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Genomic organization of the rDNA cistron of the teleost fish *Cyprinus carpio*

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ABSTRACT

The seasonal adaptation of the teleost *Cyprinus carpio* to the cyclical changes of its habitat demands physiological compensatory responses. The process involves profound nucleolar adjustments and remarkable changes in rRNA synthesis, which affects ribosomal biosynthesis. In this context, we have demonstrated that the synthesis of several proteins involved in ribosomal biogenesis as protein kinase CK2, ribosomal protein L41 and nucleolin, as well as U3 snoRNP, are differentially regulated in summer-acclimatized carp compared to the cold-season adapted fish.

To understand the mechanisms involved in the seasonal regulation of rRNA gene transcription, we have been studying the carp rDNA cistron structure. Because the *cis*-elements that regulate the expression of the tandem organized ribosomal genes are located in the non-transcribed intergenic spacer (IGS), we analyzed the primary structure of the carp rDNA gene IGS. The gene organization is similar to that described from other vertebrate species, including numerous repetitive sequences, the transcription start site, and some potential *cis*-elements such as ribosomal enhancers, proximal terminator and transcriptional terminators.

Ribosomal DNA is a remarkable case of gene duplication and has been used as a model to test the concerted evolution theory. We performed sequence comparison analyses of 18S rRNA coding sequences from carp with different species, data with which an unrooted phylogram was constructed.

Key terms: ribosomal gene, rRNA; rDNA; fish; carp

INTRODUCTION

The process of protein synthesis, which is mediated by ribosomes, is essential for cell growth, proliferation, and adaptation to changing environments. The ribosomal biogenesis is a very complex process that involves the synthesis and assembly of four different ribosomal RNA (rRNA) molecules and of 80 different proteins that takes place in the nucleolus of eukaryotic cells. The transcription of a 45S rRNA precursor (pre-rRNA) takes place in this nuclear compartment, along with its processing and maturation in the 18S, 5.8S and 28S rRNAs and the assembly of the pre-ribosomal particle. The ribosome synthesis gives rise to the characteristic ultra-structural organization of the nucleolus into domains that contain a minimum of three distinctive components: the fibrillar center, the dense fibrillar component, and the granular component (Lazdins *et al.*, 1997). Nucleolar structure is largely dependent upon the process of ribosome assembly. The nucleolus is disassembled during mitosis, at which time pre-rRNA transcription and processing are suppressed (Hernandez-Verdun *et al.*, 2002). Actinomycin D treatment affects the structure and integrity of the nucleolus in HeLa cells, resulting in a segregation of nucleolar components and rRNA transcription repression (Schofer *et al.*, 1996), along with changes in the relative abundance of a subset of nucleolar proteins (Andersen *et al.*, 2002). It is

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known that in the human meiotic oocyte, the nucleolus shows spontaneous segregation of its components due to temporary inactivation of the ribosomal genes expression (Mirre *et al.*, 1980).

The habitat of the carp (*Cyprinus carpio*) undergoes seasonal changes that compel the fish to develop adaptive strategies for sensing the cyclical fluctuation of the physical parameters (*e.g.* temperature, photoperiod) and to transduce them into molecular signals through mechanisms that involve the neuroendocrine system (Figueroa *et al.*, 1994; Kausel *et al.*, 1998; 1999; López *et al.*, 2001). One of the most outstanding cellular features of this adaptive process is the reorganization of the nucleolus architecture. In the cells of winter-adapted fish, the nucleolar structure is segregated and the fibrillar components are densely packed and separated from the granular component and the associated chromatin (Sáez *et al.*, 1984; Vera *et al.*, 1993). Concurrently, the transcription of ribosomal genes by RNA polymerase I and the processing of the rRNA precursors decrease dramatically during the cold season (Vera *et al.*, 1993). During summer, however, the nucleolar structure reflects the active synthesis and processing of rRNA, *e.g.* the fibrillar and granular components are intermingled (Sáez *et al.*, 1984; Vera *et al.*, 1993).

The natural segregation of the carp nucleolar components and the down regulation of rRNA transcription during the adaptation of the fish to the cold season are analogous to the blocking of rRNA synthesis and nucleolar segregation obtained *in vitro* with Actinomycin D. Thus, the carp acclimatization constitutes a valuable model to study the regulation of ribosome biogenesis, which is closely synchronized at multiple steps, including the regulation of pre-rRNA synthesis at different stages of the process, *e.g.* chromatin remodeling, transcriptional activation, initiation, elongation and termination (Leary and Huang, 2001). All these regulatory steps involve interactions between *cis*-acting elements of rDNA genes and *trans*-acting protein factors. To approach the study of the regulation of rRNA synthesis during the seasonal adaptation of the carp, it is necessary to know the structure of the gene that encodes for the rRNA precursor and the *cis* elements located at the regulatory region of the carp ribosomal cistron. In this report we describe the primary sequence arrangements of the carp ribosomal cistron potentially involved in the initiation and termination of the transcription, and complement the previous carp rDNA sequences we have described previously (Vera *et al.*, 1997; Joseph *et al.*, 1999).

MATERIALS AND METHODS

Animal and tissues:

Male carp were captured and maintained at summer- (20-22º C) and winter- temperatures (8-10º C), and the tissues dissected and frozen as described (Vera *et al.*, 1993).

Genomic library screening

A 72bp fragment, coding for a partial sequence of the 5.8S rRNA (Vera *et al.*, 1997) was used to screen a λFIX II carp genomic library (Stratagene, USA), which yielded 100 hybridizable clones under stringent conditions (Grossberger, 1987). Six recombinant λ-clones were chosen to be purified by three additional screenings. Two (λCRR11 and λCRR13 with inserts of 14kb and 16kb, respectively) were analyzed by restriction mapping, and their nature confirmed by Southern blot using the 5.8S probe. In addition, a 177bp fragment corresponding to the D3 region of the carp 28S rRNA (Michot *et al.*, 1990) was used as probe in Southern blot analyses.

Figure 1 depicts the sub-cloning strategies used for sequence analyses of both λCRR11 and λCRR13 clones. A 5kb *Xba* I fragment from the λCRR11 clone, hybridizing with the 5.8S and 28S probes, was sub-cloned (pCRR5X) into the pUC19 vector and sequenced. A 2kb *Eco* RI-*Pst* I fragment from the λCRR13 clone, overlapping the 5' end of the pCRR5X clone

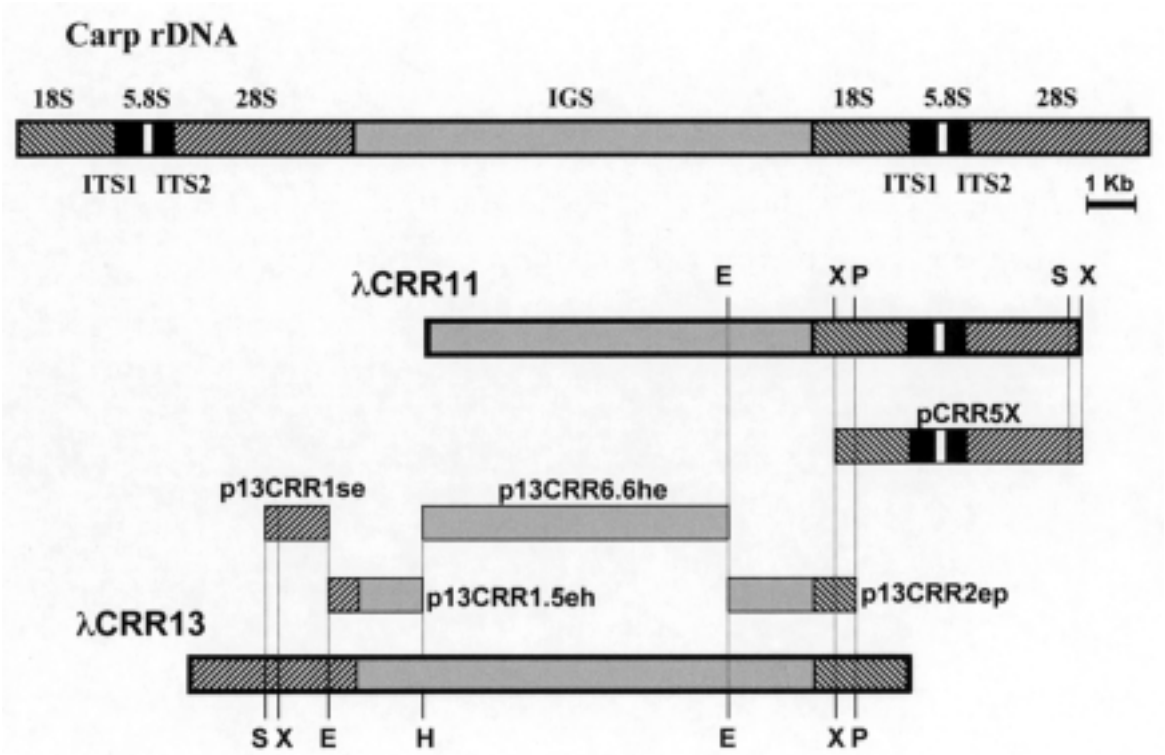


Figure 1
Schematic representation of the carp rDNA cistron (A) and sub-cloning of the lambda clones (B). The restriction enzymes used for the sub-cloning are represented as follows: *Eco* RI (E), *Pst* I (P), *Sal* I (S) and *Xba* I (X).

was sub-cloned (p13CRR2ep) into the pUC19 vector and sequenced. A 6kb *Hind* III-*Eco* RI fragment from the lCRR13 clone, flanking the 5' end of the p13CRR2ep, was subcloned (p13CRR6.6he) into pUC19 vector and partially sequenced. A 1.5kb *Eco* RI-*Hind* III fragment, flanking the 5' end of the p13CRR6.6he, was sub-cloned (p13CRR1.5eh) into pUC19 vector and sequenced. Finally, a 1kb *Sal* I-*Eco* RI fragment, flanking the 5' end of the p13CRR1.5eh, was sub-cloned (p13CRR1se) into pUC19 vector and sequenced.

Transcription start site

To determine the transcription initiation site, we performed a ribonuclease protection assay (RPA) using the RPA III kit (Ambion, USA) according to the manufacturer's instructions. Briefly, a 743bp *Sma* I-*Not* I fragment from the p13CRR2ep clone was sub-cloned (p13CRR0.7sn) into the pBS SK+ vector. From the p13CRR0.7sn clone linearized with *Pst* I, using T3 RNA polymerase (Promega, USA), a riboprobe was generated by *in vitro* transcription and simultaneously radio-labeled with [α-^{32}]CTP. Total kidney RNA (2µg) extracted from both winter- and summer–acclimatized carp (Sambrook and Russell, 2001) were hybridized with the riboprobe (100.000cpm) and treated with a nuclease mix (RNAse A/RNAse T1). The RPA products were fractionated in a denaturing 7% polyacrylamide gel and exposed to an X-Omat film (Kodak, USA).

Phylogenetic analysis

The 18S rRNA genes from table II were aligned using ClustalX (Thompson *et al.*, 1997) with default gap penalties. The alignment was subsequently optimized

manually. To build the phylogenetic tree, an analysis based on the Neighbor-Joining method was performed using the software MEGA v2.1 (Kumar *et al.*, 2001) with 5,000 replicates using only bootstrap values over 50.

RESULTS AND DISCUSSION

The down regulation in the synthesis and processing of rRNA (Vera *et al.*, 1993) and in the transcription of the ribosomal protein L41 during the adaptation of the carp to the cold season environment conditions (Molina *et al.*, 2002) suggest the existence of a complex and coordinated mechanism to modulate the ribosome biogenesis during the acclimatization process. In this study we described the carp rRNA cistron sequence in order to dissect the structure and organization of its most important features that are potentially involved in the carp seasonal regulated rRNA expression.

The rRNA genes are represented in multiple copies throughout the genome of eukaryotic cells, varying in size and copy numbers from species to species (Long and Dawid, 1980; Cortadas and Pavon, 1982). These genes are clustered among many chromosomal loci, ordered in tandem in a "head to tail" manner. Each copy encodes for a polycistronic RNA precursor which is processed into three mature rRNAs (18S, 5.8S, and 28S), separated by two internal transcribed spacers (ITS-1 and ITS-2), and flanked by two external transcribed spacers (5'-ETS and 3'-ETS). Between each coding unit there is a non-transcribed DNA segment called an intergenic spacer (IGS) and containing a number of repeated sequences, some of which enhance the rDNA promoter activity (Paule, 1998).

In most of eukaryotic species, the length diversity of rDNA genes is represented by the IGS variation, ranging from 2kb in yeast to over 20kb in mammals (Paule, 1998). Our results show that the complete carp rRNA gene is contained between both genomic clones (lCRR11 and lCRR13). The size of the carp rDNA cistron is approximately 15kb, of which 8.3kb correspond to the non-coding sequence (IGS) and 6.7kb to the transcribed region (GenBank accession numbers AF133089 and AY260899).

Among different species, the nucleotide sequence of IGS represents the most variable region of the rDNA genes and contains the *cis*-elements that regulate transcription mediated by RNA polymerase I (Fath *et al.*, 2001). The regulatory sequences of rDNA promoters are arranged in the same order and general position relative to the +1 transcription site, even though they are quite divergent in sequence (Paalman *et al.*, 1995). RPA analyses of the carp rDNA gene localize the transcription start site at an A residue 418bp upstream of the 18S rRNA coding sequence (Figs. 2 and 3). Secondary protected fragments were found at +135, +141 and a strong-labeled signal at +165 that could correspond to partially processed transcripts (Fig. 2). The protected fragments observed from RPA analyses performed with RNA extracted from summer- and winter-adapted carp presented the same size. Nevertheless, stronger signals were found in summer-acclimatized carp RPA products, suggesting that seasonal changes in rRNA transcription are not mediated by a potential alternative promoter and/or by a differential processing of the precursor at the 5'ETS. These results are consistent with the diminished transcription and processing of rRNA previously reported by *in vitro* transcription experiments in pituitary glands from winter-adapted carp (Vera *et al.*, 1993).

Despite the lack of conservation in the rDNA regulatory elements, using *in vitro* assays to study transcriptional activity in different species reveals a core promoter within approximately –40 to +5 with respect to the transcription start site, which is sufficient to drive transcription initiation (Doelling and Pikaard, 1995; Radebaugh *et al.*, 1997). In mammals and *Xenopus* rDNA genes, a second regulatory element is the upstream promoter element (UPE), located around 150bp upstream from the core promoter (Paule, 1998). The upstream binding factor (UBF) binds UPE and enhances the rRNA transcription by recruiting the core promoter factors (Xie and Rothblum, 1992). An alignment of the

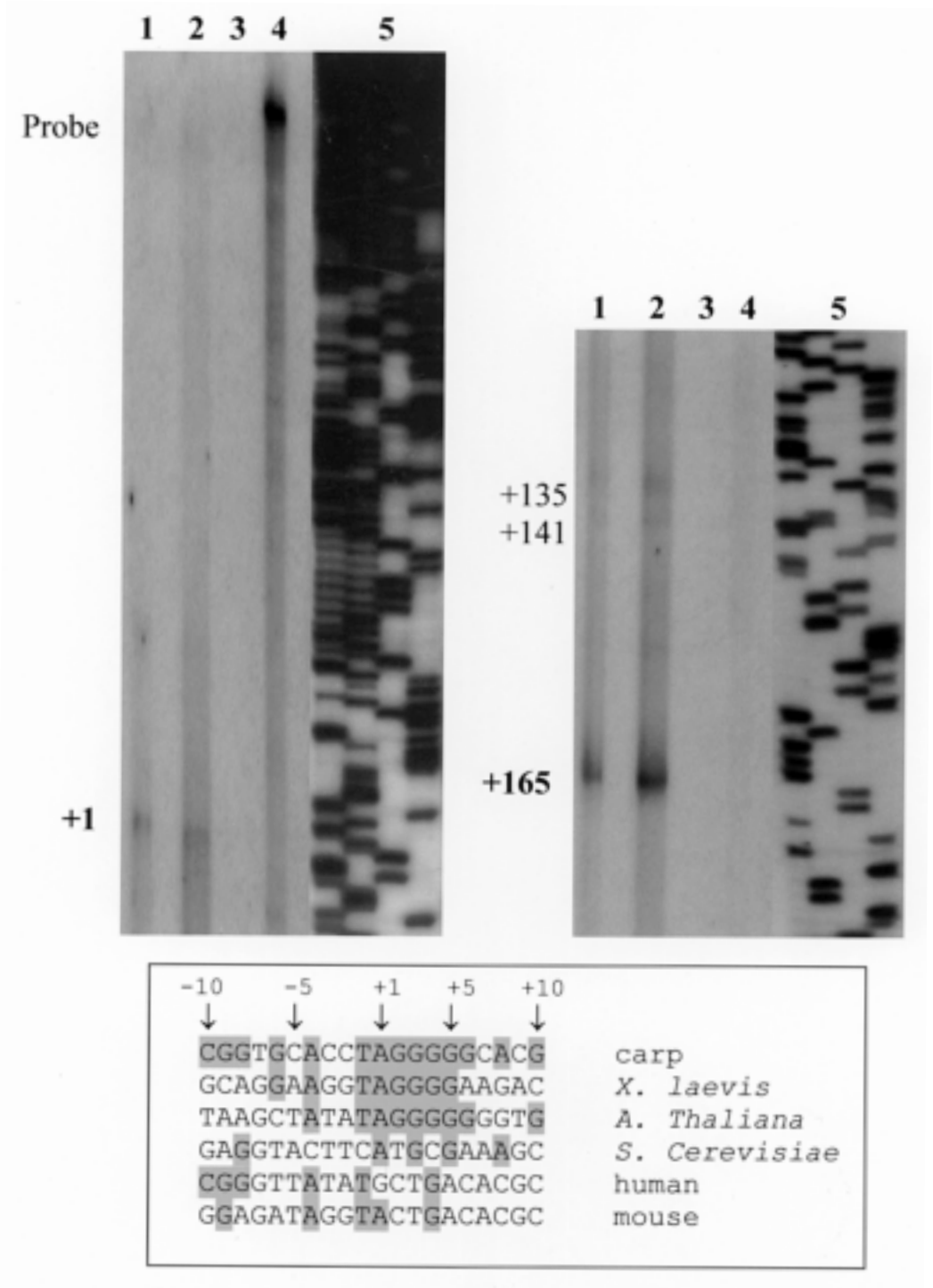
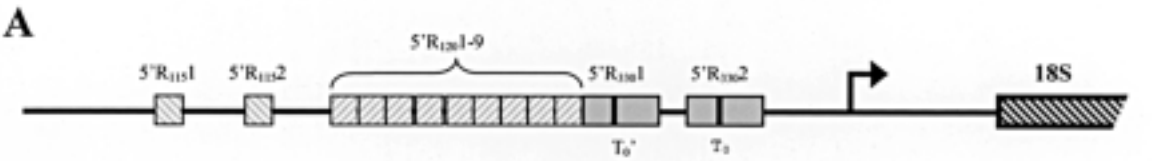


Figure 2
Transcription initiation site determined by ribonuclease protection assay (RPA). Lane 1 shows the riboprobe; Lane 2 shows negative control (without RNA); Lane 3 shows the RPA product of RNA from summer-acclimatized carp; and Lane 4 the RPA product of RNA from winter-acclimatized carp. A non related sequence used as a molecular size marker is on lines denoted by the number 5. Sequence comparison of the region surrounding the transcription start site of different species is shown at the bottom.



B

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-3780  ...cgcgggcgtcgggtccccgtgggctgaataactctggggacccgtagagggcctcggggcctaccgaacgcgaccgccccgagtc
-3690  gcgggctgtccggtcccgagttatgagggcacgaatcccaaagtttgggggtccgtctctcggcctcccgtgggtttagggcctggg
-3600  gcgaatcggcgtctagaggatccccaagaggggcccgctctaatttaccccgggtttgaagtgtcgtttgccccgtttacaacgtc
-3510  gtggctgagattacggccttggccagattttgtgacgtttgatcgccgttgcagtatgggtccgtatcctccgggggcccctcgggtc
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  271  ttcccccgagcgggggaggtcgccgcacgtcgccctatgagcggccgcgccgcgtgggtcccctccgggggatacccacagcggagcga
  361  gacccgcgtgtaggaaaccagcgcgctgcggtggtccatcccacgccccgtccgaggg
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Figure 3

Schematic representation and nucleotide sequence of the region upstream of the 18S rRNA including the 5'ETS and a partial sequence of the IGS. (A) The localization of the repeat sequences represented by boxes, transcription start site (arrow) and 18S rRNA are schematized on the top. (B) The sequence numeration is related to the transcription start site denoted by italic bold uppercase letter. The $5'R_{115}$ and the $5'R_{330}$ repeats are shadowed; the region comprising the $5'R_{120}$ repeats is underlined and the repeats alternate between bold and normal letters. Dark shadowed letters denote putatives T_0 and T_0'.

region upstream to the carp +1 rDNA with other species was unable to detect major transcriptional controlling *cis*-elements, *e.g.* the core promoter or the upstream promoter (UPE). In most eukaryotic species, the region surrounding the transcription start site is the only conserved sequence (Perna *et al.*, 1992). Surprisingly, the alignment with *X. laevis* and *A. thaliana* depicts a core sequence identity up to 6bp downstream of the transcription start site (Fig. 2). The remaining IGS contains repeated sequences that regulate the transcription developed through evolution by repetitive duplication and truncation (Paule and White, 2000 and references within).

The rDNA promoter sequence repeats, called ribosomal enhancers, contain *cis*-elements that stimulate transcription mediated by the core promoter. The size and number of those sequence repeats present a high variability among different species, 60 and 81bp repeats in *Xenopus* (Reeder, 1984), 240 and 330bp repeats in *Drosophila* (Grimaldi and Di Nocera, 1988), 140bp repeats in mouse (Kuhn *et al.*, 1990), several different sized repeats in rat (Cassidy *et al.*, 1986), a 140bp repeat in *Acanthamoeba* (Paule *et al.*, 1991), and even a single 200bp in yeast (Elion and Warner, 1986). In some species, notably in *Xenopus*, this enhancer activity passes through the binding of transcription factors which stimulate the transcriptional machinery in the core promoter (Caudy and Pikaard, 2002). Additionally, it has been proposed that if those repeats are too distant from the +1 site, they inhibit transcription because they compete with the promoter for transcription factors as UBF (Pape *et al.*, 1989; Pikaard *et al.*, 1989).

In the carp IGS we found several different repeat elements, with notorious variations in sequence and size (Figs. 3 and 4). Two direct 330bp repeats at positions -443 and -1064 (5'R_{330}1 and 5'R_{330}2, respectively); nine 120 bp direct repeats were detected at positions -2177, -2064, -1937, -1811, -1686, -1563, -1423, -1310 and -1202 (5'R_{120}1 to 5'R_{120}9); and two direct 115bp repeats at positions -3733 and -2694 (5'R_{115}1 and 5'R_{115}2, respectively). Downstream of the 3'end of carp 28S rRNA we found five repetitions of the sequence GGGTGACCAGTGG, potentially representing the transcription terminators (3'T_1 to 3'T_5), (Figure 4). Four of those repeats are included in two larger sequence repeats (179bp) located at 199bp

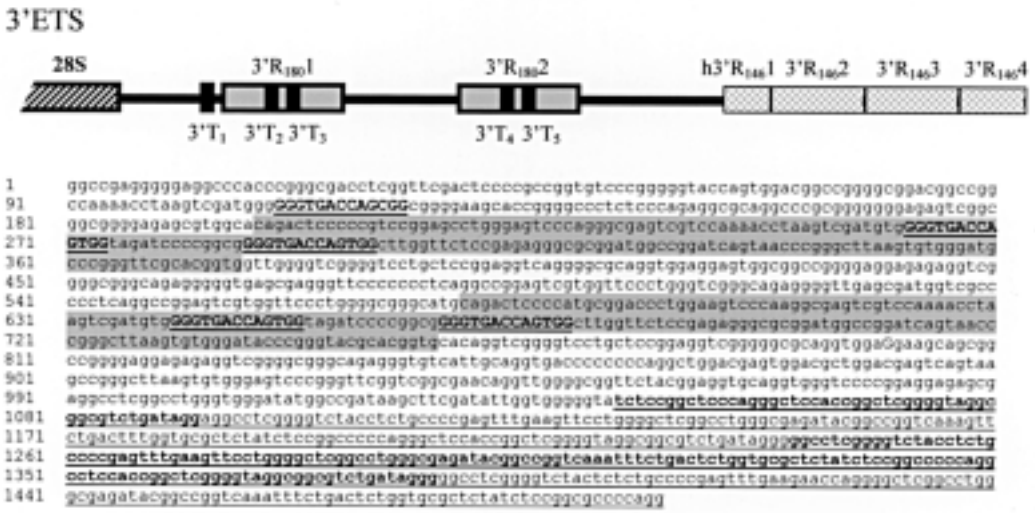

Figure 4
Schematic representation and nucleotide sequence of the region downstream of the 28S rRNA. (A) Repeat sequences are represented by boxes. (B) The 3'R_{180} repeat sequence is shadowed. The 3'R_{146} and h3'R_{146} repeats are underlined and the repeats alternate between bold and normal letters. Putative transcriptional terminators T_1 to T_5 are in bold letters and underlined.

and 580bp from the 3'-end of the 28S (3'R_{180}1 and 5'R_{180}2). The same sequence repeat was found upstream of the +1 embodied in the direct repeats 5'R_{330}1 and 5'R_{330}2, probably corresponds to the proximal terminators T_0 and T_0' (Figure 3). Another sequence repeat of 146bp was found at 1094, 1141, and 1387bp downstream from the 28S rRNA (3'R_{146}1 to 3'R_{146}3). Half of this repeat was also found 1044bp downstream of the 28S, (h3'R_{146}4) at the beginning of the repeat sequence series (Fig. 4). A summary of the sequence comparison performed within all repeat elements is showed in Table I. The identities were as high as 98% within h3'R_{146} repeats and as low as 56.4% for the 5'R_{120} repeat. Nevertheless, no evident identities were found among the different type of repeat sequences (data not shown).

Upstream of the UPE is the proximal terminator (T_0) that significantly stimulates rRNA transcription. In carp IGS, the sequence GGGTGACCAGTGG, which probably corresponds to the transcriptional terminator, was found upstream of the +1 and downstream of the 28S rRNA. This observation strongly suggests that this element could be the proximal terminator (T_0), which in other species is involved in transcription initiation stimulation (Labhart, 1994, 1995). Interestingly, carp rDNA shows two T_0 elements integrated in a large sequence repeat, and to our knowledge, the carp is the only specie to show this feature, a fact that could represent an additional mechanism for transcription initiation control in eurythermal fish.

It is well known that the nucleolus build-up is organized in the loci containing rDNA arrays (Scheer and Weisenberger, 1994), nevertheless, the mechanisms governing the changes in rRNA expression and their relationship with the morphological cyclic changes of the nucleolus under the acclimatization process remains a major question. The putative *cis*-elements described here can be used as an important structural basis to future studies on the seasonal transcriptional regulation of the carp rDNA.

Ribosomal DNA (rDNA) is a remarkable case of gene duplication and has been used as a model to test the concerted evolution theory (Hillis and Dixson 1991; Reed *et al.*, 2000). Due to their moderate rate of sequence evolution, coding- and non-coding (ITS1 and ITS2) transcribed sequences of the rDNA are significant tools for phylogenetic and population studies (Reed *et al.*, 2000). Inversely, phylogenetically-supported folding models have been used in fish, including the carp, to discern functional structural features that were preferentially preserved among different species (Joseph *et al.*, 1999).

As expected, 18S rRNA coding sequences comparison reveals a high degree of conservation through the evolution (data not shown). Therefore, an unrooted phylogram was constructed to analyze the relationship of the *C. carpio* 18S rRNA gene with all other available 18S rRNA sequences from fish, as well as non-related taxa, such as plants, yeast, bacteria, and humans (Table II and Fig. 5

TABLE I

Sequence comparison within all repeat sequences detected at the 5' and 3'-regions of the carp rDNA cistron IGS. Each sequence repeat was compared with the sequence corresponding to the first repeat (first row) of the same group.

	1	2	3	4	5	6	7	8	9
5'R_{120}	100	91.8	79.8	56.4	72.4	80.2	73.0	77.8	64.7
5'R_{110}	100	89.0	-	-	-	-	-	-	-
5'R_{330}	100	91.7	-	-	-	-	-	-	-
3'R_{180}	100	95.0	-	-	-	-	-	-	-
h3'R_{146}	100	98.0	98.0	-	-	-	-	-	-
3'R_{146}	100	98.7	71.4	-	-	-	-	-	

TABLE II

Species and GenBank accession numbers of the full length sequences used for the phylogenetic analysis of the 18S rRNA. (*) Zebrafish 18S rRNA gene sequence was compiled from non-assembled contigs available at the non-processed zebrafish sequence database (http://trace.ensembl.org/perl/ssahaview?server=danio_rerio). (**) Spotted green pufferfish 18S rRNA gene sequence is available under EMBL accession number DS42722.

	Organism	GenBank Accession Number	Common name
1	*Cyprinus carpio*	AF133089	Carp
2	*Danio rerio*	*	Zebrafish
3	*Tetraodon nigroviridis*	**	Spotted green pufferfish
4	*Xenopus laevis*	X02995	African clawed frog
5	*Homo sapiens*	X03205	Human
6	*Mus musculus*	X00686	House mouse
7	*Arabidopsis thaliana*	X16077	Thale cress
8	*Saccharomices cerevisiae*	Z75578	Baker's yeast
9	*Escherichia coli*	Z83205	-
10	*Polyodon spathula*	AF188371	Mississippi paddlefish
11	*Dalatias licha*	AY049827	Kitefin shark
12	*Mitsukurina owstoni*	AY049840	Goblin shark
13	*Ictalurus punctatus*	AF021880	Channel catfish
14	*Oncorhynchus mykiss*	AF308735	Rainbow trout
15	*Squatina californica*	AY049858	Pacific angelshark
16	*Pristiophorus cirratus*	AY049849	Longnose sawshark
17	*Rhinobatos productus*	AY049852	Shovelnose guitarfish
18	*Oryzias latipes*	AB105163	Japanese rice fish
19	*Clupea harengus*	X98845	Atlantic herring
20	*Megalops atlanticus*	X98846	Tarpon
21	*Elops hawaiensis*	X98841	Hawaiian ladyfish
22	*Fundulus heteroclitus*	M91180	Killifish
23	*Sebastolobus altivelis*	M91182	Longspine thornyhead
24	*Squalus acanthias*	M91179	Spiny dogfish
25	*Latimeria chalumnae*	L11288	Coelacanth
26	*Salmo trutta*	X98839	Brown trout

The unrooted phylogram shows that fish group in three clades, two of which correspond to the class Actinopterygii and one to the class Elasmobranchii. It is interesting to observe that the different species that constitute each fish clade are sorted according to their order. Furthermore, the distribution of the different species within the cladogram is supported by phylogenetic trees built using different genes (Zardoya and Meyer, 1996; Ishikawa, 2000; Wittbrodt *et al.*, 2002)

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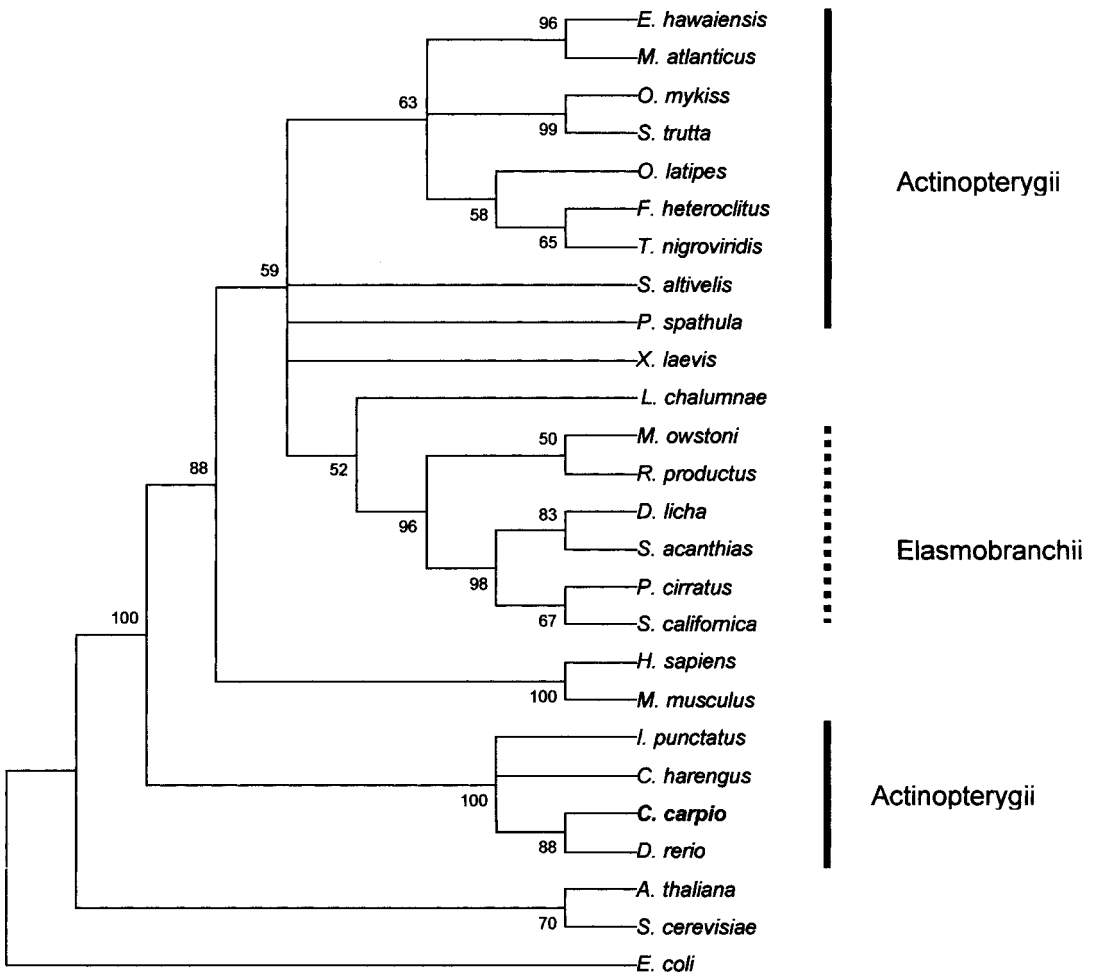

Figure 5
Neighbor-joining phylogenetic tree from full-length 18S rRNA sequences. The bootstrap values are included for each branch.

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