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Biochemical and Biophysical Research Communications 301 (2003) 152–158
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An increased expression of nucleolin is associated with a physiological nucleolar segregation

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Received 11 December 2002

Abstract

Nucleolar segregation is the most striking cellular phenotypic feature of cold-acclimatized carp and depicts the cyclical reprogramming that the physiology of the fish undergoes between summer and winter, where a clear differential expression of some nucleolar related genes occurs. We characterized carp nucleolin, a nucleolar protein involved in multiple steps of ribosome biogenesis, and evaluated its expression upon fish acclimatization. We show that the carp cDNA deduced amino acid sequence exhibits the same tripartite structural organization found in other species. Nevertheless, we observed that nucleolin mRNA expression was strongly induced in the cold-adapted carp as was the nuclear protein content, assessed by immunocytochemistry in liver sections. The physiological up-regulation of nucleolin in the cold-acclimatized carp, where rRNA transcription and processing are depressed concomitantly with the nucleolus segregation, is consistent with the notion that nucleolin plays a fundamental role in repressing rRNA synthesis.

Keywords: Nucleolin; Nucleolus; U3 snoRNA; Ribosomal protein L41; Acclimatization; Carp; Fish; Adaptation

The carp (*Cyprinus carpio*) habitat undergoes seasonal cyclical changes that require the fish to make physiological and biochemical adjustments. These are triggered by sensing the gradual shifts in physical parameters (temperature and photoperiod) coupled with their concurrent transduction into molecular signals that can be implicated in the control of gene expression. In this complex adaptive response, the neuroendocrine system appears to play a critical role [1–4]. As a consequence of the seasonal environmental variations, as described by Sáez et al. [5], a generalized nucleolar rearrangement occurs. Upon winter acclimatization, the carp nucleolar components are fully segregated and rRNA transcription is dramatically affected [6,7]. The nucleolar molecular reprogramming is associated with a clear differential expression of ribosomal protein L41 [8], which follows the same pattern as protein kinase CK2β that transcribes at higher levels in the summer-acclimatized carp with respect to the winter-adapted fish [9]. This distinctive ultra-structural feature observed in cold-adapted fish with its silenced rRNA transcription is comparable to the arrest of the RNA pol I activity and concurrent nucleolar segregation obtained in vitro with actinomycin D [10,11]. Thus, the nucleolar reorganization occurring upon seasonal acclimatization of an eurythermal fish constitutes an invaluable physiological model to approach the understanding of nuclear dynamics and regulation of ribosome biogenesis.

Ribosome biogenesis is the major function associated with the nucleolus [10]. This nuclear compartment organizes around the chromosomal regions that code for ribosomal RNAs (5.8S, 18S, and 28S) providing a structural frame for an active transcription of these genes by RNA pol I [12,13]. During rRNA transcription, the pre-rRNA precursor associates with different proteins and ribonucleoproteins involved in the modification, maturation, and assembly of the pre-ribosomal

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doi:10.1016/S0006-291X(02)02978-9

particles [14]. Nucleolin, involved in multiple steps of ribosome biogenesis, is the most abundant non-ribosomal nucleolar protein [15,16]. Accordingly, it interacts with nascent pre-rRNA near the transcription initiation site [17–19] to favor the assembly and maturation of the primary rRNA processing complex [20].

Nucleolin is organized in functional domains [21]. The N-terminal contains highly charged acidic sequence repeats that vary in number depending on the species, separated by basic stretches [22]. This protein segment has many potential cdc2 and CK2 protein kinase phosphorylation sites [23–25] and a bipartite nuclear localization sequence [26]. The N-terminal region has been implicated in chromatin remodeling [27,28] and pre-rRNA processing mediated by U3 snoRNP [29]. The central region of nucleolin contains four conserved RNA binding domains (RBDs) [22]. These nucleolin RBDs interact with nascent pre-rRNA through a co-operative association between RBD1 and RBD2 [19]. There is evidence to suggest that nucleolin binds to a short stem-loop structure that forms a minimal RNA binding site in the 5′-ETS rRNA [18,30]. Furthermore, it has been observed that nucleolin induces the repression of RNA polymerase I transcription [31]. The C-terminal is a glycine–arginine rich domain (GAR/RGG domain) that binds with low affinity and non-specifically to RNA [32], but also interacts with several ribosomal proteins [33].

In order to understand the underlying molecular mechanisms of the physiological nucleolar segregation in winter-adapted carp previously associated with a silencing of rRNA synthesis, we characterized a cDNA encoding for carp nucleolin and assessed its expression upon fish acclimatization. Our findings show that nucleolin increases as a result of the physiological response of the fish to the cold season in agreement with the recent evidence that nucleolin down-regulates rDNA transcription [31].

Materials and methods

Animals and tissues. Male carp weighing 1000–1500 g were caught during winter and summer and maintained in a fixed 3×4 m cage submerged 2 m in an affluent of the same river with temperatures of 18–20 °C (summer) and 8–10 °C (winter). Tissue sections for immunocytochemistry were prepared and stored as described in [1].

Isolation of a carp nucleolin cDNA. A specific probe for carp nucleolin was obtained by PCR amplification using primers corresponding to sequences of exons 8 (NUCE8S: 5′-CGTGAAGAA CCTGCCGTACT-3′) and 13 (NUCE13AS: 5′-GAAGTCTACG AAACCAAA-3′), derived from a partial genomic sequence of carp nucleolin (J.P. Bachellerie, personal communication). A unique 700 pb fragment was obtained and cloned in pGEM-T vector system I (Promega, USA).

The cDNA encoding for carp nucleolin was isolated from a Uni-Zap XR vector carp liver cDNA library (Stratagene, USA) using the 700 pb fragment labeled with [α-^{32}P]dCTP as probe [34]. The largest isolated cDNA (2.0 kb) clone (pFNUC) included amino acids 160 to 693 according to the expected sequence.

5′RACE. The full-length 5′-region of carp nucleolin cDNA including the transcription start site was obtained using the FirstChoice RLM-RACE kit (Ambion, USA) [8,35,36]. Briefly, a RT-PCR was performed with an adapter and gene specific primers (NUCext: 5′-CCTCGTCTTCTTCAGATTCC-3′ and NUCinter: 5′-CTTCGCTTT CACCATTCCTG-3′) using as template 5′-full-length cDNAs. The 5′-RACE RT-PCR product was cloned into the pGEM-T easy vector and fully sequenced.

Northern blots. The hybridization of Northern blots of fractionated RNA (25 μg) from winter- and summer-acclimatized carp was performed according to [37] with a 1492 pb radioactive labeled PCR product (cNUC) as probe (using primers J2FW: 5'-TGCACCT GCCAAGGCAACT-3' and NUCE13AS). The membranes were also hybridized with specific U3 snoRNA [6] and L41 probes [8].

Construction of a carp nucleolin RBD1–4 expression vector. The nucleotide sequence comprising RBD1–RBD4 of carp nucleolin was amplified by PCR using Vent DNA polymerase (New England Biolabs, England) and primers 5R1NDE: (5′-GGAATTCCATATGACT GATGGCGAAGGTTTCAGT-3′) and 3R4BHI (5′-CGGGATCC TAGCCGCCTTCGCCCTTGGGCTT-3′) containing *Nde*I and *Bam*HI restriction sites (underlined). The PCR product was cloned in the corresponding restriction sites of pET15b plasmid (Novagen, Germany). This clone was named pcNUCRBD1–4.

Expression and purification of recombinant protein. BL21(DE3) plysS competent cells were transformed with the constructed pcNUCRBD1–4, as described [19]. Cells grown at 37 °C in 500 ml Minimal Medium (100 mg/l ampicillin, 20 μg/l chloramphenicol) were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h. Concentrations were estimated with Bradford reagent (Bio-Rad protein assay) and checked into a 10% SDS–PAGE.

Preparation of nucleolin polyclonal antibodies. Specific carp anti-nucleolin antibodies were prepared by Eurogentec S.A. (Belgium) immunizing two rabbits with recombinant NUCRBD1–4 protein.

Western blots. Liver and brain protein extracts from winter-acclimatized carp were prepared as described in [1], and then fractionated by SDS–PAGE, and electro-transferred to Optitran membranes (Schleicher & Schuell, Germany). These membranes were immunostained according to the fabricant instructions using the recombinant anti-nucleolin antibody against the RBD1–4 of the carp protein (pcabRBD1–4). Detection was made with HRP-labeled secondary antibodies.

Immunocytochemistry. Liver tissue sections were prepared and stored as reported in [1] and immunostained with pcabRBD1–4 polyclonal antibody. The chromatin distribution was evidenced by DAPI staining (according to fabricant instructions, KPL, USA).

Quantification of nucleolin in the liver sections. Assessment of immunolabeling in liver sections from winter- and summer-acclimatized carp was performed using the program Image Pro Plus 3.0 in six individuals [38]. Student's *t* test was used to assess differences. $P < 0.001$.

Results

A full length *C. carpio* nucleolin cDNA sequence (2784 bp) was attained (Fig. 1A; GenBank Accession No: AY166587). Because the RLM-RACE approach considers all the capped mRNAs, we were able to locate the transcription start site (+1) at 108 bp upstream from the ATG start codon. The stop codon was situated at position 2186. From this cDNA sequence we derived a 693 amino acid protein that displays a significant percentage of identity with different species. While the nu-

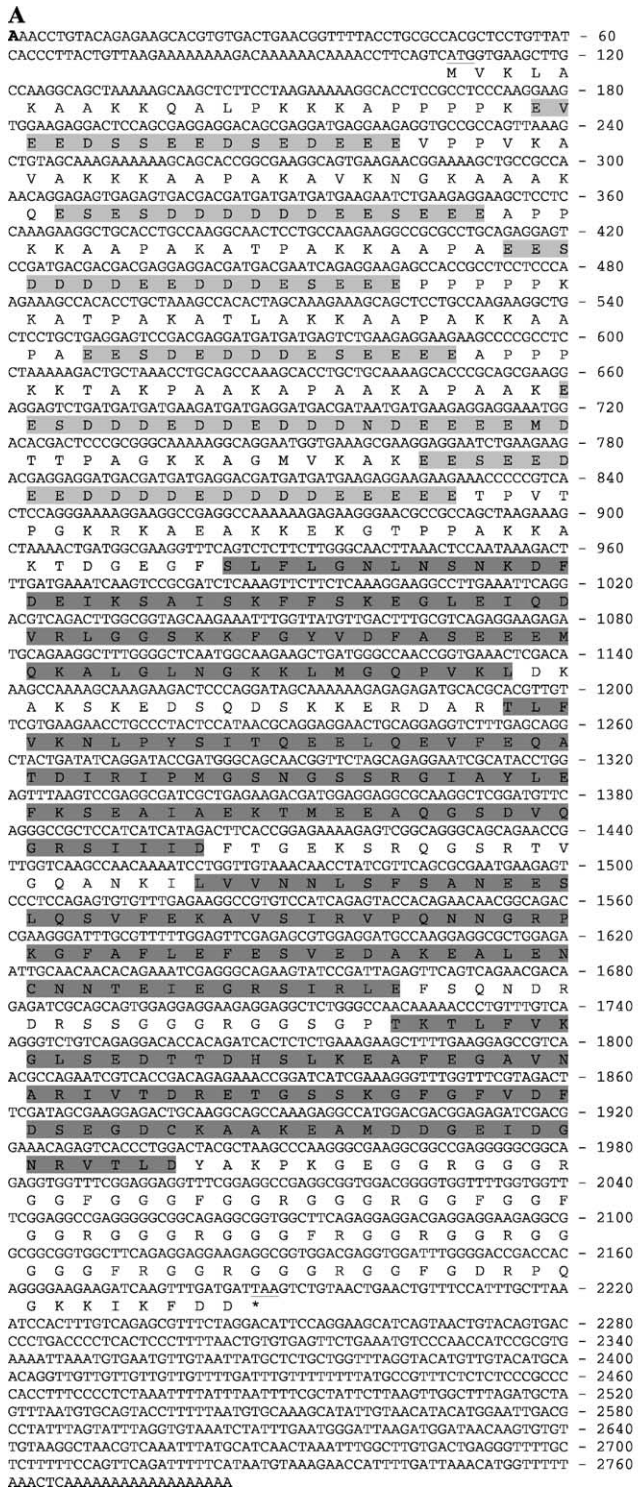

Fig. 1. (A) Carp nucleolin cDNA (deposited in GenBank Accession No. AY166587) and its derived amino acid sequence. The transcription start site and the stop codon are underlined. Acidic regions are highlighted in light gray and RNA binding domains (RBDs) in dark gray. (B) Schematic representation of nucleolin from *C. carpio* (A) and other species: B, *X. laevis*; C, *G. gallus*; D, *R. norvegicus*; E, *M. musculus*; F, *H. sapiens* (Swiss-Prot Bank Accession Nos. P20397, P15771, P13383, P09405, P19338, respectively). (▯) Acidic domains, (▭) RBDs, (▭) GAR domain, and (▯) NLS.

cleolin N-terminal region from other species shows four or five acidic segments, carp nucleolin comprises six highly acidic repeats (Fig. 1B). As in the nucleolin from other species, these segments, which exhibit potential serine phosphorylation sites, are separated by basic amino acid stretches. There are four characteristic RNA binding domains in the central carp nucleolin region, all of which display a high percentage of identity with re-

Table 1
Percentage of amino acid sequence identity of RBD domains of nucleolin from different species

Nucleolin	RBD1	RBD2	RBD3	RBD4
C. carpio	100	100	100	100
X. laevis	67	61	76	76
G. gallus	57	55	72	76
R. norvegicus	52	59	70	80
M. musculus	53	55	67	83
H. sapiens	52	59	70	82

The percentage of identity was calculated by comparison of each RBD domain against its counterpart in carp nucleolin.

spect to the RBDs from other species (Table 1). The C-terminal shows a long glycine–arginine rich sequence (GAR/RGG domain) that comprises 63 amino acid residues.

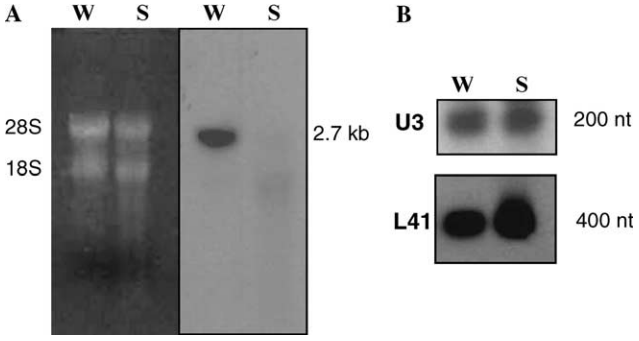

Fig. 2. Gene expression of carp nucleolin, U3 snoRNA, and ribosomal protein L41. (A) Fractionation of carp liver RNA in 1.5% agarose denaturing gel stained with EtBr (0.5 μg/ml) and Northern blot hybridization analysis of nucleolin, and (B) Northern blot hybridization analyses of U3 snoRNA and ribosomal protein L41 from summer (S)- and winter (W)-acclimatized carp.

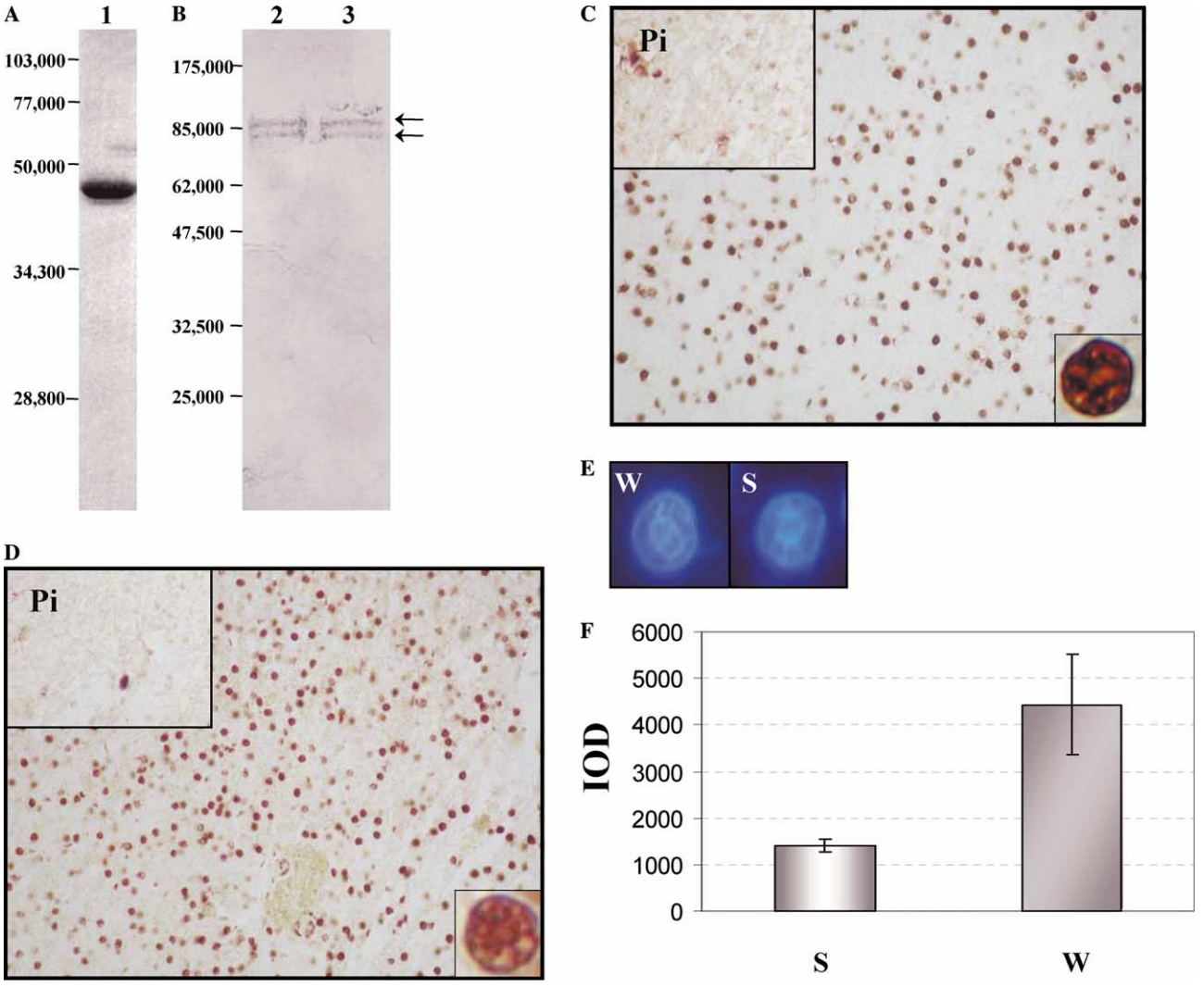

Fig. 3. Carp nucleolin immunodetection with polyclonal pcabRBD1–4 antiserum. (A) SDS–PAGE fractionation of purified recombinant RBD1–4 protein (0.6 μg). (B) Western blot analysis. Lanes 2 and 3: 20 μg total proteins from liver and brain of winter-acclimatized carp, respectively. (C) and (D) Immunostaining of liver sections from winter- and summer-adapted carp, respectively (Pi: pre-immune serum). (E) DAPI staining of hepatocyte nuclei from winter (W)- and summer (S)-acclimatized carp. (F) Semi-quantitative analyses of the immunolabeling are represented graphically. The histograms depict the mean total pixel values (±SD) in three different individuals corresponding to each season. The analyses rendered significant differences at Student's t test $p < 0.001$. Columns depict the mean integrated optical density (IOD).

As shown in Fig. 2A, the carp nucleolin mRNA can be detected as a unique band of about 2700 nt that is notoriously stronger in the winter-acclimatized carp than the almost negligible signal attained with RNA from the warm-season adapted carp. The seasonally regulated expression was also confirmed by RT-PCR assays (data not shown).

Concurrently, and as a reference, hybridization of winter- and summer-acclimatized fish liver RNA was performed with specific probes for carp U3 snoRNA and the L41 ribosomal protein (Fig. 2B). As expected [8], L41 transcription is clearly stronger in summer-fish when compared to the winter-carp. However, U3 snoRNA transcription displays a similar pattern in both seasons.

The nucleolin content was assessed by immunocytochemistry analyses. A polyclonal antibody raised against the RBD regions of carp nucleolin was obtained with a purified recombinant RBD1–4 protein (Fig. 3A). Immunoblots of liver and brain protein extracts from winter-adapted fish rendered two bands with a molecular mass of approximately 85 kDa (Fig. 3B).

Figs. 3C and D depict liver sections from winter- and summer-acclimatized carp immunostained with the polyclonal carp RBD1–4 nucleolin antibody. In both seasons nucleolin is found essentially in the nucleus. Nevertheless, a higher concentration of nucleolin was found in the nucleus of winter-adapted carp when compared to the summer-acclimatized fish. Furthermore, in both adaptive conditions, the nucleolin labeling was preferentially distributed around the nucleus envelope and surrounding the nucleolus overlapping with the distribution of the heterochromatin in the carp hepatocyte nuclei (Figs. 3C–E). Quantification of the signals confirmed the significant differences in nucleolin content between both seasonal adaptive states (Fig. 3F).

Discussion

Ribosome biogenesis is a complex process that occurs mainly in the nucleolus and involves an ordered coordination between rRNA transcription, maturation, and packing with ribosomal proteins.

We have documented that rRNA synthesis is not constitutive in the carp [7]. As a result, when the nucleolar components segregate as part of the physiological compensation that a cold environment imposes to the fish, rRNA expression decays dramatically [6]. Concurrently, within the molecular events associated with ribosomal biogenesis, protein kinase CK2β transcription and ribosomal L41 protein expression decline in the winter-acclimatized carp [8,9]. These observations are consistent with the knowledge that the nucleolar morphology is closely related to the state of ribosome biogenesis [39] and that segregation reveals a transient inactivation of the rRNA gene expression [40].

We report the first entire cDNA sequence of a fish nucleolin, a non-ribosomal protein that plays different roles in the ribosome production pathway. In fish, only a partial trout nucleolin cDNA sequence comprising the first two RBDs and the start of RBD-3 has been reported (GenBank AF249730). The deduced amino acid sequence of carp nucleolin exhibits the same tripartite structural organization found in other eukaryotic species. Nevertheless, in the N-terminal domain an additional acidic segment was found. Thus, carp nucleolin displays six acidic segments with multiple potential sites for phosphorylation while nucleolin from *Xenopus*, chicken, rat, mouse, and human contain four or five. It has been proposed that phosphorylated nucleolin may interact with histone H1 in order to modulate the condensation of certain forms of DNA [27,41]. Thus, the concurrence of heterochromatin localization and nucleolin immunolabeling in the nucleus of carp hepatocytes (Figs. 3C–E) may be interpreted as an association favored by the multiple acidic sequences contained in the carp nucleolin.

Furthermore, the N-terminal domain of nucleolin interacts with U3 snoRNP [29]. The nucleolar U3 snoRNP or small subunit processome (SSU) is required for 18S pre-rRNA processing in all organisms [42]. We have shown previously [6] that in pituitary tissue, the U3 snoRNA content is lower in winter-adapted carp. However, we did not detect differences in snoRNA in liver cells from fish adapted either to summer or winter (Fig. 2B), and this result was confirmed by competitive RT-PCR assays (data not shown). The pituitary gland plays a distinctive and relevant role in the complex process that transduces the periodic changes of the external milieu into signaling molecules that can be implicated in the control of gene expression [1–4]. Thus, U3 snoRNA may behave differently in both tissues.

The central and the C-terminal domains of carp nucleolin show the same features described by [22] in other vertebrates species, showing a high level of similarity, up to 50%.

Western blot analyses using a carp anti-nucleolin antibody rendered two bands with similar mobility (Fig. 3B) both in liver and brain tissue extracts. In *Xenopus laevis*, a second form of the protein with an additional N-terminal acidic segment has been also reported [43,44].

Our results show that the level of nucleolin mRNA is strongly up-regulated in the winter-acclimatized carp (Figs. 3C and D). Conversely, other genes implicated in nucleolar functions, i.e., L41 ribosomal protein, CK2β, and 5.8S rRNA, are clearly down-regulated in the cold season [6–9]. Concurrent with the up-regulation of nucleolin mRNA transcription in the winter-acclimatized fish, we observed that during the cold season, carp hepatocytes contained 3.5 times the amount of protein found in summer (Fig. 3F). Interestingly, nucleolin im-

munolabeling scatters, preferentially around the nucleus envelope and surrounding the nucleolus, this completely overlaps with the distribution of the heterochromatin in the carp hepatocyte nuclei (Figs. 3C–E). Using electron microscopy, we previously observed that the peripheral organization of the nucleolar chromatin appears to be a classic feature of the winter-segregated nucleolus [5,6]. Although the level of nucleolin protein is usually associated with the level of cell proliferation, i.e., high level of protein in fast growing cells, it appears that in carp liver this may not be the case. As shown in Figs. 3C and D, under the same level of magnification, summer-adapted fish tissue has more cells per field than the winter-obtained tissue. This has been a recurrent observation in liver tissue from warm-acclimated carp and deserves further studies.

Nucleolin associates to the path of ribosome biogenesis, from transcriptional control of rDNA to the assembly of the ribosomal particles [22]. While earlier reports suggested that nucleolin could block transcription elongation, thus establishing a bridge between the nascent pre-rRNA transcript and chromatin [45,46], a specific repression of RNA polymerase I transcription was recently observed in *Xenopus* oocytes in the presence of an excess of nucleolin through an interaction of nucleolin with the RNA polymerase I transcriptional machinery or the rDNA promoter sequence [31]. The up-regulation of nucleolin that occurs in the cold-acclimatized carp where rRNA transcription and processing are depressed [6,7,47] concomitantly with the appearance of a segregated nucleolus supports the evidence that physiologically, nucleolin is very relevant in the down-regulation of rRNA transcription.

Acknowledgments

We thank Margaret Snook for critical reading of the manuscript. This work was supported by Grants 1000-061 from FONDECYT, RDFPC11584 from CNRS-CONICYT, 66-00, and 93-01 from the Research Fund of UNAB.

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