperphosphorylation and retention in the cytoplasm suggested that phosphorylation is involved in its localization. Indeed, *xnf7* is phosphorylated *in vivo* at numerous sites throughout the molecule. It acquires a basal level of phosphorylation when it enters the GV during oogenesis and a second level of hyperphosphorylation during oocyte maturation (Li et al., 1993; Li, X., and L. D. Etkin, unpublished observations). Sites throughout the entire molecule most likely including threonines at both site 1 (threonine 103) and site 2 (threonines 209, 212, and 218) are phosphorylated *in vivo* during oocyte maturation (Reddy et al., 1991; Li, X., and L. D. Etkin, unpublished observations).

We have two lines of evidence indicating that phosphorylation of these two sites within the *xnf7* protein is important in the function of the CRD sequence. The first is that mutations (thr to ala) of both sites results in early entry into the nucleus. The second is that changing the threonines of the second site to glutamic acids results in the retention of *xnf7* in the cytoplasm. The fact that the two phosphorylation sites can substitute for one another in the functioning of the CRD suggests a redundancy of function. This also implies a mechanism involving cross-talk between the phosphorylation sites and the CRD sequence. The *xnf7*ΔCRD protein is phosphorylated during oocyte maturation suggesting that deletion of the CRD does not affect the phosphorylation of the protein.

When a second NLS is added to the COOH-terminal end of NFκB it enters the nucleus suggesting that cytoplasmic retention may be due to a mechanism in which IκB binds to the NFκB molecule and masks the NLS (Begg et al., 1992). Therefore, the cytoplasmic retention of NFκB may not involve anchoring to a subcellular structure or the cytoskeleton. Our data showing that the protein produced from the *xnf7*-IC-NLS construct in which a second NLS is added to the COOH-terminal end of the molecule is retained in the cytoplasm before the MBT strongly suggests that *xnf7* retention is due to the physical interaction of the CRD with a fixed anchor protein or subcellular structure. Our model (Fig. 8) is that the phosphorylation of one of the two domains (at site 1 or site 2) results in a structural change in the molecule that exposes the CRD. This altered structure permits interaction of the CRD with the cytoplasmic anchor. When the embryo reaches the blastula stage, *xnf7* is dephosphorylated and the protein is released from the anchor and is free to enter the nucleus. The changes in phosphorylation of *xnf7* indicates that a signal transduction pathway involving both kinase and phosphatase activities regulates the localization of proteins such as *xnf7*. The elucidation of this pathway will be important in furthering our understanding of early developmental events.

There are several other late shifting proteins such as nucleolin, a component of the nucleolus (Messmer and Dreyer, 1993), and PwA33, a potential RNP protein that is a member of the B-box zinc finger family (Bellini et al., 1993), that have been cloned and characterized. PwA33 possesses CRD sequence that is 97% identical to *xnf7*, however, it is not known whether PwA33 is a phosphoprotein. Nucleolin does not possess a domain that has sequence homology to the *xnf7* and PwA33 CRD sequence but is hyperphosphorylated when located in the cytoplasm and dephos-

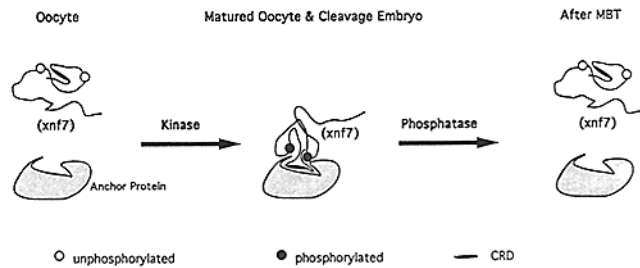


Figure 8. *Xnf7* is retained in the cytoplasm during early cleavage stages. During this time it is phosphorylated at sites 1 and 2 and bound to an anchor protein through the CRD. Near the time of the MBT *xnf7* is dephosphorylated resulting in its release from the anchor protein and the utilization of the NLS. It accumulates in the nuclei at a rapid rate.

phorylated when it enters the nucleus at the MBT (Messmer and Dreyer, 1993). In nucleolin there may be a structural motif similar to the *xnf7* CRD that functions in cytoplasmic retention or alternatively there may be several independent mechanisms involved in the late shifting proteins which results in their retention properties. The answer to this question will have to await the cloning of the genes for other late shifting proteins in amphibians. However, it is evident that cytoplasmic retention is an important strategy used to regulate the function of many transcription factors, oncoproteins, and other nuclear proteins during development and cellular differentiation.

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