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The Complete Mitochondrial Genome of the Hagfish *Myxine glutinosa:* Unique Features of the Control Region

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Abstract. The complete sequence of the mitochondrial DNA of the hagfish *Myxine glutinosa* has been determined. The hagfish mtDNA (18,909 bp) is the longest vertebrate mtDNA determined so far. The gene arrangement conforms to the consensus vertebrate type and differs from that of lampreys. The exceptionally long (3628-bp) control region of the hagfish contains the typical conserved elements found in other vertebrate mtDNAs but is characterized by a large number of putative hairpins, which can potentially fold into a highly compact secondary structure that appears to be unique to hagfish. The comparison of the mtDNAs of two *M. glutinosa* specimens, excluding the control region, shows a 0.6% divergence at the nucleotide level as a sample of intraspecies polymorphism.

Key words: Hagfish — *Myxine glutinosa* — Cyclostomes — Mitochondrial DNA — Control region

Introduction

The phylogenetic relationships among cyclostomes, i.e., hagfish and lampreys, are not fully understood. Most anatomical data support the paraphyly of cyclostomes (Lovtrup 1977; Janvier 1996), as did a recent analysis of the protein-coding genes of the mitochondrial DNA (mtDNA) of *Myxine glutinosa* (Rasmussen et al. 1998). In contrast, most other molecular data tend to support the monophyly of cyclostomes (Stock and Whitt 1992; Mallatt and Sullivan 1998). The conflict between data will probably be solved only by the determination of additional DNA sequences related to the node under study, as well as by comparison of mitochondrial genome maps. In this respect, the complete nucleotide sequences of the mtDNA of two lampreys, *Petromyzon marinus* (Lee and Kocher 1995) and *Lampetra fluviatilis* (Delarbre et al. 2000), and the sequence of the protein-coding genes of the mtDNA of a hagfish, *Myxine glutinosa* (Rasmussen et al. 1998), had been reported previously. The 12S and 16S rRNAs and the tRNA sequences (except *trnP*) had also been determined (Rasmussen et al. 1998; unpublished data). But the complete mitochondrial DNA sequence of the hagfish and its precise genomic map could not be reported due to severe technical difficulties in the determination of the sequence of the control region.

The use of a combination of PCR-based techniques has made it possible to obviate these difficulties and thus to determine the complete sequence of the mtDNA of another specimen of *Myxine glutinosa.* The present study thus allows the description of the complete mitochondrial sequence of *Myxine glutinosa* and its genomic organization, pointing to its belonging to the consensus vertebrate type in contrast with lampreys. It shows a highly distinctive control region potentially forming a densely packed structure. Finally, it allows for the first time a detailed comparison between the mitochondrial

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Table 1. PCR primers used in the determination of the nucleotide sequence of *Myxine glutinosa* mitochondrial genome[a]

	Sequences (5′–3′) of PCR primers	Location (bp)
1:	CGTGATCTGAGTTCAGACCGG	
	GGAGACCCCAAAAAAGAGAG	18,682–942
2:	CCACGATTCCGATATGATCAACT	
	CCRATRTCYTTGTGWTTAGTAGA	832–2,610
3:	ATCACTATGTATCTTTCCCGATG	
	GCATCTGGGTARTCKGAGTADCGKCG	2,558–3,900
4:	CACTTCTGAATTATGTTTACAGGA	
	TCAAAGGTTTTTAGGGGAATCAC	3,800–4,908
5:	TGTGGCGCAAAYCAYAGYTTTATRCC	
	CGTGAAAGCCKGTGGCKACAAARAAKG	4,847–6,454
6:	CTTTACTCCAAGGGATAGAATAC	
	GGGRGCYTCTACRTGRGCTTTDGG	6,362–8,085
7:	GGTTAGGTTGCTTTTCTGCCCTG	
	GTGCTGARACAGGAGTTGGGCCYTCTAT	8,000–9,715
8:	GAAAATCAGCTCAATTCATACTTCAC	
	GGCTGCTGATTGTTTTTGGGGGC	9,644–10,884
9:	CTCTTCCCTCTTAACCTCCGCC	
	GTTGATTTACAAAATCAATGTTTTG	10,755–12,574
10:	AGTACCTGAACTCACGTTACCCCCCTTTCTAACC	
	TAAGGGTAAGACTAACCCTTTATGTGTCTGAGATG	12,480–16,383
11:	AAAGYATRRYAYTGAARATGYTMAG	
	GGCATTTCACCCCTACTCGAG	16,315–17,600
12:	ATYAAAYCTYGTACCTTTTGCATCAT	
	CTAYAYTTTATYTKTCCTTTCGTACTAA	17,441–18,768

[a] IUB code: R, A/G; Y, C/T; M, A/C; W, A/T; D, G/A/T; K, G/T.

genes of two hagfish specimens, thereby providing information on the extent of the intraspecies polymorphism of a cyclostome species.

Materials and Methods

DNA Isolation

The *Myxine glutinosa* (*Myxine 2*) specimen was collected from Kristineberg on the Swedish west coast. The animal was anesthesized, killed, and dissected. After collection, various tissues were immediately frozen in liquid nitrogen and stored at −78°C. Total DNA was prepared from muscles by proteinase K digestion according to conventional procedures (Hogan et al. 1994).

PCR and Sequencing of mtDNA

Overlapping fragments of the mtDNA were obtained by PCR from total DNA. The sequences of the primers used for PCR amplification and their positions in the genome are listed in Table 1. The general PCR conditions were the following: 300 ng total DNA, 200 μ*M* dNTP, and 500 n*M* concentrations of primers. The enzyme was *Pfu* Exo+ polymerase (Stratagene). The thermal cycles were 3 min at 94°C, followed by 50 cycles of 1 min at 94°C, 1 min at 48 to 55°C, depending upon the primers, and 5 min at 72°C. The last cycle was ended by incubation for 10 min at 72°C. For long or problematic PCR of the control-region DNA, we used the Expand Long Template PCR System (Roche Molecular Biochemicals) in a final volume of 50 μl with 300 ng total DNA, 500 μ*M* dNTP, 500 n*M* concentrations of primers, 2.25 m*M* $MgCl_2$ and detergents (buffer 3 supplied in the kit), and 250 ng of T4 gp 32 (Q.BIOgene), with the following conditions: 3 min at 95°C and 50 cycles of 1 min at 95°C, 1 min at 58 to 68°C, 5 min at 68°C, and a final cycle of 10 min at 68°C. The PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN), phosphorylated with polynucleotide kinase (Pharmacia), and ligated at the *Eco*RV site of dephosphorylated KS Bluescript vector (Stratagene) using the Rapid DNA Ligation kit (Roche Molecular Biochemicals). XL1Blue competent bacteria (Stratagene) were transformed with the ligation products. Several recombinant clones were selected for each PCR product and plasmid DNA was recovered using the QIAprep Spin Miniprep Kit (QIAGEN). The cloned PCR products were sequenced using the Sequenase Version 2.0 DNA Sequencing Kit (USB) with [$\alpha - {}^{33}$P] dATP and M13 (−40) and KS reverse primers. The entire sequence was determined on both strands using primer walking.

The search for hairpins in the nucleotide sequence of the control region was made using program DNA Strider 1.2.

Results and Discussion

Nucleotide Sequence and Genomic Organization of the mtDNA of Myxine glutinosa

The mtDNA of *Myxine glutinosa* of the specimen studied in the present paper (*Myxine 2;* accession number AJ404477), is 18,909 bp in length, the longest vertebrate mtDNA determined so far, and includes the usual genes for 13 proteins, 22 tRNAs, and 2 rRNAs (Table 2). Its exceptional length is due to a 3628-bp-long control region. The nucleotide composition of the major coding strand is as follows: A, 30.2%; C, 24.7%; G, 12%; and T, 33.1%. These values are very similar to those determined for two other cyclostomes: *Petromyzon marinus* (Lee

Table 2. Localization of the genes in the mitochondrial genome of *Myxine glutinosa*[a]

			Size		Codons	
Gene	From	To	bp	aa	Start	Stop
nad1	1	957	957	318	ATG	TAG
trnI (*gat*)	957	1,024	68			
trnQ (*ttg*)	1,026	1,096	71(L)			
trnM (*cat*)	1,101	1,169	69			
nad2	1,180	2,226	1,047	348	ATG	TAA
trnW (*tca*)	2,225	2,289	65			
trnA (*tgc*)	2,289	2,352	64(L)			
trnN (*gtt*)	2,355	2,420	66(L)			
trnC (*gca*)	2,421	2,486	66(L)			
trnY (*gta*)	2,491	2,556	66(L)			
cox1	2,564	4,117	1,554	517	ATG	AGG
trns (*tga*)	4,105	4,175	71(L)			
trnD (*gtc*)	4,179	4,244	66			
cox2	4,247	4,936	690	229	ATG	AGA
trnK (*ttt*)	4,929	4,993	65			
atp8	4,995	5,159	165	54	ATG	TAA
atp6	5,153	5,839	687	228	ATG	TAA
cox3	5,842	6,626	785	261	ATG	TA-
trnG (*cct*)	6,626	6,692	67			
nad3	6,714	7,061	348	115	ATA	TAA
trnR (*tcg*)	7,064	7,128	65			
nad4L	7,130	7,420	291	96	ATC	TAA
nad4	7,414	8,790	1,377	458	ATG	TAA
trnH (*gtg*)	8,786	8,849	64			
trnS (*gct*)	8,850	8,910	61			
trnL (*tag*)	8,912	8,982	71			
nad5	8,983	10,788	1,806	601	ATA	TAA
nad6	10,784	11,287	504(L)	167	ATG	AGA
trnE (*ttc*)	11,288	11,354	67(L)			
cob	11,355	12,512	1,158	385	ATG	TAA
trnT (*tgt*)	12,533	12,598	66			
trnP (*tgg*)	12,602	12,667	66(L)			
Noncoding	12,668	16,295	3,628			
trnF (*gaa*)	16,296	16,361	66			
rrnS	16,362	17,223	862			
trnV (*tac*)	17,224	17,292	69			
rrnL	17,293	18,833	1,541			
trnL (*taa*)	18,834	18,907	74			

[a] *atp6* and *-8,* ATP synthase subunits 6 and 8; *cox1–3,* cytochrome *c* oxidase subunits I to III; *cob,* cytochrome *b; nad1–6,* NADH dehydrogenase subunits 1 to 6; *rrnS* and *rrnL,* small and large subunits of ribosomal RNA genes; *trn,* transfer RNA genes with anticodons in parentheses. (L) The gene is located on the L strand of the mtDNA.

and Kocher 1995) and *Lampetra fluviatilis* (Delarbre et al. 2000).

The molecular map of the mtDNA of *M. glutinosa* is found to be identical to the consensus vertebrate mtDNA type (reviewed by Boore, 1999; Saccone et al. 1999). This genomic organization differs from that of the mtDNAs of *Petromyzon* and *Lampetra,* which have the control region between *cob* and *nad6* and *trnT* and *trnE* present in the middle of this control region (Lee and Kocher 1995; Delarbre et al. 2000). The absence, in the mtDNA of *Myxine glutinosa,* of the origin of replication of the light strand between *trnN* and *trnC,* as already reported (Delarbre et al. 1997), was confirmed and was found to be a trait common to all cyclostomes studied so far. The location of the actual origin of light strand replication remains unknown in cyclostomes.

Although the control region is much longer in *M. glutinosa* than in *L. fluviatilis,* the rest of the mitochondrial genome is more compact (15,281 bp) than in the latter animal (15,517 bp). Most tRNAs are individually shorter, with the total length of the 22 tRNAs genes of *Myxine* being 51 bp shorter; *rrnS* and *rrnL* are 41 and 75 bp shorter; and the protein-encoding genes are, in total, 45 bp shorter.

The potential secondary structures of the 22 tRNAs are shown in Fig. 1. All of them, except for *trnS1,* are able to fold into a cloverleaf secondary structure. In addition, there is potential for additional pairing in the D-arm of tRNAs Q, N, C, L1, and S2 as well as in the T-arm of P and S1.

Most properties of the protein-coding genes have been described earlier (Rasmussen et al. 1998), and only some additional points are mentioned below. The 5′ limits previously assigned to *nad1, nad3, nad4L,* and *nad6* have been reassigned on the basis of their alignment with the mtDNA sequences of other animals. At the beginning of *nad2* and *nad6,* there exist two consecutive codons, ATG and ATA, each coding for methionine; we chose the first ATG as the initiation codon for the two genes (the first codon for *nad2* and the second codon for *nad6*), keeping in mind that ATA could not be excluded as the genuine initiation codon. The initiation codon of *cox1* is ATG, as in *Xenopus laevis* (Roe et al. 1985) and human (Anderson et al. 1981), whereas it is GTG in most vertebrates, including the other two cyclostomes *L. fluviatilis* and *P. marinus.* Concerning stop codons, AGG or AGA is used in *cox1, cox2,* and *nad6.* The stop codon of *cox3* was, however, found to be incomplete and should be generated by polyadenylation of the terminal TA. Some genes of the mtDNA of *Myxine* are found to overlap, in contrast with the same genes of *Lampetra* and *Petromyzon: trnS (tga)* overlaps *cox1* by 13 bp, *trnK* overlaps *cox2* by 8 bp, and *trnH* overlaps *nad4* by 5 bp. In addition, *atp8* and *atp6* overlap by 7 nucleotides (nt), whereas the overlap is 10 nt in *Lampetra* and *Petromyzon;* the overlap of *nad4L* and *nad4* is the same in all cyclostomes studied (7 nt); and the overlap of *nad5* and *nad6* is more extensive (16 nt) in *Lampetra* and *Petromyzon* than in *Myxine* (5 nt). Several noncoding sequences are dispersed between tRNA genes and protein-coding genes of *M. glutinosa:* 20 nt between *cob* and *trnT,* 10 nt between *trnM* and *nad2,* and 7 nt between *trnY* and *cox1.* These "spacers" are absent from the mtDNA of the lampreys *Lampetra* and *Petromyzon.*

Intraspecies Polymorphism

The nucleotide sequence of the mtDNA, excluding the control region and *trnP,* of another specimen of *M. glu-*

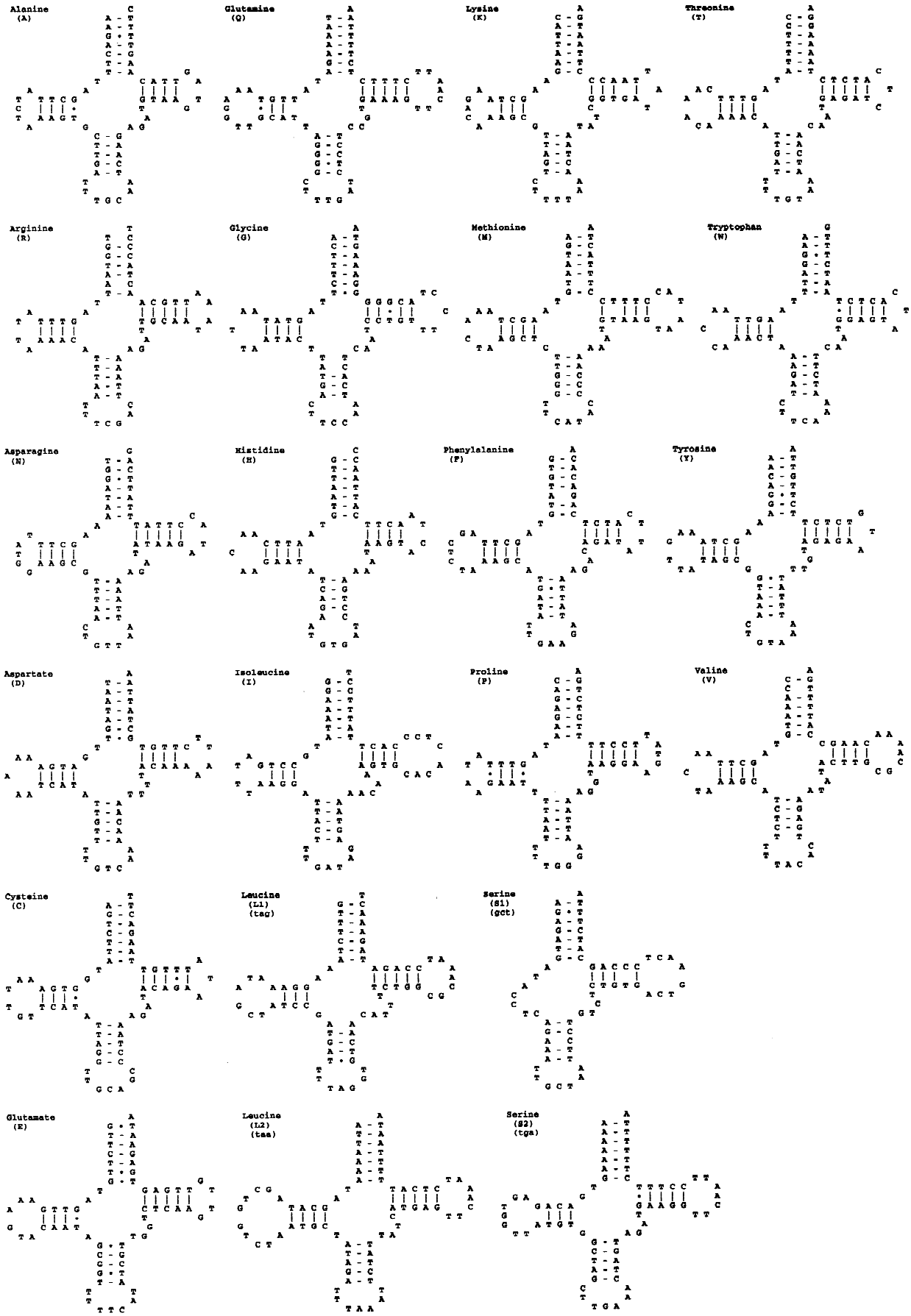

Fig. 1. Potential secondary structures of the 22 tRNAs. AT and GC pairings are indicated by *bars*, and GT pairings by *dots.*

tinosa (*Myxine 1*), also caught in Kristineberg on the Swedish west coast, had been determined previously (Rasmussen et al. 1998; unpublished data). The availability of two independently determined 15,197-bp-long DNA sequences amenable to alignment and comparison, derived from animals belonging to the same species and living in the same environment, allowed us to sample intraspecies polymorphism. The overall divergence at the nucleotide level between the two sequences is about 0.6%. More precisely, the comparison of the tRNA genes (without *trnP*, the sequence of which had not been determined in *Myxine 1*) shows only two differences: one nt in *trnG* and one nt in *trnS* (*gct*). The percentage of divergence between the tRNA genes of the two hagfish is thus as low as 0.13%. The 12S and 16S rRNAs sequences show a 0.2 and 0.3% divergence over lengths of 862 and 1541 nt, respectively. The protein-coding genes are found to be more divergent, since they show an overall 0.68% divergence at the nucleotide level and 0.44% divergence at the amino acid level, for total lengths of 11,372 nt (including stop codons) and 3778 amino acids, respectively. Nucleotide substitutions are mainly transition type (85%), either C–T or T–C (41.2%) and A–G or G–A (43.8%), and of these, 87% are synonymous. *atp8* and *nad4L* are identical at the nucleotide level and the nucleotide substitutions observed in *cox1, atp6,* and *cox3* are synonymous. *nad1, nad4,* and *nad6* show one different amino acid; *nad2* and *cob,* two different amino acids; and *nad5, cox2,* and *nad3,* three different amino acids. *nad3* and *cox2* appear to have the highest percentage of divergence. *atp8* was usually the fastest-evolving mitochondrial gene (Pesole et al. 1999) but actually showed no intraspecies polymorphism in *Myxine.* The nonsynonymous nucleotide substitution rate in *cox1* is the lowest among protein-coding genes, as already shown for mammals (Pesole et al. 1999): the 13 substitutions found in *cox1* are all silent. There is no way of relating the overall low polymorphism (about 0.6% of divergence at the nucleotide level) to any parameter linked to the genetic distance between the two specimens studied, especially since the evolutionary rate of mtDNA of *M. glutinosa* could not be determined. Only as a matter of comparison, the divergence between two anciently split species, *Branchiostoma lanceolatum* (Spruyt et al. 1998) and *Branchiostoma floridae* (Boore et al. 1999), was found to be 1.36% along the entire nucleotide sequence. Anyhow, the extent of polymorphism gives an estimate of the margin of error introduced by using a single hagfish specimen in phylogenetic analyses.

Structure of the Control Region

The control region of the mtDNA of *M. glutinosa* is between *trnP* and *trnF,* as in the "consensus" vertebrate gene arrangement (Boore 1999). Using two primers (PCR 10 in Table 1), one matching to the 3′ end of *cob* and the other to the 5′ end of *rrnS,* we failed to obtain a full-length PCR product of the control region. PCR products of different lengths—870, 650, and 530 bp—were repeatedly obtained and cloned for sequencing. The longest PCR product, about 2000 bp, was obtained only once. We cloned the latter PCR product and no 2000-bp clone was obtained. We obtained five smaller overlapping clones of different lengths (1450, 1260, 870, 650, and 530 bp), including *trnT* and *trnP* in the 5′ part and *trnF* in the 3′ part, all lacking the 3′ part of the control region. The sequences of the 870-, 650-, and 530-bp clones obtained from the 2000-bp PCR product were found to be identical to the clones obtained from the PCR products of the same sizes. Using the same primers, we obtained a unique 637-bp clones localized near *trnF* in the 3′ end of the control region. From the sequence of this clone we designed two new primers on both strands. Using these primers and six primers localized in the already determined sequence, we amplified and sequenced the second part of the control region. The 3′ part of the sequence of the control region was deduced from the sequences on both strands of five independent clones, resulting from PCRs carried out using different primers. Two clones including nt 1321 to 1700 (Fig. 2) overlap by 300 bp and two clones including nt 2050 to 3628 overlap by 62 bp. The fifth clone, localized in the middle of the sequence, overlaps the other clones by 282 bp on one side and 65 bp on the other side. Finally, we verified, by PCR using internal and external primers and also by cloning and sequencing, that the control region had entirely been sequenced.

The complete DNA sequence of the control region is depicted in Fig. 2. This 3628-bp-long noncoding sequence is much longer than the control region of all other vertebrates. Indeed, the length of the control region of mammals is usually about 1000 bp (reviewed by Sbisa et al. 1997). It is 1227 bp for the chicken (Desjardins and Morais 1990) and 928 and 1050 bp for fish (Chang et al. 1994; Delarbre et al. 1998). Among other vertebrates, the longest control region sequenced so far is that of *Xenopus* (2134 bp) (Wong et al. 1983) and the shortest is that of *Branchiostoma* (129 bp) (Spruyt et al. 1998; Boore et al. 1999). The control regions of the two lampreys *Petromyzon* and *Lampetra* (Lee and Kocher 1995; Delarbre et al. 2000) have an intermediate size, with a length of 690 and 642 bp, respectively.

The 5′ part of the control region, extending over the first 1666 nt, contains many repeated sequences. First, the core sequence of the control region contains two identical and adjacent, 298-bp-long, repeated sequences and a third one which differs from the two previous ones by only 7 bp. Second, large fragments of the aforementioned sequence are found associated with three novel sequences, each repeated twice, resulting in two additional, 450-bp-long, repeated sequences. Long repeated sequences are often found in the 5′ part of the control

Fig. 2. Nucleotide sequence of the control region of the mitochondrial DNA of *Myxine glutinosa*. The potential regulatory elements (TAS and CSBI) are *double-underlined* and are in *boldface*. The long repeated sequences are indicated as rep I, II, and III, and the short repeats as rep 1, 2, 3, 4, 5, and 6. The palindromic sequences and the nucleotides involved in the stems of the hairpins are *underlined* with *solid* or *dashed lines*. The two C-rich sequences are *underlined* with *dots*.

region (reviewed by Sbisa et al. 1997). Four identical termination-associated sequences (TAS) (Doda et al. 1981), TACATTTTAT, are located within these repeated sequences. These termination-associated sequences are identical to part of TAS of *Sorex araneus* (Fumagalli et al. 1996). TAS sequences involved in the termination of the D-loop (reviewed in Shadel and Clayton 1997) are also located within the 5′ end long repeats of the sturgeon (Brown et al. 1996) and shrews (Fumagalli et al. 1996), for example. Using the DNA Strider application, we found 28 possible hairpins in the 5′ part of the noncoding sequence of the hagfish, with a maximum of two mismatches in the hairpin stems. The hairpin stems are composed mainly of stretches of G and C and are thus able to form highly stable secondary structures, and nearly all of the G nucleotides in this part of the control region are involved in the stems of the hairpins. In four regions with potential stem–loop structures, the formation of alternative secondary structures involving sequences adjacent and complementary to the 5′ part of the putative hairpin structure are possible. Furthermore the putative hairpins could recombine with the complementary stems of identical putative hairpins, potentially generating an even more complex secondary structure. Hairpins had also been found at the beginning of the control region of the mtDNA of some animals, but in a lower number compared to *Myxine:* as examples, one hairpin only was evidenced in the 5′ part of the control region of the chicken (Desjardins and Morais 1990), two in *Scyliorhinus canicula* (Delarbre et al. 1998), and three in *Dinodon semicarinatus* (Kumazawa et al. 1998).

In the second part of the control region of the hagfish, six imperfect and short repeated sequences, 20 nt long (the sixth of them was only partial, being 14 bp long), are observed. The presence of short repeats in this part of the control region had already been reported in several mammals (reviewed by Sbisa et al. 1997). Compared with the first part of the control region of the hagfish, only two palindromes and nine possible hairpins are found. At nt 2303 of the control region, a conserved sequence block (CSBI), AGGACATA, a potentially regulatory sequence of the control region (reviewed by Shadel and Clayton 1997), can be identified. This sequence is identical to the CSBI of several mammals (reviewed by Sbisa et al. 1997). We could not identify the CSBII and CSBIII sequence blocks. As already discussed (Sbisa et al. 1997), CSBI seemed to be the only genuinely essential regulatory element, since it was detected in all mammals, whereas the CSBII and CSBIII sequences were not conserved in *Bos taurus* (Anderson et al. 1982) and *Glis glis* (Sbisa et al. 1997), as examples. Interestingly, a TATAA sequence flanked by eight C residues, located downstream of the hagfish CSBI sequence, has a location compatible with that of a conventional CSBII sequence and might play the same role as CSBII, although its sequence differed from the consensus sequence of CSBII. In favor of this hypothesis, Dairaghi and Clayton (1993) have shown that a short stretch of C could act as a potential regulatory element in bovine mtDNA, although its sequence differed from the consensus sequence of CSBII. A similar C-rich region, consisting of the central TATAA sequence flanked by 10 C residues, was found to be located in the middle of the hagfish control region. C-rich sequences have also been reported in the control region of *Pelomedusa subrufa* (Zardoya and Meyer 1998) and *Dinodon* (Kumazawa et al. 1998) and were supposed to facilitate the formation of the D-loop. The CSBI sequence of *Myxine* is followed by two 14-bp-long palindromes which can associate to create a hairpin. Such a secondary structure may potentially be associated with replication of mtDNA (Shadel and Clayton 1997). Three A/T-rich sequences that have the potential to form secondary hairpin structures are found to be located at the end of the control region, just upstream of *trnF*. These putative hairpins may be involved in the regulation of H-strand transcription starting at a position adjacent to *trnF* (reviewed by Taanman 1999). We could not identify other regulatory sequence elements in the D-loop of *Myxine*, like promotor elements of the major transcription sites, and we may deduce that these sequences are very divergent from the already known sequences of mammalians.

The sequence of the control region of *M. glutinosa* suggests structural features indicative of a potentially highly compact, heat-stable secondary structure. It may be said that such a structure of a control region is so far unique among craniates and the difficulties encountered in PCR amplification, cloning, and sequencing may be due to these stable secondary structures. The presence of such structures could potentially serve as signals for regulating replication and transcription of the mtDNA of the hagfish.

The uniqueness of the control region of *M. glutinosa* appears to strengthen the differences between hagfish and lampreys. However the character of the control region could be an autapomorphy and means nothing about the relationships of hagfish and lampreys. The determination of the mtDNA of a different hagfish is needed to determine if the aforementioned characters are unique to *Myxine* or are characteristic of hagfish. Then further phylogenetic analysis will be necessary to answer the question of the relationships among cyclostomes.

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