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Molecular Characterization and Functional Commonality of Nucleophosmin/Nucleoplasmin in Two Cyprinid Fish

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Abstract Nucleophosmin/nucleoplasmin has been studied mostly in mammals and amphibians. To clarify the characteristics and function of nucleophosmin/nucleoplasmin in teleost fish, we cloned a full-length cDNA sequence from two cyprinid fish, *Carassius auratus gibelio* and *Carassius auratus*. Molecular characterization and multiple sequence alignments suggested that they are the homologs of nucleophosmin. RT-PCR and Western blot detected a specific expression in gonads, and immunofluorescence localization revealed their distribution in oogenic and spermatogenic cells. Furthermore, a sperm decondensation function was demonstrated by immunodepletion and in vitro sperm decondensation experiments. The data suggest that the cloned nucleophosmin should share expressional and functional characterization with nucleoplasmin and therefore provide novel evidence for a functional commonality of nucleophosmin and nucleoplasmin in fish.

Keywords Oogenesis · Spermatogenesis · Sperm decondensation · *Carassius auratus gibelio* · *Carassius auratus*

Introduction

Nucleophosmin/nucleoplasmin has been studied for many years, and most of the studies are concentrated in mammals and amphibians. Their molecules contain acidic domains, multiple potential phosphorylation sites, and a putative nuclear localization signal (NLS) (Eirin-Lopez et al. 2006), and their functions are multiple and diverse (Burns et al. 2003).

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Nucleophosmin has been found in the nucleoli of somatic cells and eggs in mammals and amphibians (Burns et al. 2003; Okuda 2002; Peculis and Gall 1992; Wang et al. 1993). It modulates diverse molecular functions, such as ribosome biogenesis, centrosome duplication, cell proliferation, and tumor suppression (Grisendi et al. 2005; Lim and Wang 2006), and plays a role as a cytoplasmic/nuclear shuttle protein (Yun et al. 2003). In addition, it can act as a histone chaperone and can facilitate histone H2A storage, since *Xenopus* nucleophosmin preferentially binds the (H3–H4)2 tetramer in vitro (Namboodiri et al. 2004). Nucleoplasmin is the most abundant protein in *Xenopus* oocytes and participates in sperm chromatin decondensation after fertilization by removing sperm nuclear basic proteins (Philpott et al. 1991). In mammals, nucleoplasmin might play different roles, because sperm decondensation still occurs after deleting nucleoplasmin (Burns et al. 2003).

It is not yet clear, however, whether the molecular and functional characteristics of nucleophosmin/nucleoplasmin are similar in teleost fish. In many fish species, such as medaka, zebrafish, tetraodon, and stickleback, only nucleophosmin cDNA sequences have been obtained during genome sequencing, according to data in GenBank and the Ensembl Genome Browser. As a sole exception, rainbow trout nucleoplasmin was found, but only mRNA abundance was tested for in eggs (Aegerter et al. 2005). In addition, Sato et al. (2002) purified a nucleoplasmin-like protein from common carp eggs. The protein could be recognized by the polyclonal antiserum against *Xenopus laevis* nucleoplasmin, but only a partial amino acid sequence was obtained. Since nucleoplasmin has not been found in most fish species studied so far, fish nucleophosmin/nucleoplasmin might have diverged functionally in a different way from those of amphibians and mammals. Further studies on the molecular characterization and functional roles of fish nucleophosmin/nucleoplasmin are therefore critical.

In this study, we cloned and characterized a nucleophosmin/nucleoplasmin homolog from two cyprinid fish species, *Carassius auratus gibelio* and *Carassius auratus*, and observed that the homolog was mostly related to nucleophosmin in molecular characterization and its expression profile was similar to that of nucleoplasmin. In addition, we provide further evidence for the functional commonality of nucleophosmin and nucleoplasmin by immunodepletion and in vitro sperm decondensation experiments.

Materials and Methods

Carassius auratus gibelio and *Carassius auratus* were maintained at the Guanqiao Experimental Station of the Institute of Hydrobiology, Chinese Academy of Sciences, in Wuhan, China. Induced spawning, artificial insemination, and embryo culture were performed as described previously (Gui et al. 1993; Zhou et al. 2000).

cDNA Cloning and Sequence Analysis

Degenerate primers NLP-forward and NLP-reverse-2, derived from a conserved fragment of the nucleophosmin/nucleoplasmin family, were used to amplify the corresponding *Carassius auratus* nucleophosmin/nucleoplasmin fragment from a Smart cDNA library made with mRNA from matured eggs. Subsequently, the full-length cDNA was obtained by RACE-PCR using N2, NLP3, and corresponding Smart primers. Based on the high homology between *Carassius auratus* and *Carassius auratus gibelio*, specific primers NT5 and NT3 were used to generate the open reading frame (ORF) sequence of *Carassius auratus gibelio* nucleophosmin/nucleoplasmin from a Smart cDNA library made with mRNA from matured eggs, and then full-length cDNA was obtained by RACE-PCR using Y-N5, Y-N3, and Smart upstream and downstream primers (Table 1).

The protein sequences used for homology comparison were obtained from the NCBI protein database. Multiple sequence alignments were performed using ClustalW 1.8 (Thompson et al. 1994), and the output was shaded using Boxshade 3.21. The acid region and phosphorylation sites were predicted using ScanProsite and NetPhos 2.0, respectively, at the ExPASy Molecular Biology Server (http://expasy.pku.edu.cn/). The nuclear location signal was predicted according to *Xenopus* nucleophosmin structure (Hingorani et al. 2000; Namboodiri et al. 2004).

Protein Expression and Antiserum Preparation

A partial cDNA fragment encoding 95 amino acids of the *Ca*-nucleophosmin *N*-terminus (Fig. 1) was fused in frame to a *His* tag by insertion between the *Eco*RI and *Hin*dIII sites in pET-32α (+) (Novagen). Recombinant protein and rabbit polyclonal antiserum were prepared according to the protocol published previously (Xu et al. 2005).

Western Blot Analysis

Protein extract preparation and Western blot detection followed the protocol described previously (Zhu et al. 2008). To observe the band shift clearly in various stages of oocytes and eggs, electrophoresis time was extended for 1.5 h (150 vol) after the bromophenol blue was run out of the gel.

RT-PCR

Temporal expression was analyzed by RT-PCR in a volume of 25 μl containing 1 ng cDNA and *Cag*-nucleophosmin or *Ca*-nucleophosmin cDNA-specific primers (Table 1). RT-PCR conditions were 94°C for 30 s, 62°C for 30 s, and 72°C for 40 s, for 30 cycles. As a control, *α-tubulin* was amplified from the same set of cDNA samples. The PCR products were separated on 1.5% agarose gel and documented by a bio-imaging system (Syngene Division, Synoptics Ltd).

Table 1 All primers used in this study

Primer name	Sequence	Use
NLP-forward	5′-AA(CT)GT(AG)GTGGA(AG)GTG AC(CT)GC-3′	Degenerate primer
NLP-reverse-2	5′-A(CT)(AG)(AG)(AT)(AC)ACTGG(AT)CCAG(AT)(AT)CC-3′	
Smart upstream primer	5′-AACGCAGAGTACGCGGG-3′	RACE-PCR
Smart downstream primer	5′-CAGAGTACT16-3′	
N2	5′-GGAACACAACAGGTGGAGT-3′	
NLP3	5′-GATTCGGAGGGTCAAAAGGTC-3′	
Y-N5	5′-TTGAGCTCGCAACCGTACAG-3′	
Y-N3	5′-GCTCTGGAAGTGGAGACAGAG-3′	
NT5	5′-TTATTTTACACTCTGTCTCC-3′	Gene cloning
NT3	5′-ATGGATCTCGAACAAATG-3′	
Nuc forward primer	5′-CAGAATTCATATGGATCTCGAACAAATGGGT-3′	Plasmid construction and RT-PCR
Nuc reverse primer	5′-GGATCCAAGCTTTTACACAACAGGTGGAGTGATCTC-3′	
α-tubulin forward primer	5′-GTGCACTGGTCTTCAGGGGTT-3′	RT-PCR
α-tubulin reverse primer	5′-GGGAAGTGGATGCGTGGGTAT-3′	

Immunofluorescence Localization

To investigate the protein distribution, we performed immunofluorescence localization of nucleophosmin in gonads and gametogenesis, as described previously (Xu et al. 2005; Xia et al. 2007). Briefly, the ovaries and testes were embedded in OCT and immediately frozen in liquid nitrogen. All the samples were sectioned 8–12 μm thick using a frozen microtome (Leica), mounted on 0.1% poly-L-lysine-coated slides, and air-dried. The sections were rehydrated in PBS for 30 min and incubated in 5% nonfat dry milk in PBS at room temperature for 1 h to prevent nonspecific binding of antibodies. The sections were then incubated in 1% nonfat dry milk in PBS containing the rabbit antiserum (1:250 dilute) at 4°C overnight and washed 5 × 5 min with PBST. After incubation in FITC-conjugated secondary antibody (goat anti-rabbit IgG, 1:100 dilute, Bierce) in darkness for 1 h, the sections were washed 5 × 10 min with PBS. Then they were stained with propidium iodide for 10 min and washed 4 × 5 min with PBS to show cell nuclear localization. Finally, the sections were observed with a Leica confocal fluorescence microscope.

Immunodepletion and In vitro Sperm Decondensation

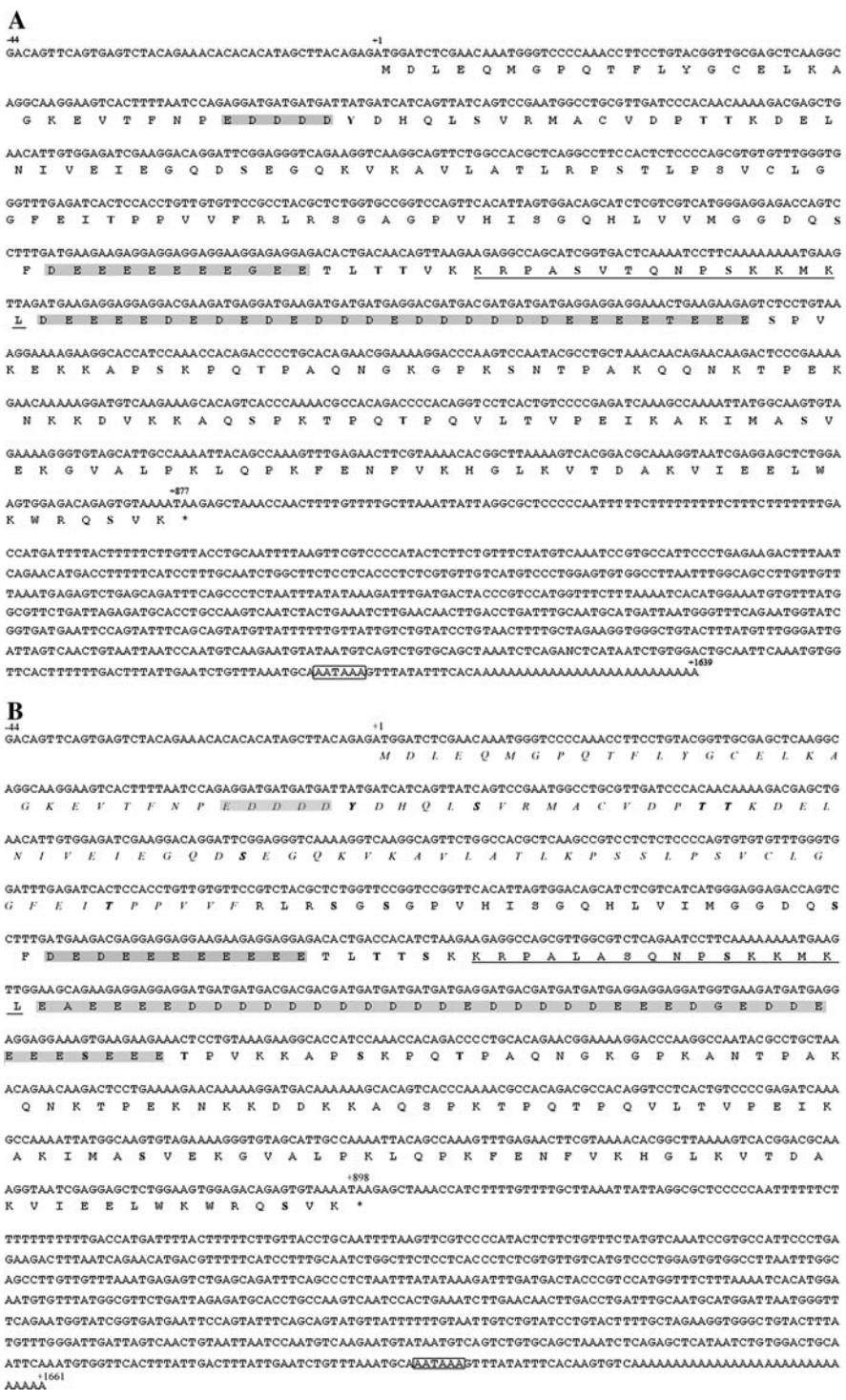
The egg extract preparation followed the protocol published previously (Li and Gui 2003). Immunodepletion was carried out according to the method described by Philpott et al. (1991), with partial improvements. Briefly, the egg extracts were mixed with the rabbit antiserum (or with pre-immune serum for controls) and incubated on ice for 45 min, then mixed with hydrated protein A-Sepharose, and incubated on ice for 20 min. Subsequently, the supernatant was separated from the protein A-Sepharose pellet by microcentrifugation (4°C, 3000*g*, 5 min). The supernatant was immunodepleted once more to remove the nucleophosmin completely. Complete immunodepletion was confirmed by a Western blot. The demembranated sperm nucleus preparation and in vitro sperm decondensation experiment were performed according to the methods described previously (Li and Gui 2003).

Results

Molecular Characterization

The full-length cDNA of *Carassius auratus gibelio* is 1683 bp. Its ORF of 879 bp encodes a mature peptide of 292 amino acids. A putative polyadenylation signal (AATAAA) was found at 19 bp upstream from the poly (A) tail (Fig. 1). The full-length cDNA of *Carassius auratus* is 1705 bp. Its ORF is 900 bp long, which encodes a mature peptide of 299 amino acids. The AATAAA signal was also found at 26 bp upstream from the poly (A) tail (Fig. 1).

Multiple alignments indicated that the deduced amino acid sequences exhibited high homology to vertebrate nucleophosmin/nuculeoplasmin and were mostly related to vertebrate nucleophosmin. As shown in Fig. 1, the identity between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-44
+1
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F D E E E E E E E E G E E T L T T V K K R P A S V T Q N P S K K M K
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L D E E E E E D E D E D E D D D E D D D D D D E E E E T E E E S P V
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AAAAA

◀**Fig. 1** Nucleotide sequences and the deduced amino acid sequences of *Cag*-nucleophosmin (**A**) and *Ca*-nucleophosmin (**B**) and their sequence alignments with other nucleophosmin/nucleoplasmin homologs (**C**). The *underlined* characters indicate the nuclear location signal; the *shaded boxes* indicate the acidic regions; the *italic* characters represent the expressed part; and the *bold* characters represent the predicted phosphorylation sites. Protein accession numbers: *Cag*-nucleophosmin EU309494, *Ca*-nucleophosmin EU309495, zebrafish-nucleophosmin BC053240, tetraodon-nucleophosmin CAG01554, human-nucleophosmin NM002520, mouse-nucleophosmin NM008722, chicken-nucleophosmin X17200, *Xenopus*-nucleophosmin (NO38) X56039, *Xenopus*-nucleoplasmin X04766, mouse-nucleoplasmin Q80W85, and trout-nucleoplasmin BX076441

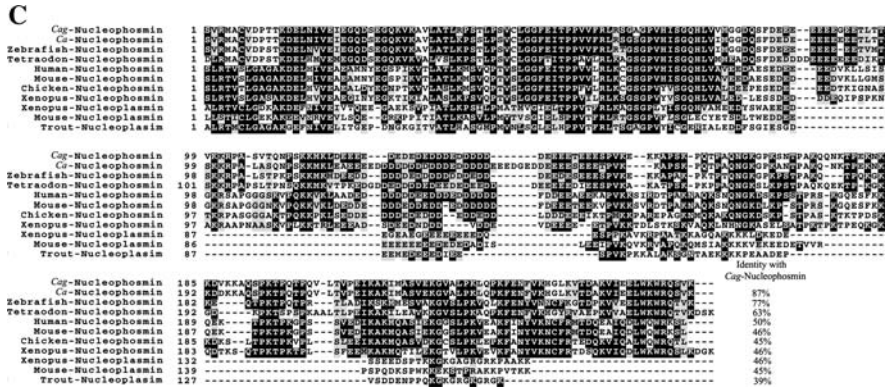

Fig. 1 continued

Carassius auratus gibelio and *Carassius auratus* nucleophosmins is 87%. The identities of nucleophosmins between *Carassius auratus gibelio* and the other two fish (zebrafish and tetraodon) are high, ranging from 77 to 63%. In comparison with nucleophosmins or nucleoplasmins from mammals and amphibians, the identities range from 50 to 45%. Significantly, the identity is only 39% between the cloned nucleophosmin and trout nucleoplasmin, the only nucleoplasmin reported in fish. We therefore referred to our cloned genes as *Cag*-nucleophosmin and *Ca*-nucleophosmin.

Both *Cag*-nucleophosmin and *Ca*-nucleophosmin contain three acidic regions and a NLS, which are typical traits of nucleophosmin (Namboodiri et al. 2004). In the amino acid sequence of *Cag*-nucleophosmin, the first acid region (A1) is from position 28 to 32, the second (A2) from 121 to 131, and the third (A3) from 154 to 182. The region from 138 to 153 amino acids is predicted to be the NLS (Fig. 1). Similarly, in *Ca*-nucleophosmin, A1 ranges from position 28 to 32, A2 from 121 to 131, and A3 from 154 to 192, and a NLS is predicted from position 138 to 153 (Fig. 1). The shortest and the longest acidic regions are A1 and A3, respectively, in both species, which is similar to other nucleophosmins (Namboodiri et al. 2004), whereas the longest acid region in nucleoplasmin is A2 (Salvany et al. 2004). This provides further molecular structural evidence that our cloned nucleophosmin/nucleoplasmin homologs are more closely related to nucleophosmin than to nucleoplasmin.

Expression Patterns in Various Tissues and Embryogenesis

RT-PCR and Western blot analysis of the adult tissues that we have examined (ovary, testis, brain, kidney, liver, heart, spleen, and muscle) revealed that *Cag*-nucleophosmin and *Ca*-nucleophosmin expression was specific to gonads (ovary and testis). Note that the estimated molecular weight of both is about 43 kDa (Fig. 2). Western blot analysis was then performed on various stages of oocytes and ovulated mature eggs to identify the expression pattern of *Cag*-nucleophosmin and *Ca*-nucleophosmin during oogenesis. This revealed obvious differences between oocytes and mature eggs in both *Cag*-nucleophosmin and *Ca*-nucleophosmin (Fig. 2). For *Cag*-nucleophosmin, the detected protein size was about 42 kDa in all stages of oocytes, increasing, however, to about 45 kDa in the mature eggs. For *Ca*-nucleophosmin, the detected protein size gradually became larger, rising from 43 to 44 kDa between oocyte stages I and IV, and became about 47 kDa in the

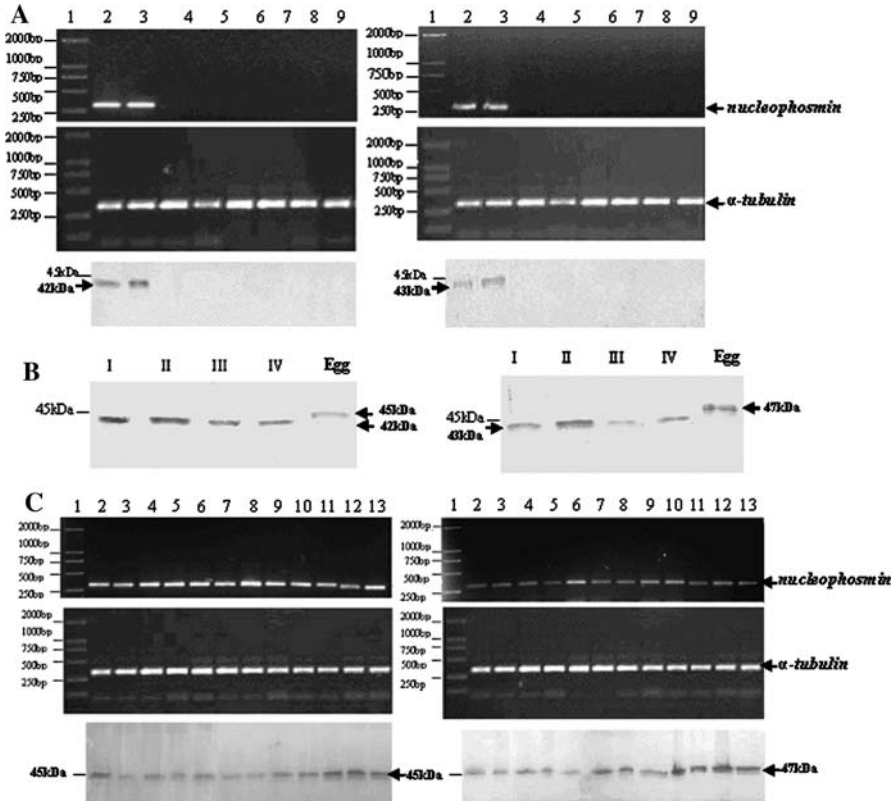

Fig. 2 Expression profile of *Cag*-nucleophosmin (*all on the left*) and *Ca*-nucleophosmin (*all on the right*). **A** RT-PCR and Western blot detection in various tissues. Lanes: 1 marker, 2 ovary, 3 testis, 4 brain, 5 kidney, 6 liver, 7 heart, 8 spleen, 9 muscle. **B** Western blot detection during oogenesis. Lanes: I and II, stages I and II previtellogenic oocyte; III, stage III vitellogenic oocyte; IV, stage IV vitellogenic oocyte. **C** RT-PCR and Western blot detection during embryogenesis. Lanes: 1 marker, 2 mature egg, 3 one-cell, 4 cleavage, 5 morula, 6 blastula, 7 gastrula, 8 neurula, 9 tail bud, 10 muscle effect, 11 heart beating, 12 prehatching, 13 hatched

ovulated mature eggs. These band shifts indicated that post-translational modifications might occur during oogenesis and oocyte maturation, and modification differences might exist between *Cag*-nucleophosmin and *Ca*-nucleophosmin.

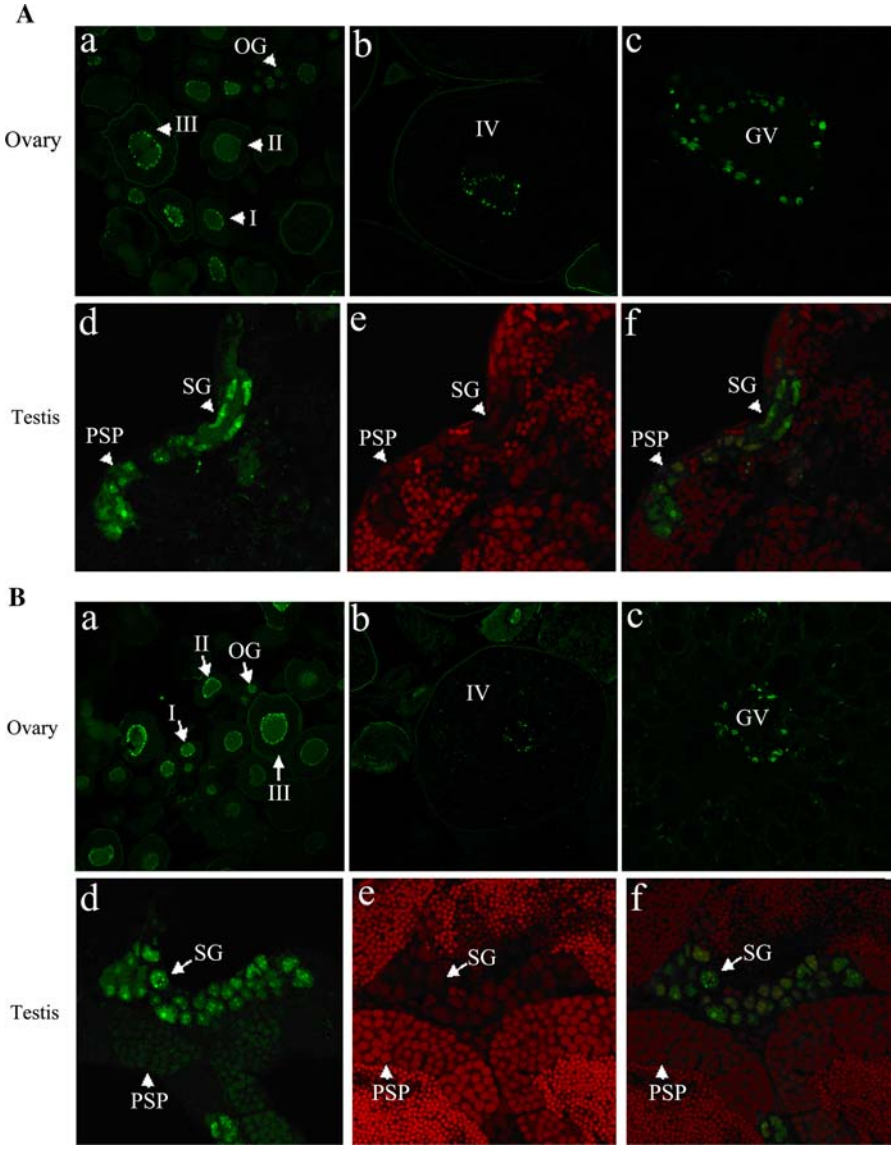

Fig. 3 Localization and distribution of *Cag*-nucleophosmin and *Ca*-nucleophosmin in ovary (a–c) and testis (d–f) of *Carassius auratus gibelio* (**A**) and *Carassius auratus* (**B**). a–d, green fluorescence immunostained by the antibody. e, red fluorescence stained by propidium iodide that shows the nuclear and chromatin position. f, overlap of the green immunofluorescence and chromatin red fluorescence. Magnification: a and b, 20×; c–f, 40×. *Arrows*: I and II, previtellogenic oocyte; III, vitellogenic oocyte; IV, fully vitellogenic oocyte; GV, germinal vesicle; SG, spermatogonia; PSP, primary spermatocytes

Nucleophosmin expression was also analyzed by RT-PCR and Western blot in embryos at various developmental stages (Fig. 2). Both mRNAs and proteins of *Cag*-nucleophosmin and *Ca*-nucleophosmin existed throughout embryogenesis.

Immunofluorescence Localization and Distribution in Oogenic and Spermatogenic Cells

The *Ca*-nucleophosmin antiserum prepared from the expressed protein was able to trace localization and distribution of *Cag*-nucleophosmin and *Ca*-nucleophosmin in oogenic and spermatogenic cells (Fig. 3). In oogenic cells, signals of both *Cag*-nucleophosmin and *Ca*-nucleophosmin were observed in the germinal vesicle region of oocytes throughout oogenesis, and the distribution of both signals changed from a widely dispersed pattern in oocyte stages I–III to a concentrated pattern in the nucleoli of stage IV oocytes. In spermatogenic cells, signals of *Cag*-nucleophosmin and *Ca*-nucleophosmin were detected strongly in spermatogonia and weakly in primary spermatocytes in both the nucleus and the cytoplasm, although the signals in the cytoplasm were much weaker.

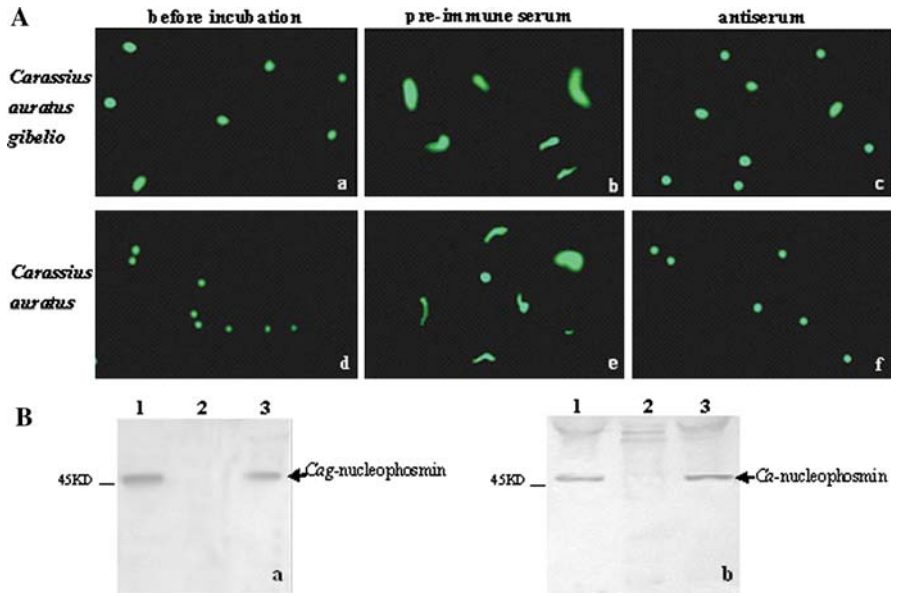

Fig. 4 Immunodepletion and sperm decondensation blockage. **A** In vitro sperm decondensation assay of *Carassius auratus gibelio* demembranated sperm nucleus in *Carassius auratus gibelio* egg extracts (a–c; 200×) and *Carassius auratus* demembranated sperm nucleus in *Carassius auratus* egg extracts (d–e; 200×). a and d, sperm nuclei in egg extracts immunodepleted using the antiserum before incubation; b and e, sperm nuclei in egg extracts immunodepleted using the pre-immune serum after 120 min incubation; c and f, sperm nuclei in egg extracts immunodepleted using the antiserum after 120 min incubation. **B** Western blot detection after complete immunodeletion of *Cag*-nucleophosmin (a) and *Ca*-nucleophosmin (b). Lanes: 1 total egg extracts, 2 egg extracts immunodepleted using the antiserum, 3 egg extracts immunodepleted using the pre-immune serum

Immunodepletion and Sperm Decondensation Blockage

Immunodepletion and in vitro sperm decondensation experiments were carried out to test whether the *Cag*-nucleophosmin and *Ca*-nucleophosmin function during sperm decondensation (Fig. 4). For both species, in egg extracts immunodepleted with pre-immune serum, the demembranated sperm nuclei underwent chromatin decondensation, changing in morphology into swollen conical forms and elongated bare and swollen spheres, very similar to changes seen in total egg extracts (Li and Gui 2003). In egg extracts immunodepleted using the antiserum, however, all the demembranated sperm nuclei remained as condensed spheres even after 120 min incubation. Note that complete immunodepletion of either *Cag*-nucleophosmin or *Ca*-nucleophosmin was confirmed by Western blot detection. These observations demonstrated that removing either *Cag*-nucleophosmin or *Ca*-nucleophosmin efficiently blocked sperm decondensation and pronucleus formation in an in vitro sperm decondensation system.

Discussion

In this study, we cloned and characterized *Cag*-nucleophosmin and *Ca*-nucleophosmin from two cyprinid fish, *Carassius auratus gibelio* and *Carassius auratus*, revealed the specific expression of both in gonads, and localized their distribution in oogenic and spermatogenic cells. Moreover, immunodepletion and in vitro sperm decondensation experiments demonstrated a sperm decondensation function for these factors. These new findings are different from all previous studies on nucleophosmin/nucleoplasmin, in that nucleophosmin was observed to be expressed in somatic tissues and nucleoplasmin was revealed to be specific to oocytes and eggs in previous studies (Eirin-Lopez et al. 2006; Shackleford et al. 2001; Wedlich and Dreyer 1988). Our data suggest that *Cag*-nucleophosmin and *Ca*-nucleophosmin are nucleophosmin homologs but share expressional and functional commonalities with nucleoplasmin.

Cag-nucleophosmin and *Ca*-nucleophosmin share many characteristics with mammalian and amphibian nucleophosmin (Grisendi et al. 2005; Namboodiri et al. 2004; Peculis and Gall 1992; Pfeifle et al. 1987; Verheggen et al. 2000). First, their sequences have a putative NLS and three acidic regions. Second, the proteins are distributed in the nucleoli of oocytes. Third, both the mRNA and proteins are expressed throughout embryogenesis. This is in contrast to nucleoplasmin, which is expressed only during early embryonic stages (Prado et al. 2004). Nonetheless, fish nucleophosmin shows low sequence identity with amphibian and mammalian nucleophosmins, and in fact has almost the same low sequence identity with both nucleophosmins and nucleoplasmins of amphibians and mammals (Fig. 1), suggesting that fish nucleophosmin might have a different functional divergence.

Cag-nucleophosmin and *Ca*-nucleophosmin are similar to *Xenopus* nucleoplasmin in both their expression pattern and their function in sperm decondensation. Specifically, they are mainly distributed in oocyte nucleoli. There are many predicted phosphorylation sites, such as 12 Ser, 8 Thr, and 1 Tyr in *Cag*-

nucleophosmin and 12 Ser, 9 Thr, and 1 Tyr in *Ca*-nucleophosmin (Fig. 1), suggesting that phosphorylation might occur during oogenesis as a necessary post-translational modification during chromatin decondensation (Banuelos et al. 2007). Moreover, sperm decondensation is blocked by a lack of either *Cag*-nucleophosmin or *Ca*-nucleophosmin in the in vitro system, consistent with the classic function of nucleoplasmin in *Xenopus* (Philpott et al. 1991) and *Drosophila* (Crevel et al. 1997).

Although it is clear that during oogenesis the accumulation of *Cag*-nucleophosmin and *Ca*-nucleophosmin is a preparation for sperm decondensation upon fertilization, the reason for their presence in spermatogonia and primary spermatocytes remains unknown. Perhaps *Cag*-nucleophosmin and *Ca*-nucleophosmin could be involved in keeping the spermatocyte chromosomes in a state of decondensation. Both *Cag*-nucleophosmin and *Ca*-nucleophosmin might then quickly degenerate, facilitating the start of chromosome condensation in primary spermatocytes to form the sperm chromosomes. From these data, it can be concluded that *Cag*-nucleophosmin and *Ca*-nucleophosmin are associated with keeping chromosomes in a state of decondensation not only in male pronucleus formation but in spermatogonia storage as well. This is consistent with the function of nucleoplasmin in undifferentiated mouse cells (Tamada et al. 2006). In previous reports, all in vitro sperm decondensation systems have used either *Xenopus* nucleoplasmin (Iwata et al. 1999) or *Xenopus* sperm (Crevel et al. 1997; Kawasaki et al. 1994). Here we used a fish sperm decondensation system to mimic the in vitro situation more precisely.

In conclusion, our findings provide novel evidence for the functional commonality of nucleophosmin and nucleoplasmin in fish. In comparison with the functional diversification in mammals and amphibians, we suggest that a functional overlapping and compensation between nucleophosmin and nucleoplasmin might exist in fish. We have noticed the presence, however, of five other nucleophosmin/nucleoplasmin genes (npm1-Dr.43659, npm2-Dr. 86275, npm2a-Dr.38495, npm3-Dr.39149, and npm4-Dr.18867; UniGene database) in another cyprinid fish (zebrafish), although their identities with *Cag*-nucleophosmin and *Ca*-nucleophosmin were calculated to be 39–41% (data not shown). Therefore, more molecular and functional data are needed before this hypothesis is confirmed.

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