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Finely Tuned Regulation of Cytoplasmic Retention of *Xenopus* Nuclear Factor 7 by Phosphorylation of Individual Threonine Residues

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***Xenopus* nuclear factor 7 (xnf7) is a maternal gene product that functions in dorsal/ventral patterning of the embryo. The xnf7 protein is stored in the oocyte nucleus germinal vesicle in a hypophosphorylated state. At oocyte maturation, xnf7 is hyperphosphorylated and released into the cytoplasm, where it is anchored until the midblastula stage, when it is dephosphorylated and enters the nucleus. We demonstrated that cytoplasmic anchoring of xnf7 was regulated by changes in the phosphorylation status of four threonines within two sites, site 1 (Thr-103) and site 2 (Thr-209, Thr-212, and Thr-218), which function in an additive manner. A mutant form of xnf7 (xnf7thr-glu) in which the threonines at sites 1 and 2 were mutated to glutamic acids to mimic a permanent state of phosphorylation was retained in the cytoplasm in oocytes and embryos through the gastrula stage. The cytoplasmic form of xnf7 was detected in a large 670-kDa protein complex probably consisting of xnf7 and several other unknown protein components. Anchoring of xnf7 was not dependent on association with either microtubule or microfilament components of the cytoskeleton, since treatment with cytochalasin B and nocodazole did not affect cytoplasmic retention. Both wild-type xnf7 and xnf7thr-glu form dimers in the yeast two-hybrid system; however, homodimerization was not required for cytoplasmic retention. We suggest that the cytoplasmic retention of xnf7 depends on the phosphorylation state of the protein whereas the cytoplasmic anchoring machinery appears to be constitutively present in oocytes and throughout development until the gastrula stage.**

Selective transport of proteins into the nucleus is an important mechanism by which the function of nuclear proteins is regulated (11, 15, 32, 38, 39, 43). It is clear that in many instances phosphorylation is a critical step in the cytoplasmic retention or nuclear translocation of a large number of proteins, as demonstrated in the cases of *Drosophila* dorsal (36, 37, 40), mammalian NF-κB (1, 3, 13, 19), yeast SWI5 (31), lamin B2 (14), and simian virus 40 T antigen (16).

Xenopus nuclear factor 7 (xnf7) is a maternally expressed putative transcription factor that is retained in the cytoplasm until the midblastula transition (MBT), when it reenters the embryonic nucleus (8, 25, 26, 29, 30, 35). xnf7 belongs to a family of proteins which possess a tripartite motif containing the RING and B-box zinc fingers and a coiled-coil motif. The core group of this family consists of xnf7 (35), rfp (41), RPT-1 (33), PwA33 (2), and SS-A/Ro (5), which all possess the simple tripartite structure consisting of one RING finger, one B box, and a coiled-coil domain. xnf7, rfp, RPT-1, and PwA33 are all nuclear proteins and may be involved in transcriptional regulation or regulation of chromatin structure. Other members such as PML (promyelocytic leukemia protein) (7, 17, 18) and T-18 (27) also have an additional B-box domain (B2), while ataxia-telangiectasia group D (23) and 1A1.3B (ovarian cancer marker) (4) possess only the B-box and coiled-coil domains. Interestingly, the product of the recently cloned breast cancer susceptibility gene *BRCA1* (28), which possesses only the RING finger domain, is closely linked to 1A1.3B, which has the B-box and coiled-coil domains, suggesting that they may have evolved from the same gene. Several of these proteins function as oncogenes when the tripartite domains are recombined with DNA segments from several other genes (12, 18, 34). Also, many of these proteins function as dimers, with the tripartite domain playing a key role in protein-protein interaction. It was recently shown that there was a correlation between the cytoplasmic localization of the BRCA-1 protein, which is normally nuclear, in cells of breast cancer patients (6), while we have shown that there are differences in the nuclear/cytoplasmic distribution of the rfp protein in different cell lines (4a). This finding suggests that nuclear/cytoplasmic partitioning may be a common mechanism of regulation utilized by this related group of proteins.

When xnf7 is released into the cytoplasm during oocyte maturation, it is hyperphosphorylated; it is dephosphorylated coincident with nuclear reentry at the MBT. We previously showed that cytoplasmic retention of xnf7 was due to the presence of a 22-amino-acid (amino acids 52 to 73) *cis*-acting cytoplasmic retention domain (CRD) whose function is regulated by the phosphorylation of threonines at two sites (site 1 [Thr-103] and site 2 [Thr-209, -212, and -218]) within the protein. This was demonstrated by mutating the threonines at the phosphorylation sites to alanines, which resulted in entry of xnf7 into the nucleus prior to the MBT. It was determined that the cytoplasmic retention of xnf7 was due to intermolecular interactions tethering the protein to a cytoplasmic anchor (25).

In the present study, we used xnf7 as a model to further characterize the phenomenon of cytoplasmic retention. Our findings suggest that the phosphorylation of xnf7 is a key ele-

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ment in its cytoplasmic retention and that there is an exquisitely finely tuned mechanism in which the phosphorylations of four individual threonines function in an additive manner in retention. In addition, the cytoplasmic form of xnf7 was associated with a large cytoplasmic protein complex of approximately 670 kDa, and its retention was not dependent on microtubule or microfilament cytoskeletal components. The ability of a mutant form of xnf7 that lacks the coiled-coil domain and cannot form homodimers to be retained in the cytoplasm indicates that homodimerization is not necessary for cytoplasmic retention.

MATERIALS AND METHODS

Expression vectors. xnf7 mRNA was synthesized in vitro from a vector that consisted of pBS, 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. The T7 viral coat peptide was used as an epitope tag to distinguish the exogenous xnf7 from the endogenous protein with polyclonal antibodies (25–27). All of the phosphorylation mutants were generated by the PCR splicing by overlapping extension technique and partially sequenced to ensure that no mutations were introduced by PCR (see reference 26 for details).

Oocyte injections and metabolic labeling of injected oocytes. To obtain oocytes, female frogs were anesthetized in 0.1% 3-aminobenzoic acid ethyl ester (Sigma), which was neutralized with sodium bicarbonate. A section of the ovary was surgically removed, and stage VI oocytes were manually defolliculated and maintained in modified Barth's saline. Oocytes were injected with 1 to 10 ng of RNA (25). Injected oocytes were incubated at 18°C in modified Barth's saline for different periods of time in the presence of [^{35}S]methionine at a final concentration of 1 mCi/ml. Usually 25 to 50 oocytes were injected for each sample. To obtain embryos, females were injected with 800 IU human chorionic gonadotropin, and eggs were collected the next day. Eggs were fertilized in vitro (9).

Analysis of protein compartmentalization. Injected and labeled oocytes were manually dissected into germinal vesicles (GV) and cytoplasms. Protein extracts were prepared by homogenization of GV and cytoplasms in a buffer consisting of 10 mM Tris-HCl (pH 8.0), 10 mM dithiothreitol (DTT), and 5 mM EDTA followed by centrifugation. Supernatants taken from 25 GV and cytoplasms were adjusted to final concentrations of 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), and 10 mM Tris-HCl (pH 7.2). Antisera (1:50 dilution of the anti-xnf7 polyclonal antibody L24 and 1:500 dilution of the polyclonal antibody against the T7 coat protein) was used to immunoprecipitate the recombinant xnf7 protein. After 1 h of incubation with the antisera, 50 μl of a 50% *Staphylococcus* A slurry (Sigma) was added to absorb the immune complexes. The precipitates were washed with washing buffers A (1 M NaCl, 0.01 M Tris-HCl [pH 7.2], 0.1% Nonidet P-40 [NP-40]), B (0.1 M NaCl, 1 mM EDTA, 0.01 M Tris-HCl [pH 7.2], 0.1% NP-40, 0.3% SDS), and C (0.01 M Tris-HCl [pH 7.2], 0.01% NP-40). The pellet was solubilized in 50 μl of 2× SDS sample buffer, boiled, and separated by SDS-polyacrylamide gel electrophoresis (PAGE) (10% acrylamide). The sample loading buffer consisted of 50 mM Tris (pH 6.8), 2% SDS, 0.1% bromophenol blue, 20% glycerol, and 100 mM DTT. Protein was detected by fluorography.

Immunoperoxidase staining of sections. Embryos were fixed in 100% methanol at 4°C overnight, embedded in Paraplast Plus (Monoject), and serially sectioned into 10-μm sections. Immunoperoxidase staining was carried out as described by Li et al. (25). Deparaffinized and hydrated sections were bleached in 6% hydrogen peroxide in methanol for 20 to 30 min. Following two 10-min phosphate-buffered saline (PBS) washes, nonspecific staining was blocked with a 1-h incubation in a blocking buffer consisting of 10% goat serum and 3% bovine serum albumin in Tris-buffered saline with 0.5% Tween 20. Sections were incubated for 1 h in a 1:50 to 1:200 dilution of polyclonal antibody L24 (35) in blocking buffer and then washed two times for 10 min each time 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 two times for 10 min each time in PBS. The color reaction was initiated with 1 mg of diaminobenzidine per ml–0.03% H_2O_2 in PBS for 5 min and was stopped by two 5-min washes with water. The sections were counterstained with either hematoxylin or azure B and mounted with Permount (Fisher) or a 1:1 dilution of PBS with glycerol.

Treatment with various inhibitors. *Xenopus* oocytes or embryos were treated by incubation in cytochalasin B (25 μg/ml) and nocodazole (10 μg/ml). Treatment of oocytes with this dose of nocodozole inhibits the microtubule-dependent translocation of several maternal RNAs to the vegetal cortex (21, 21a, 46), while treatment with this dose of cytochalasin B affects anchoring of these RNAs (21, 46).

Size exclusion chromatography. Dejellied eggs were homogenized in an equal volume of EB buffer (44) plus 5 mM DTT, 1 mM ATPγS, 0.5 μM okadaic acid, and protease inhibitors (50 ng each of leupeptin, chymostatin, and pepstatin per ml; Boehring Mannheim Biochemicals) (22). The homogenate was centrifuged at 40,000 rpm at 4°C for 1 h in a Beckman Ti50 rotor. The clear layer between the lipid and yolk was removed and stored at 4°C for 1 day for thiophosphorylation. This material was fractionated by fast protein liquid chromatography (FPLC) on a Superose 6 gel filtration column at 4°C in EB buffer containing 20 mM NaF, 50 μM ATPγS, and 1 μM DTT. Fractions of 0.5 ml were collected and analyzed by SDS-PAGE and Western blotting (immunoblotting). Bio-Rad gel filtration standards were used to calibrate the column.

Two-hybrid analysis of xnf7 dimerization. A fragment of the xnf7 cDNA encoding the RING finger, the B box, and the coiled-coil domain was subcloned into the vector pGBT9 (Clontech), containing the GAL4 DNA binding domain, and the vector pGAD424 (Clontech), containing the GAL4 activation domain. *Saccharomyces cerevisiae* SFY526 (Clontech) was used as the reporter. Yeast cells were grown at 30°C in rich medium (YEPD; 1% yeast extract, 2% Bacto Peptone, 2% glucose, 0.1 mg of adenine per ml) or synthetic minimal medium for selection and transformation. β-Galactosidase activity was assayed according to the standard protocol (42). The filters were left at room temperature for 10 min to 2 h for color to develop. β-Galactosidase activities of transformed yeast strains were quantitated by growing cells to mid-log phase. Cells were collected by centrifugation and permeabilized, and β-galactosidase activity was determined according to the standard protocol (42). The β-galactosidase activity of each transformant was compared with the activity of wild-type xnf7.

RESULTS

Exogenous xnf7thr-glu mutant proteins are differentially retained in the cytoplasm of injected oocytes. Previously we showed that wild-type exogenous xnf7 translocated very efficiently into the GV of oocytes and was retained in the cytoplasm of embryos until the MBT through a tethering mechanism that anchored it to a cytoplasmic anchor (24–26). Retention in the cytoplasm involved a 22-amino-acid CRD sequence and the phosphorylation of site 1 (threonine 103) and site 2 (threonines 209, 212, and 218) of xnf7. To demonstrate the role of phosphorylation, we had mutated the three threonines at site 2 to glutamic acids (xnf7thr-glu2) which mimic phosphorylation and found that the mutant protein was retained in the cytoplasm of embryos past the MBT (25). In addition, mutations of site 1 and site 2 to alanines (which prevents phosphorylation) resulted in the early entry of xnf7 into the nucleus prior to the MBT (25).

We wanted to determine if the retention mechanism existed in oocytes and whether there were differences in the ability of xnf7 mutated at either phosphorylation site 1 or site 2 to be retained in the cytoplasm. To test this, we constructed the xnf7 mutants xnf7thr-glu1, in which the threonine at phosphorylation site 1 (Thr-103) was changed to glutamic acid, xnf7thr-glu2, in which the threonines at site 2 (Thr-209, -212, and -218) were changed to glutamic acids, and xnf7thr-glu, in which all four of the threonines at sites 1 and 2 were changed to glutamic acids (Fig. 1A). Synthetic mRNAs made from these constructs were injected into stage 6 oocytes. Newly synthesized proteins were labeled by incubation of injected oocytes in the presence of [^{35}S]methionine for 6 to 24 h, and their distribution was determined by dissection of oocytes into GV and cytoplasms. The protein was extracted, immunoprecipitated with either the T7 epitope tag (against exogenous xnf7) or L24 (against both exogenous and endogenous xnf7) antibody, and separated by SDS-PAGE; the protein was detected by fluorography.

Figure 1B shows an analysis of the distribution of the exogenous xnf7 protein after immunoprecipitation with the T7 epitope tag antibody. After 6 h, the wild-type exogenous protein xnf7-8 (lanes 1 and 2) was efficiently accumulated in the GV, while the mutant proteins xnf7thr-glu1 (lanes 3 and 4) and xnf7thr-glu2 (lanes 5 and 6) showed only a low level of accumulation in the GV. On the other hand, even after 24 h of incubation, the xnf7thr-glu (lanes 7 and 8) was retained in the cytoplasm, with no detectable accumulation in the GV. It was also evident that the xnf7thr-glu migrated more slowly on the gel than did the site 1 or site 2 mutant protein. This shift to a new xnf7 isoform was identical to that seen with the endogenous xnf7 at oocyte maturation and suggests that a structural

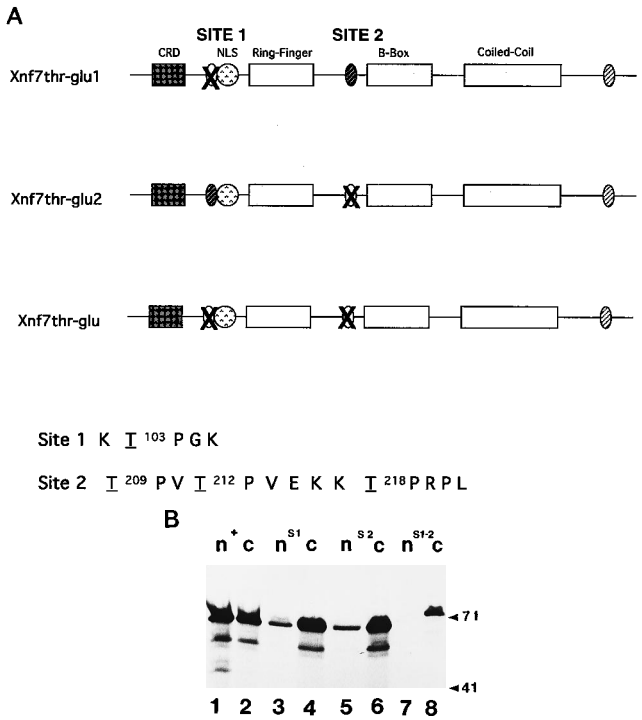

FIG. 1. Accumulation of xnf7 phosphorylation mutants in the GV of stage 6 oocytes. (A) Diagrams of mutant xnf7 molecules used in these experiments. In xnf7thr-glu1, the threonine at site 1 (residue 103) is mutated to glutamic acid; in xnf7thr-glu2, the threonines at site 2 (residues 209, 212, and 218) are mutated to glutamic acids; in xnf7thr-glu, all threonines at sites 1 and 2 are mutated to glutamic acids. The amino acid sequences of sites 1 and 2 are shown below, with the mutated amino acids underlined. RING finger and B box are the two zinc finger domains. NLS, nuclear localization signal. (B) In vitro-transcribed mRNA made from the constructs shown in panel A was injected into stage 6 oocytes, which were subsequently incubated with [^{35}S]methionine for 10 h. Proteins were extracted and immunoprecipitated with the T7 antibody, which recognizes only the exogenous xnf7. Lanes 1 and 2, GV (n) and cytoplasm (c), respectively, of oocytes injected with wild-type xnf7-8 (+); lanes 3 and 4, GV and cytoplasms from oocytes injected with xnf7thr-glu1 (site 1 [S1]); lanes 5 and 6, GV and cytoplasms from oocytes injected with xnf7thr-glu2 (S2); lanes 7 and 8, GV and cytoplasms from oocytes injected with xnf7thr-glu (S1-2). Sizes are indicated in kilodaltons.

change similar to that seen in the endogenous hyperphosphorylated form of xnf7 (30) occurred in xnf7thr-glu. This change did not occur when only one of the phosphorylation sites was mutated to glutamic acid.

The next experiment consisted of a time course to determine the kinetics of accumulation of the mutant proteins xnf7thr-glu1 and xnf7thr-glu2 in the GV. Figure 2 shows that wild-type xnf7-8 accumulated in the GV within 6 h (lanes 1 to 4), at which time there was very little mutant protein xnf7thr-glu2 in the GV (lanes 5 and 6). At 20 h, xnf7thr-glu2 reached wild-type levels in the GV (lanes 7 and 8). xnf7thr-glu1 showed a similar time course of accumulation (lanes 11 to 16). These results clearly demonstrate that the retention mechanism exists in stage 6 oocytes and that xnf7thr-glu1 and xnf7thr-glu2 accumulate more slowly in the GV than the wild-type protein xnf7-8. Our interpretation is that the phosphorylation of either site 1 or site 2 alone permits a loose association of the xnf7 protein with the cytoplasmic anchor. Also, we found that the rates of synthesis and stability of all of these mutant proteins were very similar, indicating that the differences in accumulation in the GV were not due to differences in protein synthesis or stability (data not shown).

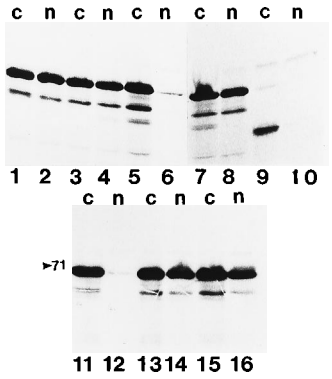

FIG. 2. Time course of accumulation of xnf7 phosphorylation mutants in the GV of stage 6 oocytes. In vitro-transcribed mRNA made from the various constructs was injected into stage 6 oocytes, which were incubated with [^{35}S]methionine for 10 h. Proteins were extracted and immunoprecipitated with the T7 antibody, which recognizes only the exogenous xnf7. Shown are the distribution of wild-type xnf7-8 between the cytoplasm (c) and GV (n) 6 h (lanes 1 and 2) and 12 h (lanes 3 and 4) after injection of mRNA, mutant protein xnf7thr-glu2 accumulation in the cytoplasm and GV after 6 h (lanes 5 and 6), mutant protein xnf7thr-glu2 accumulation in the cytoplasm and GV after 20 h (lanes 7 and 8), distribution of a mutant xnf7-1 protein that lacks the N terminus including the nuclear localization signal after 24 h (lanes 9 and 10), accumulation in the cytoplasm and GV of xnf7thr-glu1 after 6 h (lanes 11 and 12), accumulation of xnf7thr-glu1 in the cytoplasm and GV after 18 h (lanes 13 and 14), and accumulation of xnf7thr-glu1 in GV and the cytoplasm after 24 h (lanes 15 and 16). Size is indicated in kilodaltons.

Double-mutant protein xnf7thr-glu inhibits the translocation of the endogenous xnf7 protein into GV. In preliminary experiments in which mutant xnf7thr-glu was overexpressed in oocytes, we noticed that although the bulk of the maternally stored xnf7 was in the GV, all of the newly synthesized xnf7thr-glu as well as the newly synthesized endogenous xnf7 was retained in the cytoplasm. To further substantiate this finding and to test the abilities of mutant proteins xnf7thr-glu1 and xnf7thr-glu2 to inhibit translocation of endogenous xnf7 into the GV, we overexpressed both wild-type xnf7-8 and mutant proteins xnf7thr-glu1, xnf7thr-glu2, and xnf7thr-glu in stage 6 oocytes. Injected oocytes were labeled for 6 to 12 h with [^{35}S]methionine, and the xnf7 protein was immunoprecipitated from isolated GV and cytoplasms by using the polyclonal antibody L24, which recognizes both the endogenous and exogenous proteins. Figure 3 shows that when the wild-type xnf7-8 (lanes 1 and 2), mutant xnf7thr-glu1 (lanes 3 and 4), or mutant

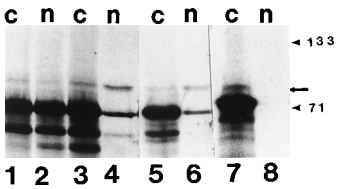

FIG. 3. Effect of phosphorylation of exogenous xnf7 on the translocation of the endogenous protein. xnf7 mRNA was injected into stage 6 oocytes, and proteins were analyzed as for Fig. 2 after 12 h except that they were immunoprecipitated with antibody L24 (35), which recognizes both exogenous and endogenous xnf7. Lanes 1 and 2, accumulation of exogenous and endogenous (arrow) xnf7 in the cytoplasms (c) and GV (n) of oocytes injected with wild-type xnf7-8 mRNA; lanes 3 and 4, accumulation of exogenous and endogenous xnf7 protein in oocytes injected with xnf7thr-glu1; lanes 5 and 6, accumulation of exogenous and endogenous xnf7 in oocytes injected with xnf7thr-glu2; lanes 7 and 8, accumulation of exogenous and endogenous xnf7 in oocytes injected with xnf7thr-glu mRNA. The difference in migration of the endogenous and exogenous xnf7 proteins is caused by the absence of 30 amino acids at the N terminus of the exogenous protein. Sizes are indicated in kilodaltons.

xnf7thr-glu2 (lanes 5 and 6) was overexpressed, the endogenous xnf7 protein accumulated in the GV. However, in oocytes overexpressing the double mutant xnf7thr-glu (lanes 7 and 8), the endogenous xnf7 protein was retained in the cytoplasm along with the exogenous protein. These results strongly support the hypothesis that the hyperphosphorylated form of xnf7 interferes with the translocation of the endogenous xnf7 protein and suggests that the mutant protein xnf7thr-glu forms dimers with the endogenous hypophosphorylated xnf7 protein.

xnf7 forms homodimers which are not required for cytoplasmic retention. Several other B-box family members such as PML and rfp form homodimers. Since overexpressed xnf7thr-glu retained the endogenous xnf7 in the cytoplasm, we suspected that xnf7 may function as a homodimer. To test this, we took advantage of the yeast two-hybrid system and placed the mutant xnf7thr-glu and wild-type xnf7-8 cDNAs within both the bait and prey vectors, which possess the GAL4 DNA binding domain and GAL4 transactivation domain, respectively (10, 42). After introducing the constructs into the appropriate yeast strain, we tested their abilities to transactivate the β-galactosidase reporter gene, which was indicative of an interaction between the molecules. The N-terminal 30 amino acids of xnf7 are acidic and have transactivation potential (26); therefore, all of the constructs used in these experiments lacked this region. The controls in Fig. 4A (samples 6 to 8) confirmed that the xnf7 molecule from these constructs could not self-transactivate the reporter gene.

Using this assay, we found that mutant xnf7thr-glu interacted with itself and also with the wild-type protein xnf7-8 (Fig. 4A, samples 2 to 4). In addition, we found that xnf7-8 also interacted with itself, indicating that it too had the ability to form dimers (sample 1). The positive control for interaction consisted of the p53 cDNA in the vector containing the GAL4 DNA binding domain (pGBT9) and the simian virus 40 large T antigen in the vector containing the GAL4 activation domain (pGAD) (sample 9). xnf7-8 or xnf7thr-glu did not interact with the GAL4 transactivation domain (samples 6 and 7), lamin (sample 8), the large T antigen, or p53 (Fig. 4B), which were used as controls for nonspecific interactions or self-activation by the xnf7 protein. Interestingly, measurements of β-galactosidase activity demonstrated that the strength of interaction between xnf7 wild-type homodimers was slightly (approximately 1.4 times) greater than that of the xnf7thr-glu mutant homodimers and very similar to that of the mutant and wild-type heterodimers (Fig. 4B). This result indicated that the degree of phosphorylation, while able to affect anchoring in the cytoplasm, did not dramatically affect homodimerization in the yeast system.

To further establish the requirement of homodimerization for cytoplasmic retention, we also tested the ability of a mutant form of xnf7 that lacked a large portion of the coiled-coil domain (xnf7Δ280-325) to form homodimers. We had previously shown that xnf7 protein made from this construct was retained in the cytoplasm until the MBT when overexpressed in injected *Xenopus* embryos (25, 26). Figure 4C shows that the level of β-galactosidase activity detected when the deletion mutant xnf7Δ280-325 was expressed in both yeast vectors is essentially the same as that in the negative controls, indicating that this mutant cannot form homodimers. In addition, there was no detectable interaction of the deletion mutant with wild-type xnf7 (Fig. 4C). Figure 4D shows the quantitation of this assay. These results demonstrate that while it is likely that xnf7 exists as homodimers, homodimerization of xnf7 was neither necessary nor sufficient for its retention in the cytoplasm.

Cytoplasmic xnf7 is associated with a high-molecular-weight protein complex. We also wanted to determine if, in addition to forming homodimers, xnf7 was associated with other proteins, some of which may play an important role in cytoplasmic retention or other biological processes. As an initial step to address this question, we determined the apparent molecular weight of xnf7 in unfertilized egg extracts when it is cytoplasmic and in oocytes when it is in the GV. These extracts were fractionated by FPLC on Superose 6 gel filtration columns (separation range, 5 to 5,000 kDa). Individual fractions were assayed for the presence of xnf7 by Western blot analysis. Figure 5 clearly shows that xnf7 from unfertilized egg extracts was present in fractions 22 to 24, which eluted with the 670-kDa protein standard. Since the predicted molecular mass of xnf7 is 80 kDa, this result suggests that xnf7 was associated not only with itself as a homodimer but also with other proteins. Interestingly, similar extracts prepared from oocytes when xnf7 is in the GV showed a large portion of the xnf7 protein associated with an extremely large complex that was in the void volume (>5,000 kDa; Fig. 5). This large complex probably consisted of xnf7 associated with the chromatin, since it is known that xnf7 is associated with the lampbrush chromosomes in oocytes (8b).

Cytoplasmic retention does not involve cytoskeletal elements. A potential mechanism by which xnf7 may be retained in the cytoplasm is through mediation of microfilament and/or microtubule cytoskeletal elements either directly or through interaction with the 670-kDa complex. To test this possibility, we treated injected oocytes with nocodazole, which disrupts microtubules, and cytochalasin B, which disrupts microfilament networks, and determined the effects of these treatments on the distribution of the endogenous xnf7 and exogenous mutant xnf7thr-glu proteins. In these experiments, we used doses of these drugs that were shown to disrupt the appropriate cytoskeletal elements (20, 21, 46) and were also shown to be effective in inhibiting the microtubule-dependent translocation and microfilament-dependent anchoring of RNAs at the vegetal cortex in oocytes. We also observed perturbations of the tubulin immunostaining patterns in oocytes treated with nocodazole, further supporting the effectiveness of this treatment (data not shown). Figure 6 shows that in oocytes treated with either nocodazole (lanes 5 and 6) or cytochalasin B (lanes 7 and 8), the xnf7thr-glu and endogenous xnf7 proteins were still retained in the cytoplasm, while both endogenous and exogenous wild-type xnf7 protein accumulated in the GV in untreated oocytes (lanes 1 to 4). These results suggest that retention does not involve an interaction with either the microtubule or microfilament components of the cytoskeleton.

xnf7thr-glu mutants are retained in the cytoplasm to different extents during development, but all forms enter the nucleus after the gastrula stage. We also tested the timing of nuclear entry of mutant proteins xnf7thr-glu, xnf7thr-glu1, and xnf7thr-glu2 during development. At stage 9, when the wild-type xnf7-8 protein had entered the nucleus, both xnf7thr-glu1 and xnf7thr-glu2 remained cytoplasmic (Table 1). However, by stage 11, the single mutants xnf7thr-glu1 and xnf7thr-glu2 had entered the nucleus whereas xnf7thr-glu was still cytoplasmic (Fig. 7; Table 1). By stage 13, xnf7thr-glu was detected within the nucleus (Table 1). These data suggest that, consistent with the results for oocytes, the single-phosphorylation-site mutants were retained in the cytoplasm during embryogenesis to a lesser degree than was the double-mutant protein xnf7thr-glu, indicating that they are more loosely bound to the cytoplasmic anchor. In addition, the fact that xnf7thr-glu entered the nucleus after stage 12 strongly suggests that the ability to anchor xnf7 in the cytoplasm is lost from the cells of the embryo following gastrulation. Western blots of the exogenous xnf7thr-glu protein at stage 14 showed that there is no apparent change

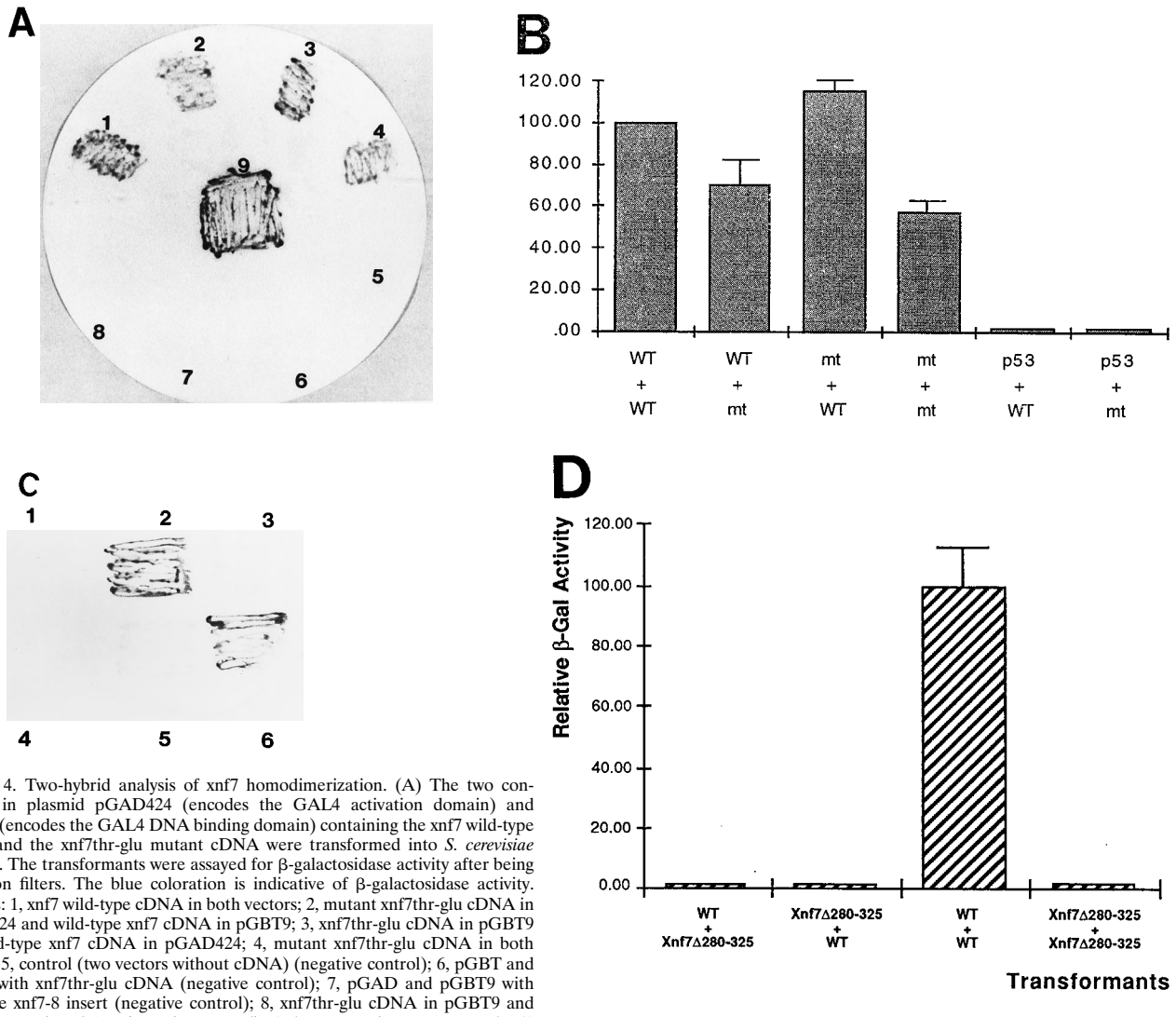

FIG. 4. Two-hybrid analysis of xnf7 homodimerization. (A) The two constructs, in plasmid pGAD424 (encodes the GAL4 activation domain) and pGBT9 (encodes the GAL4 DNA binding domain) containing the xnf7 wild-type cDNA and the xnf7thr-glu mutant cDNA were transformed into *S. cerevisiae* SFY526. The transformants were assayed for β-galactosidase activity after being grown on filters. The blue coloration is indicative of β-galactosidase activity. Samples: 1, xnf7 wild-type cDNA in both vectors; 2, mutant xnf7thr-glu cDNA in pGAD424 and wild-type xnf7 cDNA in pGBT9; 3, xnf7thr-glu cDNA in pGBT9 and wild-type xnf7 cDNA in pGAD424; 4, mutant xnf7thr-glu cDNA in both vectors; 5, control (two vectors without cDNA) (negative control); 6, pGBT and pGAD with xnf7thr-glu cDNA (negative control); 7, pGAD and pGBT9 with wild-type xnf7-8 insert (negative control); 8, xnf7thr-glu cDNA in pGBT9 and lamin cDNA in pGAD (negative control); 9, large-T-antigen cDNA and p53 cDNA in the two vectors (positive control). (B) Bar graph comparing the relative β-galactosidase activities of several of the xnf7 constructs used for panel A. WT, wild-type xnf7; mt, mutant xnf7thr-glu. WT + mt and mt + WT represent samples 2 and 3, respectively, from panel A. This analysis represents the results of four different experiments. All values are relative to the wild-type–wild-type interaction. We believe that the differences between wild-type and mutant xnf7 interaction when the cDNAs are in opposite vectors (WT + mt and mt + WT) may be due to differences in conformations of the two fusion proteins. (C) Analysis of the ability of the xnf7Δ280-325 mutant to form homodimers. Samples: 1, xnf7Δ280-325 activation vector and wild-type xnf7 in the DNA-binding-domain vector; 2, wild-type xnf7 in both vectors; 3, xnf7Δ280-325 DNA-binding-domain vector and wild-type xnf7 in the activation vector; 4, wild-type xnf7 tested with p53 (negative control); 5, xnf7Δ280-325 in both vectors; 6, p53 in the DNA-binding-domain vector with simian virus 40 large T antigen in the activation vector (positive control). (D) Quantitation of xnf7Δ280-325 activation vector with wild-type xnf7 (WT) in the DNA-binding-domain vector, xnf7Δ280-325 DNA-binding-domain vector and wild-type xnf7 in the activation vector, wild-type xnf7 in both vectors, and xnf7Δ280-325 in both vectors.

in the state of the protein that could account for its entry into the nucleus (data not shown).

We tested the effects of mutations in the individual threonines at site 2 on the timing of nuclear entry of xnf7. Table 1 shows that mutations of threonines 209, 212, and 218 (xnf7thr-glu^{209}, xnf7thr-glu^{212}, and xnf7thr-glu^{218}) individually did not affect the timing of entry; however, mutation of threonines 209 and 212 (xnf7thr-glu$^{209\text{-}212}$) together resulted in a delay in entry similar to that for mutations of all three threonines at this site. This result, in combination with the differential effects of xnf7thr-glu, xnf7thr-glu1, and xnf7thr-glu2, suggests that the effect of phosphorylation on cytoplasmic anchoring is additive.

DISCUSSION

The retention of xnf7 in the cytoplasm before the MBT illustrates an important level of regulation of nuclear protein function and is one of the few reported examples of cytoplasmic retention utilized in a developing system. We have used xnf7 as a model system whereby we can establish some of the basic principles involved in this process.

xnf7 is phosphorylated at oocyte maturation and remains predominantly in the hyperphosphorylated state until the MBT, when it is dephosphorylated coincident with nuclear entry (30). This tight correlation between hyperphosphorylation and retention in the cytoplasm suggested that phosphorylation is involved in its localization. We previously identified a *cis*-acting domain, the CRD, located between amino acids 53 and 74, that is required for the retention of xnf7 in the cytoplasm (25). Indeed, we showed that phosphorylation of site 1

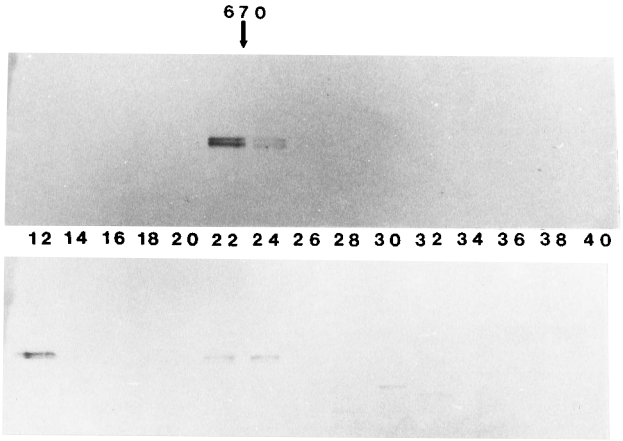

FIG. 5. Gel filtration of unfertilized egg extract (upper panel) and oocyte extract (lower panel) through a Superose 6 column. Extracts were passed through a Superose 6 column and assayed for the presence of xnf7 protein on Western blots of the different fractions. The arrow indicates the fractions in which the 670-kDa protein standard eluted from the column.

(Thr-103) and site 2 (Thr-209, -212, and -218) within the xnf7 protein is important in the function of the CRD sequence.

In the present study we have demonstrated that, in addition to being detected in embryos, the components of the retention mechanism are present in stage 6 *Xenopus* oocytes and that their function is dependent on the proper state of phosphorylation of the xnf7 protein. We have performed a detailed analysis of the effect of phosphorylation and found that it is additive in that phosphorylation of site 1 or site 2 alone was only partially effective in retention, while the double mutant xnf7thr-glu was retained in the cytoplasm until at least 24 h in oocytes and until stage 12 in embryos. Further evidence is the differential cytoplasmic retention of xnf7 mutated at one or two of the three threonines at site 2. Therefore, a critical component of the cytoplasmic retention phenomenon is the state of phosphorylation of xnf7 and the presence and activity of both kinases and phosphatases that regulate the precise state of its phosphorylation. Studies are currently under way to identify such components.

This mechanism provides an extremely sensitive means to regulate the function of a protein through the stepwise phosphorylation of a number of individual phosphorylation sites. In the case of xnf7, we know that complete phosphorylation of the four threonines within sites 1 and 2 results in a structural change detectable by the generation of new isoforms. It is

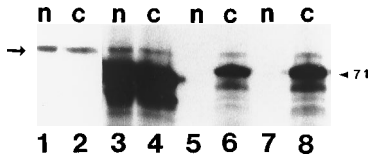

FIG. 6. Cytoplasmic anchoring of xnf7 does not involve microtubule and microfilament cytoskeletal elements. Oocytes were injected with xnf7 mRNAs and analyzed as described for Fig. 3. In some cases, they were incubated with cytochalasin B or nocodazole. Lanes 1 and 2, accumulation of endogenous xnf7 protein in GV (n) and cytoplasms (c) from noninjected oocytes after 20 h of incubation; lanes 3 and 4, accumulation of endogenous (arrow) and exogenous wild-type xnf7-8 protein in GV and cytoplasms after 20 h; lanes 5 and 6, accumulation of endogenous and exogenous xnf7thr-glu mutant protein into GV and cytoplasms after 20 h of incubation in nocodazole; lanes 7 and 8, accumulation of endogenous and exogenous xnf7thr-glu mutant protein in GV and cytoplasms after 20 h of incubation in cytochalasin B. Size is indicated in kilodaltons.

TABLE 1. Nuclear localization of wild-type and mutant proteins in embryos

Protein	Localization[a] at stage:											
	7		7.5		8.5 (MBT)		9		11		13	
	N	C	N	C	N	C	N	C	N	C	N	C
xnf7-8 (wild type)	−	+	−	+	+	+	+	−	+	−	+	−
xnf7thr-glu1	−	+	−	+	−	+	−	+	+	+	+	−
xnf7thr-glu2	−	+	−	+	−	+	−	+	+	+	+	−
xnf7thr-glu1-2	−	+	−	+	−	+	−	+	−	+	+	+
xnf7thr-glu^{209}	−	+	−	+	+	+	+	−	+	−	+	−
xnf7thr-glu^{212}	−	+	−	+	+	+	+	−	+	−	+	−
xnf7thr-glu^{218}	−	+	−	+	+	+	+	−	+	−	+	−
xnf7thr-glu$^{209\text{-}212}$	−	+	−	+	−	+	−	+	+	+	+	−

[a] Presence (+) or absence (−) in nucleus (N; GV) or cytoplasm (C).

likely that phosphorylation of the individual residues produces a more subtle change, as evidenced by the differential effect on cytoplasmic retention. These differences may be due to the effects on protein-protein interaction in the association of xnf7 with components of the 670-kDa cytoplasmic complex observed when xnf7 was in the cytoplasm.

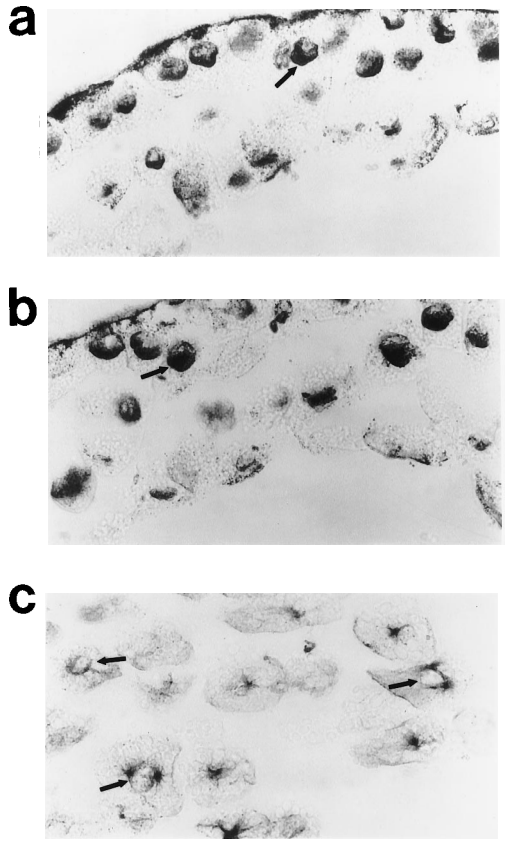

FIG. 7. Different xnf7 phosphorylation mutants are retained in the cytoplasm to different extents during development. Synthetic mRNAs from the different xnf7thr-glu mutants were injected into fertilized eggs. The developing embryos were analyzed by immunostaining with the T7 epitope tag antibody to analyze the distribution of the exogenous protein at stage 11 (gastrulation). The arrows point to the nuclei. (a) Stage 11 embryos injected with the mutant xnf7thr-glu1 mRNA; (b) embryos injected with xnf7thr-glu2 mRNA; (c) embryos injected with xnf7thr-glu mRNA.

Interestingly, using the yeast two-hybrid system, we found that phosphorylation did not drastically alter the extent of the interaction between the xnf7 wild-type or mutant homodimers. This result suggests that homodimerization of xnf7 is not sufficient for the cytoplasmic retention of the molecule, since the unphosphorylated forms of xnf7 may also exist as homodimers. Our finding that xnf7 mutants lacking a large portion of the coiled-coil domain did not form homodimers (this study) yet were retained in the cytoplasm when overexpressed in embryos also indicates that homodimerization was not necessary for cytoplasmic retention (25, 26). It is likely that the additive effect of phosphorylation of the different sites of xnf7 is the key factor in cytoplasmic retention due to the increased strength of the interaction of the protein with the anchor machinery through the *cis*-acting CRD domain at amino acids 52 to 73. Therefore, the ability of xnf7 to homodimerize was neither necessary nor sufficient for retention in the cytoplasm.

The fact that xnf7 was found in a large protein complex when anchored in the cytoplasm raises the question of the identities of the other components of the complex. We feel that the constituents of this complex represent maternal components that are also anchored within the cytoplasm along with xnf7. Thus, it is possible that the isolation and characterization of proteins associated with xnf7 when it is in the cytoplasm may identify some of the components of the anchor machinery as well as proteins that may play important roles in other biological processes during early development. We have recently identified at least three other proteins that interact with xnf7 in the cytoplasm and are likely to be components of the protein complex (45).

Overexpression of the xnf7thr-glu mutant in oocytes inhibits the entry of the endogenous xnf7 protein into the GV. Although anchoring of xnf7 did not require dimerization, it is likely that xnf7 molecules exist as dimers within the anchor complex and that tethering of the dimers within the large cytoplasmic protein complex is responsible for the ability of the xnf7thr-glu mutant to retain the endogenous xnf7 protein. We have also shown that this same phenomenon occurs in embryos and that the overexpressed xnf7thr-glu protein interacts with the endogenous xnf7, retaining it in the cytoplasm well past the MBT and thus acting as a dominant negative mutant. In fact, we recently showed that when xnf7thr-glu was overexpressed in embryos, it resulted in dorsal/ventral body axis defects, suggesting that xnf7 likely interacts with other gene products or regulates genes that are critical in the establishment of dorsal/ventral polarity (8a). Our model is that the constitutively phosphorylated (xnf7thr-glu mutant) form of xnf7 interacts with the cytoplasmic anchor machinery as a monomer and functions as a dominant negative mutant by retaining the second monomeric molecule of xnf7, regardless of its state of phosphorylation, through homodimerization.

We also observed that the single site 1 and site 2 mutants were retained in the cytoplasm to a lesser degree than the double mutant xnf7thr-glu. Interestingly, the endogenous xnf7 protein was not retained in the cytoplasm with the single mutants, whereas it was retained with the double mutant (Fig. 3). Our interpretation is that the single site 1 and site 2 mutants interact with the anchor machinery aberrantly because they assume a conformation different from that of the double mutant. This anomalous interaction with the anchor machinery probably precludes their ability to interact properly with the endogenous xnf7 protein, causing it not to be retained in the cytoplasm. The xnf7thr-glu mutant, on the other hand, assumes a conformation different from that of the single mutants, binds more tightly to the anchor, and is able to interact with the endogenous xnf7 protein, retaining it in the cytoplasm.

It is possible that xnf7, like NF-κB (1) and *Drosophila* cactus, is retained in the cytoplasm by masking of the nuclear localization signal with another protein. However, we favor the hypothesis that xnf7 is tethered to a cytoplasmic anchor, since we found that a second nuclear localization signal at the C terminus of xnf7 did not cause nuclear translocation prior to the MBT (25). The exact nature of the anchor is not known; however, we observed that xnf7 did not enter nuclei prior to the MBT in embryos treated with nocodazole or cytochalasin B, thus eliminating the microtubule and actin microfilament cytoskeletal components as potential anchors. Consistent with this finding, Li et al. (26) showed that cytoplasmic retention was independent of the association of xnf7 with the mitotic spindle in embryos. We are currently using both biochemical and genetic approaches involving fractionation and two-hybrid screens in yeast strains in an attempt to identify the components of the cytoplasmic anchoring machinery.

In addition to examples of cytoplasmic retention used to regulate the function of nuclear proteins in many other systems, there are a number of maternal nuclear proteins in *Xenopus* cells that are retained in the cytoplasm during early embryogenesis and enter nuclei at specific developmental stages (8). It is likely that many of these proteins play important roles in regulating such processes as cell cycle, DNA replication, and transcription. Additionally, the aberrant nuclear/cytoplasmic distribution of proteins such as BRCA-1 in disease states such as cancer (6) indicates the importance of understanding the mechanisms involved transport of proteins into the nucleus as well as mechanisms involved in cytoplasmic retention.

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