

Mitochondrial DNA content and mitochondrial gene transcriptional activities in the early development of loach and goldfish

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ABSTRACT The mitochondrial DNA (mtDNA) content of the mature eggs and embryos of loach and goldfish at early developmental stages were detected by means of dot hybridization. The transcription of mitochondrial cytochrome oxidase subunit I and II (COI and COII) genes during their early development was also detected by Northern hybridization. The experimental results showed that the mtDNA content of the mature egg as well as that of the embryos during the period from fertilized egg up to hatching stage in both fishes is maintained at a constant level, giving an average value of 7.40×10^7 molecules or 1.33 ng for every embryo in loach and an average value of 1.87×10^8 molecules or 3.31 ng for every embryo in goldfish. In both fish embryos, the COI and COII transcripts declined gradually after fertilization until late-blastula stage and then increased in early gastrula stage. This indicated that the transcription of mitochondrial genomes of these two freshwater fishes, which belong to different families, might be activated at the beginning of gastrulation. The steady-state amounts of mitochondrial messenger transcripts existing in the embryos during the early development in both fishes seemed to be regulated by both their half-lives and the transcriptional level of the mitochondrial genomes. The results showed that the transcription of the mitochondrial genome in the early developmental process in loach and goldfish was not regulated by a gene dosage mechanism.

KEY WORDS: *mitochondrial DNA, transcription, embryo, development, loach, goldfish*

Introduction

Vertebrate mitochondrial genomes are circular duplex DNA molecules of approximately 16.4 kb. Each strand of the duplex («heavy» and «light») could be distinguished through alkaline cesium chloride gradient centrifugation. The heavy strand encodes 2 rRNAs, 14 tRNAs and 12 proteins; and the light one contains 8 tRNAs and one protein gene (Clayton, 1984, 1991). All 13 proteins encoded by the mitochondrial genome are subunits of enzymes in the respiratory chain or ATPase complexes (Chomyn *et al.*, 1986; Fearmley and Walker, 1986; Mariottini *et al.*, 1986).

Vertebrate oocytes synthesize large quantities of mitochondria which will provide energy for cellular activities by means of oxidative phosphorylation for supporting the development of the fertilized egg (Dworkin and Dworkin-Rastl, 1991). The mtDNA molecules are gradually accumulated during the oogenesis of mouse and *Xenopus*, and remain at a constant level for some time after the egg is fertilized and developing (Chase and Dawid, 1972; Webb and Smith, 1977; Piko and Taylor, 1987). However, the activation of mitochondrial transcription and subsequently mitochondrial RNA accumulation also occurs during this period (Piko and Taylor, 1987;

Meziane *et al.*, 1989). This is in agreement with the increasing requirement of energy during the embryo development (Barth and Barth, 1954).

Structure analyses of the mitochondrial genomes of common carp (*Cyprinus carpio*, family Cyprinidae (Araya *et al.*, 1984; Wu *et al.*, 1991; Wang and Wu, 1992) and rainbow trout (*Salmo gairdneri*, family Salmonidae) (Davidson *et al.*, 1988) suggested that the mitochondrial genomes of fishes may have a similar gene order and gene content as those of amphibians (Roe *et al.*, 1985) and mammals (Brown, 1985). However, nothing was known concerning the mtDNA content of fish eggs and embryos and the transcriptional regulation of fish mitochondrial genomes during their embryonic development. The purpose of this study was to obtain information on these issues. In this paper, we report the results of an analysis of the mtDNA content of eggs and embryos of two kinds of teleosts of different families (Loach, *Paramisgurnus dabryanus*, family

Abbreviations used in this paper: mtDNA, mitochondrial DNA; COI, cytochrome oxidase subunit I; COII, cytochrome oxidase subunit II.

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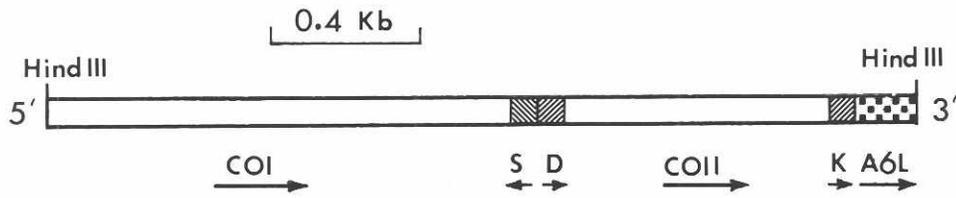


Fig. 1. Structure of the 2.4 kb HindIII fragment from carp mitochondrial genome (Wang, 1989). The fragment contains a 1.2 kb portion of the cytochrome oxidase subunit I (COI) gene (total length 1545 bp), the intact 0.69 kb cytochrome oxidase subunit II (COII) gene and the intact 165 bp A6L gene encoding a subunit of ATP synthase (F_0) on the inner membrane of the mitochondrion. These protein-coding genes are interrupted by three tRNA genes. S: tRNA^{Ser}; D: tRNA^{Asp}; K: tRNA^{Leu}. The arrow indicates the polarity of the genes.

Cobitidae and goldfish, *Carassius auratus*, family Cyprinidae) and the detection of transcriptional activities of COI and COII genes during their early development by using a cloned common carp mtDNA as a probe (see Fig. 1).

Results

Mitochondrial DNA content of the mature eggs and the embryos at early developmental stages in loach and goldfish

Different dilution series of pBCCOI plasmid DNA containing a 2.4 kb HindIII fragment cloned from common carp mtDNA were dotted

on a nylon membrane as standards. The mtDNA content of the mature eggs, fertilized eggs and embryos of loach and goldfish at early and late blastula, early and late gastrula, neurula, eye cup, tail bud, and hatching stages were detected by dot hybridization on the same membrane (Fig. 2).

In Table 1, it can be seen that the mtDNA content of a loach mature egg is 1.37 ng, of a fertilized egg 1.33 ng, and of a hatching stage embryo 1.33 ng. The average content of mtDNA of the loach embryo in its early developmental stages is 7.40×10^7 molecules or 1.33 ng per embryo. The mtDNA content of a goldfish mature egg is 3.57 ng, of a fertilized egg 3.51 ng and of a hatching stage embryo

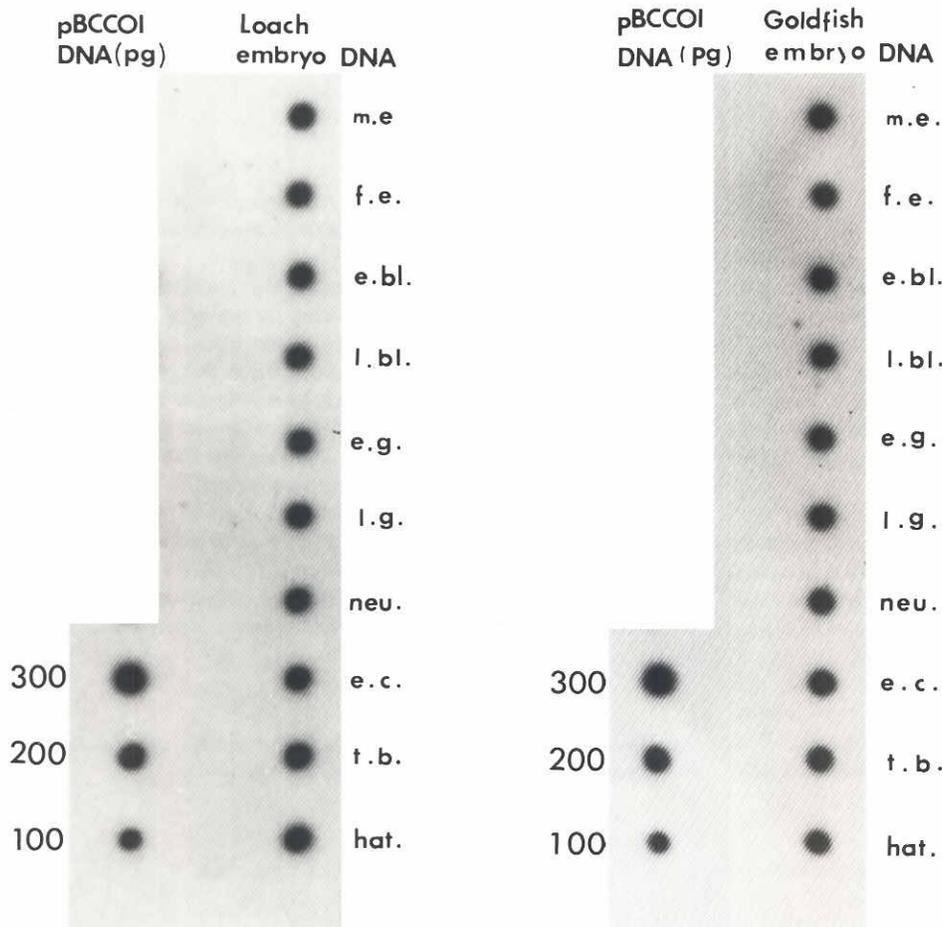


Fig. 2. Dot hybridization of loach and goldfish mtDNA from various developmental stages with the carp mitochondrial 2.4 kb HindIII fragment as a probe. The different dilution series of pBCCOI plasmid (total length 6.8 kb or 1.34×10^5 molecules/pg of DNA) containing the same insert of mtDNA was also dotted as a standard for comparison. DNA from one loach embryo and 0.6 goldfish embryo was added per dot. Abbreviations: m.e., mature egg; f.e., fertilized egg; e.bl., early blastula; l.bl., late blastula; e.g., early gastrula; l.g., late gastrula; neu., neurula; e.c., eye cup stage; t.b., tail bud stage; hat., hatching stage.

TABLE 1

MITOCHONDRIAL DNA CONTENTS OF LOACH AND GOLDFISH EMBRYOS

Developmental stages	mtDNA molecules per loach embryo		mtDNA molecules per goldfish embryo	
	(No. $\times 10^{-7}$)*	(Mass, ng)**	(No. $\times 10^{-8}$)*	(Mass,ng)**
Mature egg	7.61 \pm 0.11	1.37	2.01 \pm 0.12	3.57
Fertilized egg	7.40 \pm 0.17	1.33	1.98 \pm 0.08	3.51
Early blastula	7.92 \pm 0.10	1.42	1.88 \pm 0.07	3.32
Late blastula	7.01 \pm 0.14	1.26	1.79 \pm 0.10	3.16
Early gastrula	7.45 \pm 0.14	1.34	1.78 \pm 0.08	3.14
Late gastrula	7.24 \pm 0.18	1.30	1.85 \pm 0.09	3.27
Neurula	7.76 \pm 0.15	1.40	1.83 \pm 0.15	3.23
Eye cup	7.08 \pm 0.21	1.27	1.08 \pm 0.06	3.18
Tail bud	7.13 \pm 0.26	1.28	1.82 \pm 0.14	3.21
Hatching	7.37 \pm 0.18	1.33	1.92 \pm 0.06	3.50
Average	7.40	1.33	1.87	3.31

*Calculated from densitometric scanning of films from three dot hybridization experiments by using pBCCOI plasmid as standards as in Fig. 2. The mean \pm SE is given.

**Calculated on the basis of the molecular weight of goldfish mtDNA is 16.2 kb or 10.69×10^6 Da (Beckwitt, 1987) and that of loach mtDNA is 16.4 kb or 10.82×10^6 Da (our unpublished data).

3.50 ng. The average value of mtDNA in the goldfish embryo in its early developmental stages is 1.87×10^9 molecules or 3.31 ng per embryo. In both loach and goldfish, the mtDNA content of their mature eggs, fertilized eggs and embryos which developed up to hatching stage is kept at a constant level. The mtDNA content of a goldfish egg is about 2.5 times higher than that of a loach egg.

Identification of mitochondrial transcripts in eggs and embryos

Mitochondrial messenger RNA transcripts of loach and goldfish eggs and embryos were detected by Northern hybridization by using a 32 P-labeled 2.4 kb HindIII fragment of pBCCOI plasmid as a probe. The total RNA samples of eggs and embryos were stained with ethidium bromide (EB) before denaturation. Two major bands representing 18S and 28S rRNAs were visible on the electrophoretic agarose/formaldehyde gels, whereas the nuclear DNA and mtDNA remained at the origin (Fig. 3a). Two clear hybridization bands of 1.6 kb and 0.7 kb in length, respectively, could be seen on the films after Northern blotting and these correspond to the COI and COII transcripts (Fig. 3b,c,d and e). The positions of the two bands on the films were in agreement with the lengths of the transcripts deduced from our sequence data (Wang, 1989). A loose hybridization band located in the 4S RNA region in the over-exposed film of goldfish seemed to correspond to mitochondrial tRNAs (Fig. 3f). The transcripts of the A6L genes in loach and goldfish eggs and embryos could not be seen clearly on the films although the probe which we used in this experiment contained the intact carp A6L gene (Wang, 1989; Wang and Wu, 1992).

Steady-state amounts of mitochondrial mRNAs for COI and COII genes during the early development of loach and goldfish

Northern hybridization experiments of total RNA samples from different developmental stages of loach and goldfish embryos with

the 2.4 kb probe are shown on Fig. 4. The steady-state amount of COI mRNA was more abundant than that of COII mRNA in loach, whereas it was less abundant than that of COII mRNA in goldfish. The relative intensities of COI and COII bands from several Northern hybridization experiments of the embryos during the early developmental stages in these two fishes were analyzed by densitometric scanning. The results are summarized in Fig. 5. After fertilization, the steady-state amounts of COI and COII transcripts of the embryos gradually decreased, and dropped to their lowest level in late blastula stage. The COI and COII transcripts of loach embryo decreased to 63% and 60%, and those of goldfish embryo decreased to 83% and 60% at the late blastula stage, respectively. From then on, the content of COI and COII transcripts in the embryos of these two fishes progressively increased after the early gastrula stage. In both fishes, the amounts of COI and COII transcripts in late gastrula embryo were higher than those in the fertilized eggs. At tail-bud stage, the amounts of COI and COII transcripts were 1.4 and 1.6 times of those in the fertilized egg in loach, and 1.6 and 1.4 times of those in the fertilized egg in goldfish, respectively.

Discussion

During the oogenesis of mouse and *Xenopus*, the mtDNA content increases proportionally to the volume of the oocyte (Webb, 1977; Piko and Taylor, 1987). In mouse, it maintains a constant level in the mature egg, fertilized egg and embryos developed up to early blastocyst stage (Piko and Taylor, 1987). In amphibians, it remains unchanged in the mature egg and in embryos during the develop-

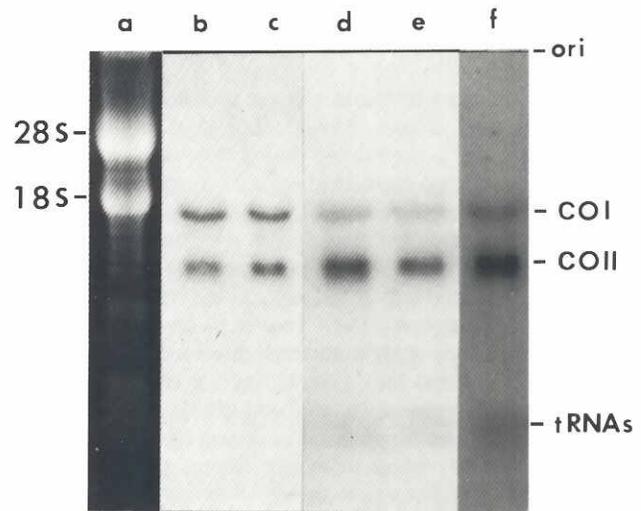


Fig. 3. Identification of COI and COII transcripts in loach and goldfish eggs and embryos with the 2.4 kb probe (see Fig. 1) by Northern hybridization. Two typical 28S and 18S cytoplasmic rRNA bands were clearly seen on the gel after electrophoresis of EB-treated RNA samples whatever the origin of the samples: eggs or embryos from loach or goldfish (lane a). Total RNA extracted from 40 mature eggs and 40 late gastrula of loach was added in lanes b and c, respectively. Lanes d and e represent the total RNA from 15 mature eggs and 15 early gastrula of goldfish. A loose hybridization band was visible in 4S RNA region (yeast tRNA marker region) in over-exposed films of goldfish, which might correspond to the mitochondrial tRNA^{Ser}, tRNA^{Asp}, tRNA^{Lys} (lane f).

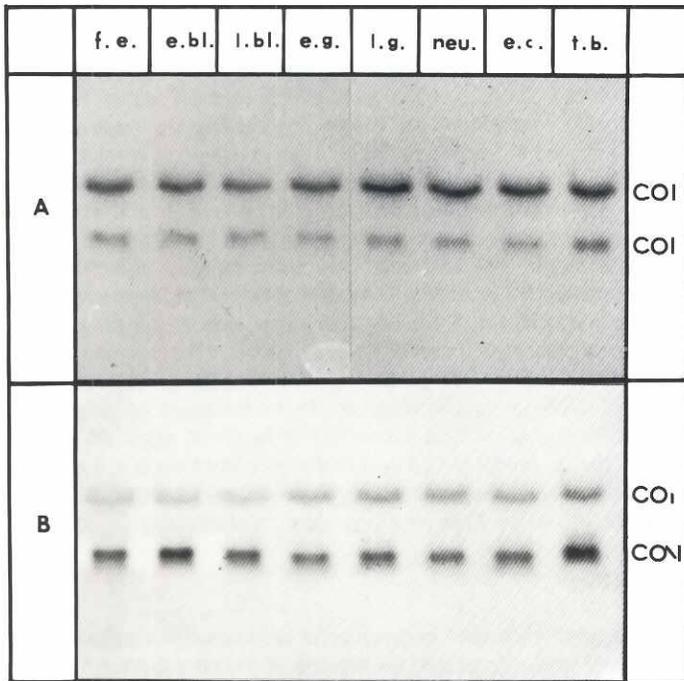


Fig. 4. Transcription of mitochondrial COI and COII genes during the early development of loach (A) and goldfish (B). Total RNA extracted from the same number of embryos (30 for loach and 15 for goldfish) of different stages was loaded to 1.4% agarose/formaldehyde gels. Hybridization was carried out with the 2.4 kb probe. The abbreviations are the same as in Fig. 2.

mental process from fertilized egg up to the swimming tadpole stage (Chase and Dawid, 1972). This implies that the large amounts of mtDNA accumulated during oogenesis in mouse and *Xenopus* are a storage product of eggs for supporting their further development after fertilization. Our results also indicate that the mtDNA contents are kept at the same level in either the mature eggs or embryos during the period of early development of loach and goldfish from fertilized egg up to the hatching stage. Therefore, the pattern of mtDNA accumulation seems to be similar in general during oogenesis and early embryonic development among mammals, amphibians and fish except that the mtDNA amounts of amphibian and fish remain unchanged during a relatively longer period of their early development (Chase and Dawid, 1972 and this paper) than that in mouse (Piko and Taylor, 1987). The mouse embryo begins to grow up to mass with the onset of trophoblastic giant cell transformation during implantation (McLaren, 1976) and so Piko and Taylor (1987) assumed that the increasing mtDNA content is necessary at that time or soon thereafter as the embryo enters a period of rapid growth. Therefore, this kind of stage-specific difference of mtDNA content accumulation in mammals, amphibian and fish seems somehow to be related to the differences in their developmental behavior.

According to our results, the mtDNA content of a goldfish egg (3.57 ng) is about 2.6 times higher than that of a loach egg (1.37 ng) and about 1700 times higher than that of a mouse egg (2.13 pg) (Piko and Taylor, 1987). This may be due to the fact that the volume

of goldfish egg is about 2 times larger than that of loach and over 1300 times larger than that of mouse. However, the mtDNA content of a goldfish egg (3.57 ng) is close to that of a *Xenopus* egg (3.8 ng) (Chase and Dawid, 1972). This kind of similarity may be caused by the different densities of the mitochondria in fish eggs and amphibian eggs.

Mitochondrial COI, COII and A6L transcripts are all encoded by the heavy strand of mtDNA which is transcribed in the form of a polycistron (Attardi et al., 1982). As was shown in Fig. 4, the steady-state amount of COI mRNA is more abundant than that of COII mRNA in the loach embryo, whereas it is less abundant than that of COII mRNA in goldfish embryos. Since the relative abundance of mitochondrial transcripts is determined by their stability (Gelfand and Attardi, 1981), our results show that the COI mRNA is more stable than COII mRNA in loach and less stable than COII mRNA in goldfish. The mechanism for this kind of stability difference between COI and COII mRNAs in both fishes is still unknown. Does it mean that the different stability of COI and COII transcripts in loach and goldfish is species-specific? An answer requires further investigation.

The probe that we used in this experiment contains the entire carp A6L gene, which has 7 nucleotides overlapping its downstream ATPase 6 gene (Wang and Wu, 1992). According to the mtDNA transcription maps of mouse and human, there is only a single transcript, RNA 14, of the two overlapping genes (ATPase 6 and A6L) which encodes the subunits of ATP synthase (F_0) and the length of RNA 14 is 0.85 kb (Montoya et al., 1981; Van Etten et al., 1982). No band corresponding to RNA 14 was clearly seen even on over-exposed films of loach (data not shown) and goldfish embryos (Fig. 3f) in our experiments. A similar result was obtained by Meziane et al. (1989). They also reported that the band corresponding to the RNA 14 transcript could not be clearly demonstrated during *Xenopus* development with the mouse A6L gene as a probe. Therefore, we think that the difficulty for detecting the RNA 14 transcript during the early development of fish and amphibians may be due to its high turn-over rate, or more likely due to the low sequence homology between the RNA 14 transcript and the A6L gene probe. A6L has limited sequence homology even among closely related species (Wang and Wu, 1992).

Relatively little is known about the transcriptional regulation mechanisms of vertebrate mitochondrial genome. William (1986) observed that the increase in mitochondrial transcripts is parallel to the increase in mtDNA copy number in rabbit striated muscle, so he suggested that gene dosage is the main regulatory event in mitochondrial gene expression in those cells (constitutive transcription). However, our results and those of others (Chase and Dawid, 1972; Piko and Taylor, 1987) indicated that mitochondrial mRNA accumulated in the absence of mitochondrial DNA accumulation during the early development of various kinds of vertebrates. Hence, it seems that the regulation of mitochondrial transcription during the early development of vertebrates is different from that of adult tissues and is not controlled by gene dosage.

The transcription patterns of mitochondrial genomes during the early development of mouse, *Xenopus* and the two fish are very similar. The amount of mRNA for COI and COII genes decreased within the period after fertilization and then increased without mtDNA content alternation (Chase and Dawid, 1972; Piko and Taylor, 1987; Meziane et al., 1989), implying that mitochondrial transcription during the early development of vertebrates may be controlled by a similar regulation mechanism. The decrease in the

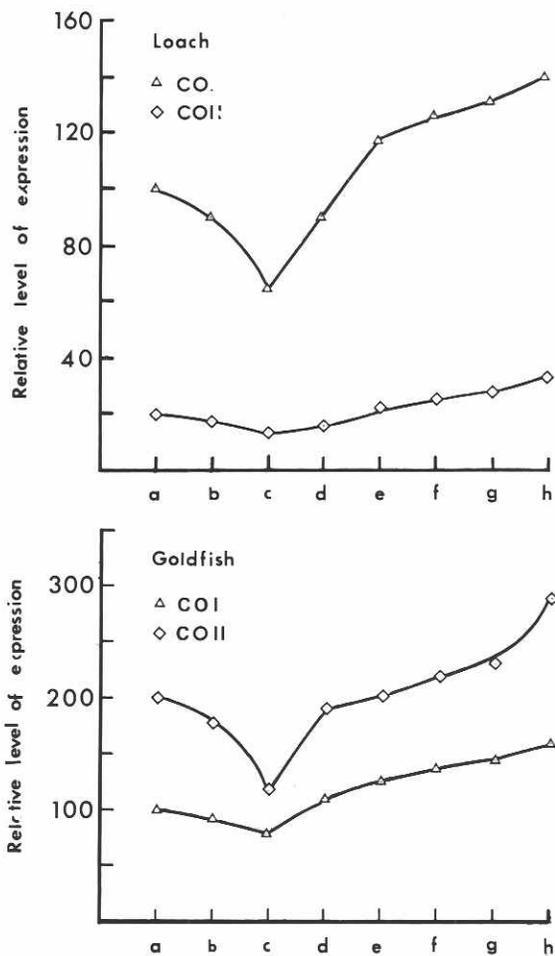


Fig. 5. Densitometric analysis of COI and COII transcripts from various developmental stages of loach and goldfish. Northern hybridization was carried out as in Fig. 4. The films were scanned with a Shimadzu scanner. The relative amounts of COI and COII transcripts are shown as percentage changes in different developmental stages over fertilized eggs, where the values of the COI transcripts in loach and goldfish fertilized eggs were set to 100. The main values from three experiments are given. Developmental stages: a, fertilized egg; b, early blastula; c, late blastula; d, early gastrula; e, late gastrula; f, neurula; g, eye cup stage; h, tail bud stage.

amount of mitochondrial transcripts during the period after fertilization might be determined by their half-lives (Gelfand and Attardi, 1981).

The time of transcriptional activation of the mitochondrial genome is different in mammals, amphibians and fish. The precise time of the activation of transcription in *Xenopus* mitochondrial genome is still not clear at the present time. Dawid (1985) showed that RNA is not synthesized before the mid-blastula transition (MBT) and is activated shortly after MBT. Meziane et al. (1989) proved that the amount of mitochondrial mRNA relative to total cellular RNA decreased after fertilization, maintained a low level up to the neurula stage, and then increased. During the early development of *Xenopus*, the total cellular RNA remains constant up to neurula stage (Denis, 1974), and so they suggested that the time for activating

mitochondrial transcription may happen at the neurula stage. It was reported that the mouse mitochondrial transcripts decreased at the 1-cell stage and 2-cell stage and increased at the 8-cell stage (Piko and Taylor, 1987), so it might be assumed that the mouse mitochondrial genome is activated at the 4-cell to 8-cell stage (Piko and Taylor, 1987). Our results show that, in loach and goldfish, the mitochondrial transcripts began to increase in the early gastrula stage. Therefore, the transcriptional activation of mitochondrial genomes in both fishes may start at the beginning of gastrulation. Since in all the aforesaid three cases, no changes of mtDNA content were observed accompanying the decrease or increase in the mRNA transcripts during their early development, it may be assumed that the steady-state amount of mitochondrial transcripts during their early development is regulated by both the half-lives of the mitochondrial transcripts and the transcriptional level of their mitochondrial genomes.

Materials and Methods

Eggs and embryos of loach and goldfish

Adult goldfish (*Carassius auratus*, family Cyprinidae) were obtained from the aquarium of the Institute of Developmental Biology, the Chinese Academy of Sciences, Beijing China. The loach (*Paramisgurnus dabryanus*, family Cobitidae) were purchased from the fish market in Beijing and cultured in the aquarium of the Institute for a few days before pituitary injection. Eggs and embryos were obtained as described by Yan (1989). The mature eggs of loach were obtained after 8 hours intraperitoneal injection of common carp pituitary glands (5 mg/kg) homogenized in Holtfreter's solution. The mature eggs of goldfish were collected from mature females during the spawning season. The fertilized eggs of both fish were obtained by artificial insemination (mixing the sperm and eggs in a Petri dish filled with clean water and shaking the dish gently to distribute the fertilized eggs and let them attach to the surface of the dish bottom evenly). The water in the dish was changed three times to wash out the surplus sperm. Both the mature eggs and pre-hatching embryos were dechorionated with a 0.25% trypsin (Sigma) solution, rinsed thoroughly in clean water two times and cultured in it at 22-24°C. Developmental stages of loach and goldfish are according to the Time Tables made by Liang et al. (1988) for loach and Li et al. (1959) for goldfish. Eggs or embryos were kept in a -70°C freezer for DNA extraction or to be used immediately for RNA extraction (see below).

Extraction of DNA and RNA from loach and goldfish

DNA was extracted from frozen-thawed eggs and embryos (10 embryos/tube) by homogenizing in 0.5 ml lysis buffer [100 mM NaCl, 10 mM Tris/HCl, 25 mM EDTA, 0.5% SDS, 100 µg/ml proteinase K (BRL), pH 8.0] through vortex. Then the homogenate was mixed with 20 ng ³H-labeled lambda phage DNA (sp. act. 4-8x10⁶ cpm/µg) as a recovery marker, incubated in 37°C water bath for 1 hr, and extracted with phenol twice. The DNA was dissolved in restriction enzyme buffer (66 mM Tris/HCl, 77 mM NaCl, 50mM MgCl₂, 100 mM DTT) and incubated at 37°C for 1 hr with 200U/ml each of the EcoRI and HindIII (Boehringer, Mannheim). RNase A and RNAase T₁ (Boehringer Mannheim) were also added, the final concentrations of both enzymes being 10 µg/ml and 5U/ml, respectively. After incubation it was extracted with phenol:chloroform once. The water phase was used for dot hybridization experiments. The number of eggs and embryos shown in this paper is corrected for the recovery rates, which were better than 70%. Lysis buffer (500 µl) was added to batches of eggs and embryos (30 for loach and 15 for goldfish/tube), homogenized by vortex. The homogenates were extracted with phenol once, and phenol: chloroform twice. The RNA recovery rate was calculated from the final volume of water phase/initial volume of water phase. We recovered 400 µl water phase per tube, so a RNA recovery rate of 80% was used to correct the number of eggs and embryos in our Northern blot experiments. Total RNA samples, containing both nuclear DNA and mtDNA, were kept in 70% ethanol in a -70°C freezer and used within two weeks.

DNA dot hybridization and RNA Northern hybridization

A recombinant plasmid pBCCOI containing 2.4 kb HindIII fragment of carp mtDNA (Wang, 1989) was used to prepare the probe in DNA dot hybridization and RNA Northern hybridization. The structure of the cloned fragment is shown in Fig. 1. The digested HindIII fragment was recovered from a low-melting-point agarose gel and labeled with ^{32}P -dCTP or dATP(ICN) by nick translation (BRL Nick-translation kit). DNA samples were dotted on Hybond-N membranes (Amersham) and hybridized with the labeled probe according to the method described in Davis *et al.* (1986). The total RNA was resolved by electrophoresis in a 1.4% denaturing agarose gel containing 2.2 M formaldehyde. Transfer, hybridization and washing of the membranes were carried out following the method described by Sambrook *et al.* (1989). The filters were exposed to autoradiographic films at -70°C . For the quantitative densitometry of autoradiographies, exposure times were chosen such that comparisons were made in the range in which the response was linear. The densitometric analysis of films was made by a dual wave TLC Scanner (Shimadzu CS-930).

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