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The Vertebrate 7S K RNA Separates Hagfish (*Myxine glutinosa*) and Lamprey (*Lampetra fluviatilis*)

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Abstract. 7S RNA sequences from the hagfish (Myxiniformes) and lamprey (Petromyzontiformes) were cloned and analyzed. In both species, 7S L RNA (also designated SRP RNA, since it represents the RNA constituent of the signal recognition particle) was clearly detectable. The sequence similarity between the two species was 86%, compared with about 75% similarity between either of these species and mammals. 7S K RNA was also cloned from the lamprey. The similarity between the 7S K RNA of the lamprey and that of mammals was 68%. Interestingly, several interspersed elements were found with nearly 100% similarity compared with mammals. In contrast to the lamprey, no 7S K RNA-related sequences were detectable among hagfish RNA, neither in northern blots nor with the PCR assay. In view of the significant conservation between the 7S K RNA of lamprey and that of mammals (human), this unexpected result clearly separates lamprey and hagfish. In addition, the lack of detectable 7S K RNA sequences in an outgroup, such as amphioxus, indicates that these results do not reflect an autapomorphy of hagfish. Therefore, our data provide additional support to the notion of a sister group relationship between Petromyzontiformes and gnathostomous vertebrates to the exclusion of Myxiniformes.

Key words: 7S RNA — Small nuclear RNA — Vertebrate evolution — Cyclostomata — Gnathostomata

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Introduction

Vertebrate phylogeny has been widely studied using morphological, physiological, and molecular criteria. As a result, extant vertebrates have been distributed into three major groups: the Myxiniformes (hagfishes), the Petromyzontiformes (lampreys), and the Gnathostomata (jawed vertebrates), reviewed by Janvier (1996). Among those three taxa, the hagfish and lampreys were frequently grouped together within the monophyletic group of Cyclostomata (Duméril 1806; Yalden 1985). Furthermore, Bjerring (1984) placed these jawless vertebrates more closely to Cephalochordata (lancelets) than to Gnathostomata. On the other hand, many characters were found in common to lampreys and the gnathostomes that occurred neither in hagfish nor in cephalochordates. Based on these lamprey–gnathostome synapomorphies, Løvtrup (1977) proposed a sister-group relationship between Petromyzontiformes (lampreys) and Gnathostomata (both together representing the "true vertebrates"), with the Myxiniformes (hagfish) in turn forming a sister group of the Vertebrata (Janvier 1981, 1996; Gee 1996). This separation of hagfish and lampreys is also supported by a recent study on two fossil lower cambrium agnathans (Shu et al. 1999).

With the growing availability of nucleic acid sequence data, the relationship between hagfish and Vertebrata has been reassessed on the basis of molecular phylogenetics. The Cyclostomata hypothesis was supported by studies of globin (Goodman et al. 1987) and ribosomal RNA genes (Stock and Whitt 1992). In addition, the analysis of the methylation patterns of chordates also seemed to establish a close relationship between

hagfish and lamprey (Tweedy et al. 1997). A different tool for reconstructing the evolutionary history of taxa originates from the use of mitochondrial DNA. Such analyses, for example, confirmed the status of the prochordates as a sister group to the vertebrates (Spruyt et al. 1998 and references therein). Recently, sequences of protein-coding genes of mtDNA were used to reexamine agnathan (jawless vertebrates) and gnathostome relationships. In contrast to the Cyclostomata hypothesis mentioned above, these results placed the hagfish (Myxiniformes) as a sister taxon to the "true vertebrates", i.e., lampreys (Petromyzontiformes) and gnathostomes (Rasmussen et al. 1988).

In contrast to ribosomal RNA sequences (see above), few attempts have been made to use the repertoire of small cellular RNA molecules for taxonomic studies. This is particularly surprising because these stable RNAs often reveal significantly diverged primary sequences among different species, yet with strict conservation of their structural domains. Only the spliceosomal U6-snRNA has been found to be remarkably conserved from yeast to mammals [75% (Brow and Guthrie 1988)]. In contrast, the sequence of the other small nuclear RNAs (snRNAs) has diverged greatly during evolution (Ares 1986; Kretzner et al. 1987; Riedel et al. 1987; Siliciano et al. 1987). The most conspicuous example of this principle of conserved structure rather than sequence is observed with the small cytoplasmic 7S L (or SRP) RNA of the signal recognition particle (Larsen and Zwieb 1991). Here, for example, no conserved block (10 nucleotides or more) can be detected between fission yeast (*Schizosaccharomyces pombe*) and human, although the proposed secondary structures of these two 7S L RNAs are largely identical (Ribes et al. 1988).

Eukaryotic cells contain a large variety of small ribonucleoprotein (RNP) particles. These particles participate in a diverse array of cellular processes in both the nucleus and the cytoplasm. Most of these RNPs contribute to the maturation of other classes of RNA molecules (such as mRNA, rRNA, and tRNA), but some have been found to be required for genome replication or for targeting of secretory proteins to the endoplasmic reticulum (reviewed by Baserga and Steitz 1993). Among the most abundant small eukaryotic RNAs, however, one species (7S K RNA) remains of unknown function. 7S K RNA is 331 nucleotides (nt) in length and has been shown to be transcribed by RNA polymerase III (Zieve et al. 1977; Reichel and Benecke 1980). Genes encoding 7S K RNA have been isolated from human (Murphy et al. 1986; Krüger and Benecke 1987), rat (Reddy et al. 1984), and mouse (Moon and Krause 1991) cells and revealed a pronounced (98%) sequence conservation among mammals. Transcriptional regulation and promoter structure of these genes has been studied extensively in vitro and in vivo (Murphy et al. 1987, 1989; Kleinert and Benecke 1988; Kleinert et al. 1990; Boyd et al. 1995; Sandrock et al. 1999).

Up to now, no information was available on the occurrence and the primary structure of 7S K RNA species in lower vertebrates. In view of the high sequence conservation observed among mammals, we started to look for 7S K (and other small) RNA sequences within lower vertebrates, using human probes. Here we describe the sequence of the 7S K RNA of the lamprey (*Lampetra fluviatilis*). Compared to mammalian 7S K RNA, this sequence shows significant conservation and clearly detects the human homologue. However, all attempts failed to identify any 7S K RNA related sequence in the hagfish (*Myxine glutinosa*). This was particularly surprising, since 7S L RNA or the spliceosomal U6-snRNA revealed no unusual loss in sequence conservation between these two species. Therefore, it appears that the 7S K RNA sequence clearly differentiates hagfish from lamprey and higher vertebrates.

Materials and Methods

Cellular RNA was isolated from frozen tissue by the guanidinium thiocyanate method (Chomczynski and Sacchi 1987). Ten to twenty micrograms of purified RNA was used as the template for reverse transcription (10 U of AMV reverse transcriptase) in 50 m*M* Tris–HCl (pH 8.3), 50 m*M* NaCl, 8 m*M* $MgCl_2$, 5 m*M* DTT, 4 m*M* d(AGCT)TP, and 0.4 pmol of the oligonucleotide primers specified in the legends to the figures. Reactions were performed for 30 min at 46°C in a total volume of 20 μl. Template RNA was removed by alkali (0.15 *M* NaOH, 68°C, 30 min) treatment and the remaining first-strand cDNA used for PCR. Amplified blunt-end fragments were cloned into the bluescript KS^+ vector (Stratagene) and sequences determined by the dideoxy chain termination method (Sanger 1981) in an automatic ALF sequencer (Pharmacia). The 3′- and 5′-sequences were obtained by the rapid amplification of cDNA ends (RACE) method, described by Frohman et al. (1988). Briefly, for 3′-ends cellular RNA was first polyadenylated in vitro and reverse transcription started with oligo-d(T) carrying at its 5′-side an oligonucleotide sequence suitable for subsequent PCR. In these cases, two rounds of PCR were performed with "nested" gene-specific primers, deduced from the central sequence amplified before. The 5′-ends were obtained by oligonucleotide ligation [T4–RNA–ligase (Tessier et al. 1986)] to the 3′-ends of first-strand cDNAs (Schaefer 1995) and subsequent PCR amplification with "nested" primers as before. Northern blots (Thomas 1980) with immobilized RNA (Hybond-N; Amersham) were hybridized with labeled probes generated from cloned cDNA fragments (Feinberg and Vogelstein 1984) using a pool of random-priming hexanucleotides (Boehringer, Mannheim) and the Klenow enzyme.

Results

The 7S K RNA Sequences in Lamprey (Lampetra fluviatilis)

On our way to characterizing in detail the 7S RNA species in lower vertebrates, first, reverse transcriptase-

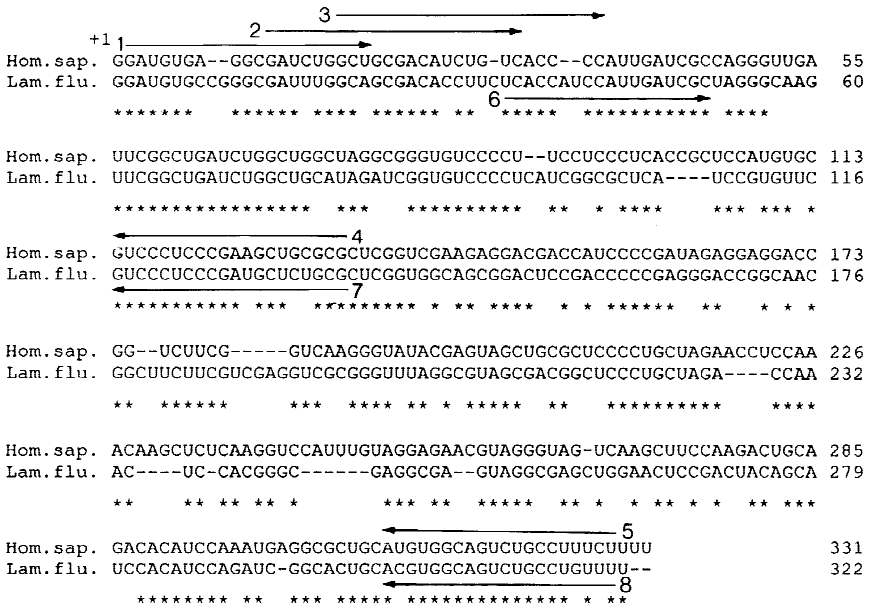

Fig. 1. Sequence comparison of the human (*Homo sapiens*) and lamprey (*Lampetra fluviatilis*) 7S K RNA sequences. Identical nucleotides are marked by *asterisks.* Position +1 identifies the initiator nt and four U residues at the 3′-end represent the authentic termination signal of RNA polymerase III transcription (White 1994). *Numbered arrows* above (human) and below (lamprey) the sequences indicate the length and position of primers used for the PCR analysis in Fig. 3.

coupled polymerase chain reactions (RT-PCR) were used to amplify 7S K-related sequences from total cellular RNA isolated from lamprey tissue. For this, oligonucleotide primers were generated which corresponded to sequences located near the 5′- and 3′-ends of human 7S K RNA, respectively. Earlier experiments in the laboratory (Kleikemper 1999) had revealed that these regions showed significant sequence conservation when comparing the 7S K RNA gene of *Xenopus laevis* with its human homologue. With these primers, internal cDNA fragments as obtained by PCR were cloned and sequenced. That analysis gave rise to the central section (about 290 bp) of the lamprey 7S K RNA gene. Subsequently, this sequence was completed with respect to its very 5′- and 3′-ends by RACE (rapid amplification of cDNA ends) reactions, as outlined under Materials and Methods. Figure 1 shows the entire 7S K RNA sequence of lamprey (lower line) in comparison with the corresponding human sequence (upper line). This result indicates that the 7S K RNA sequence has undergone some changes during vertebrate evolution. In addition to a slight increase in length of the human 7S K RNA (331 nt, vs. 322 nt in lamprey), both sequences contain fairly short insertions/deletions which encompass 37 residues and further differ by a total of 66 nt exchanges. Furthermore, near the 5′- and 3′-ends several nt blocks are detectable that are highly conserved (near 100%) between both species, whereas significantly divergent sequences appear to be clustered within the central parts of the RNAs. Together, an overall homology of about 68% is observed between the two 7S K RNA sequences. In view of the large phylogenetic distance between lamprey and human, this finding is in good agreement with the strict sequence conservation observed among mammalian 7S K RNA genes.

No 7S K RNA-Related Sequence Is Detectable in Hagfish (Myxine glutinosa)

Randomly primed oligonucleotide probes representing almost the entire lamprey 7S K RNA sequence were used for northern blot hybridization with RNA from different organisms. As expected, the probes clearly were able to detect specifically the HeLa cell 7S K RNA (Fig. 2, lane 1) and their own RNA, as internal control (lane 3). Interestingly, however, these lamprey probes did not detect any 7S K RNA-related sequence among total cellular RNA from hagfish (Fig. 2, lane 4). In this case, only a weak hybridization signal was obtained with much larger RNA molecules, migrating in the range of ribosomal 18S RNA (see 18S rRNA). With lane 2 of Fig. 2, an RNA sample of a cartilaginous fish (the shark *Mustelus asferias*) was included in this northern blot analysis. As before, cross-hybridization to rRNA was again observed with the 7S K probes, but to a much stronger extent than observed with lamprey or hagfish. In addition, the RNA from shark also revealed a specific hybridization signal with an RNA clearly comigrating with the 7S K RNA molecules. Although its exact sequence remains to be determined, a partial 7S K cDNA was recently cloned from shark. An analysis of that fragment clearly identified a well-conserved (both to lamprey and to man, at

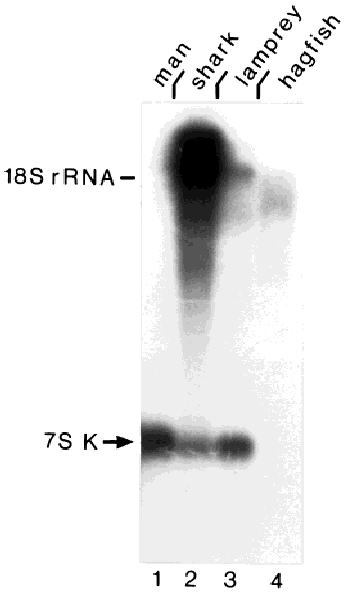

Fig. 2. Northern blot hybridization of 7S K RNA. Twenty micrograms of total cellular RNA isolated from HeLa cells (lane 1), shark (lane 2), lamprey (lane 3), or hagfish (lane 4) tissue was separated in 2% agarose gels containing 0.67 *M* formaldehyde and then transferred to nylon membranes. After hybridization with randomly primed DNA probes, the membrane was exposed to Fuji X-ray films for 16 h, using a Cronex intensifier screen. The position of large ribosomal RNA (18S rRNA) is indicated, as observed in the ethidium bromide-stained gel.

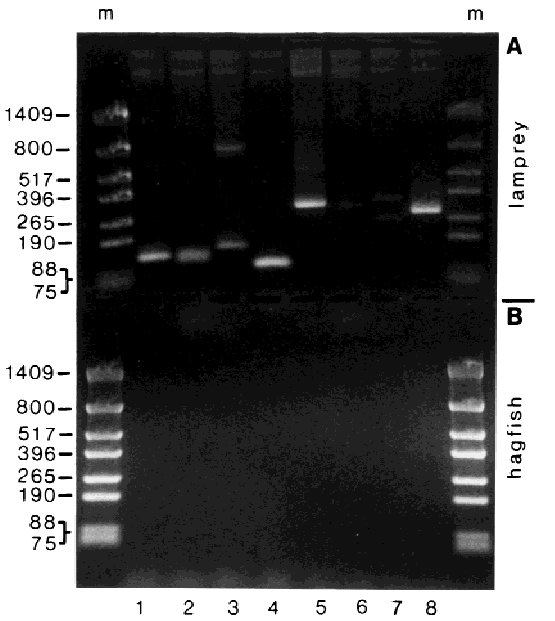

Fig. 3. RT-PCR analysis with different pairs of 7S K RNA-specific primers. **A** Ten micrograms of cellular RNA isolated from frozen lamprey tissue was used for reverse transcription and PCR amplification (40 cycles) with sets of primers deduced either from the human (lanes 1–3 and 5–7) or the lamprey (lanes 4 and 8) 7S K RNA sequence. Combinations of the primers indicated in Fig. 1 were 1 + 4 (lane 1), 2 + 4 (lane 2), 3 + 4 (lane 3), 6 + 7 (lane 4), 1 + 5 (lane 5), 2 + 5 (lane 6), 3 + 5 (lane 7), and 6 + 8 (lane 8), respectively. Electrophoresis of fragments was in 2% agarose gels, stained with ethidium bromide. **B** Same analysis as in A, but with 10 μg of hagfish RNA as template. m, marker DNA fragments.

about 85%) 7S K RNA sequence in that cartilaginous fish. In summary, the lamprey 7S K RNA sequence did not allow the detection of a 7S K RNA in hagfish.

Furthermore, RT-PCR analyses were performed, which should be much more sensitive than northern blots. For this, total cellular RNA from hagfish was used as the template for reverse transcription and cDNA amplification with different sets of 7S K-specific primers—deduced from either the human or the lamprey sequence, respectively, as indicated in Fig. 1. A lamprey RNA control was included in that experiment. The result of these PCRs is shown in Fig. 3. As expected, with lamprey RNA all 7S K primer combinations gave positive results, i.e., a 7S K cDNA band of the predicted length (Fig. 3A). No differences were observed if human (lanes 1 and 5) or lamprey (lanes 4 and 8) primers were applied. In contrast, with hagfish RNA neither of these primer combinations, irrespective of whether the oligonucelotides represented human or lamprey sequences, resulted in an amplified cDNA fragment (Fig. 3B).

To exclude the possibility that the negative results with hagfish RNA were due to extraction efficiencies, the RNA samples were first analyzed by hybridization with different probes. For this, northern blots were loaded with the same amount of total RNA of HeLa cells, lamprey, or hagfish and the blots probed simultaneously with human U6 RNA and 7S L RNA gene sequences. As is evident from Fig. 4A, with either of these probes the three RNA samples gave strong hybridization signals, corresponding to authentic 7S L (299-nt) and U6 (106-nt) RNA, respectively. However, human RNA (Fig. 4A, lane 1) showed slightly stronger signals compared to lamprey (lane 2) or hagfish (lane 3). Therefore, prior to the analysis presented in Fig. 4B, the concentration of all three RNA preparations was adjusted in accordance with the U6 hybridization signals of Fig. 4A. Now, these three RNA preparations were reexamined, but with lamprey 7S K probes (as in Fig. 2) in combination with human U6 RNA probes (as in Fig. 4A). This time, the U6 hybridization signals were almost-identical. Yet, again, the lamprey 7S K probe was not able to detect any corresponding hagfish sequence (Fig. 4B, lane 3), whereas specific hybridization was clearly obtained again with the human (lane 1) and the lamprey (lane 2) 7S K RNA. It should be noted that the absence of 7S K RNA in hagfish is in agreement with the failure to amplify genomic hagfish DNA fragments, as opposed to lamprey, with the different sets of 7S K primers (data not shown). Besides, in terms of the biological role of 7S K RNA, a nonexpressed gene would have made no difference.

By itself, the nonexistence of 7S K RNA might constitute an autapomorphy of hagfish, which does not allow us to draw any conclusions on the relationships with lamprey. Therefore, another group of relevance was

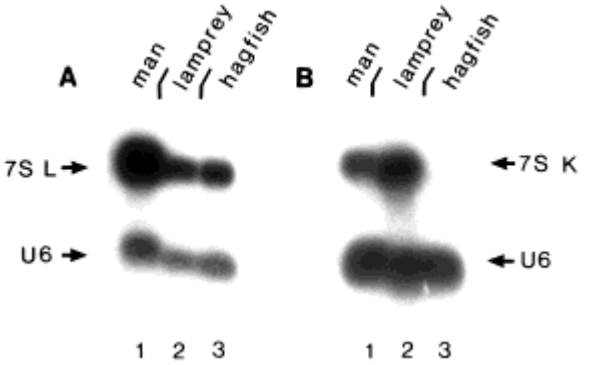

Fig. 4. Northern blot hybridization of low molecular weight RNA species. **A** Twenty micrograms of cellular RNA from HeLa cells (lane 1), lamprey (lane 2), or hagfish (lane 3) tissue was hybridized simultaneously with random probes obtained from cloned human 7S L or U6 RNA sequences. Details of northern blots, hybridization conditions, and film exposure were as in the legend to Fig. 2. **B** Same analysis as in A, however, in this case simultaneous hybridization was with labeled probes derived from the lamprey 7S K and the human U6 RNA sequences. Furthermore, prior to electrophoresis, the amount of RNA applied was normalized for the intensity of the U6 signals (determined by PhosphoImager quantification) obtained in A. It should be mentioned that even prolonged exposure (48 h) of the blot in part B did not reveal any 7S K RNA signal of hagfish.

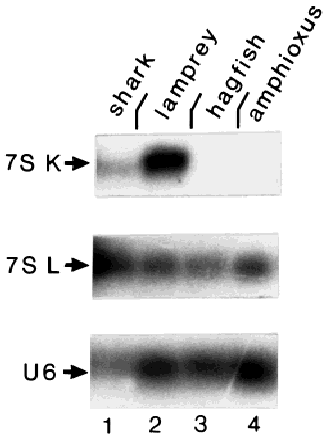

Fig. 5. Comparative northern blot analysis of U6 and 7S RNA sequences in prochordates (*B. lanceolatum*) and lower vertebrates. Twenty micrograms of RNA isolated from frozen tissue of shark (lane 1), lamprey (lane 2), hagfish (lane 3), and amphioxus (lane 4) was separated in agarose gels, blotted, and hybridized as before. Labeled randomly primed oligonucleotide probes were obtained from either lamprey (7S K) or human (7S L, U6) cDNA.

tested and we selected the lancelet amphioxus (*Branchiostoma lanceolatum*) as an outgroup. As shown in Fig. 5, as with hagfish (lane 3), a northern blot of total RNA from amphioxus did not give any signal with the highly specific probe of lamprey 7S K RNA (lane 4), whereas shark and lamprey RNAs (lanes 1 and 2) were found to be positive again. As before, all RNA samples analyzed allowed the immediate detection of 7S L and U6 RNA sequences, respectively. This result indicates that the absence in hagfish of 7S K RNA does not reflect an autapomorphic state of that taxon.

7S L and U6 RNA Sequences Are Closely Related Between Lamprey and Hagfish

For the interpretation of the unexpected result that the two jawless craniate taxa revealed such a dramatic difference with respect to the sequence of one small stable RNA, we examined to what extent comparable small RNA sequences in general have diverged between these two organisms. Although the hybridization results shown above for 7S L and U6 RNA seem to indicate significant homology between the respective small RNAs of hagfish and lamprey, it is evident that such analyses cannot provide conclusive results as to the degree of divergence that occurred during evolution. Therefore, cDNA sequences derived from reverse transcription and PCR amplification of both 7S L RNAs were generated with one set of primers corresponding to the 5′- and 3′-ends of human 7S L RNA. As shown in Fig. 6, both lamprey

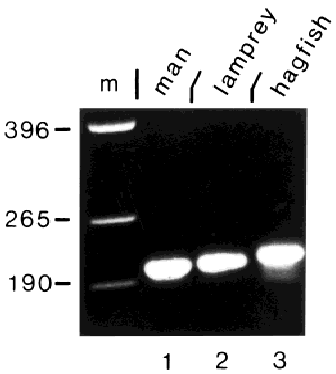

Fig. 6. Agarose gel electrophoresis of 7S L RNA-specific PCR fragments. Ten micrograms of cellular RNA was subjected to RT-PCR with two oligonucleotide primers deduced from the human 7S L RNA sequence. The 5′-primer corresponded to the sequence between +49 and +68 and the 3′-primer was complementary to the sequence between +230 and +249 (see Fig. 7A).

(lane 2) and hagfish (lane 3) RNA molecules gave rise to a prominent PCR fragment, corresponding in size exactly to that obtained with HeLa cell control RNA in lane 1. Furthermore, it appeared that the relative amount of cellular 7S L RNA was similar among the three species, although no rigorous control for quantitative PCR was included in this reaction. Additional RACE experiments allowed us to supplement the sequence of these internal fragments with the authentic 5′- and 3′-ends of both 7S L RNAs. The graphic presentation of the resulting full-length 7S L RNA sequences in Fig. 7A [shown in comparison to those of *Schizosaccharomyces pombe* (sp-7sl) and *Homo sapiens* (hs-7sl)] reveals a close relationship of this RNA species between lamprey (la-7sl) and hagfish (hag-7sl), with an apparent homology of about (83%). Again, long stretches of extremely well-

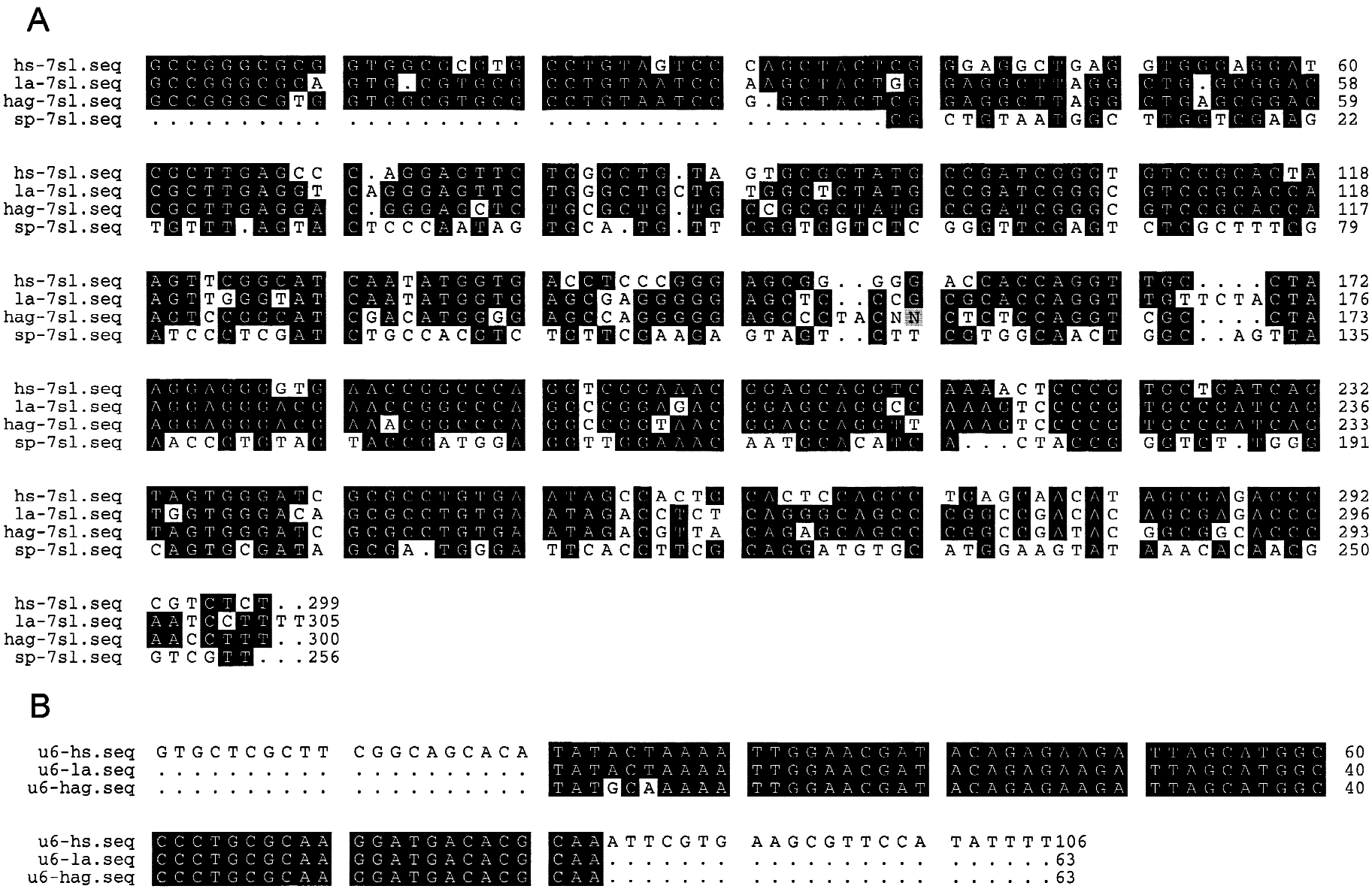

Fig. 7. Comparison between lamprey and hagfish cDNA sequences cloned from 7S L or U6 RNA, respectively. **A** PCR products as presented in Fig. 6 were cloned and sequenced. These central regions of the 7S L RNA genes were supplemented with the accompanying ends obtained by 5′- and 3′-RACE reactions, as outlined under Materials and Methods. To demonstrate the divergence of 7S L RNA between yeast and mammals, the *S. pombe* (*bottom*) and human (*top*) 7S L RNA sequences are included, as determined by Rödicker et al. (1999) and Kleinert et al. (1988), respectively. Abbreviations used to assign 7S L RNA sequences: hs, *Homo sapiens;* la, lamprey; hag, hagfish; sp, *Schizosaccharomyces pombe.* **B** The central fragments of lamprey (U6-la) and hagfish (U6-hag) U6 RNA are compared, as obtained by RT-PCR with end-standing human primers (corresponding to the 5′- and 3′-gaps). Due to the far-reaching identity with the human U6 sequence at the top (U6-hs), the 3′- and 5′-ends were not determined further.

conserved regions are detectable among these two RNAs, as evident, for example, for the sequences between positions +1/+69, +93/+123, or +178/+266, respectively. Furthermore, strong conservation of both sequences with human 7S L RNA (top) is observed, with homologies of 75.6% (lamprey) and 75.5% (hagfish), respectively. In contrast, the *S. pombe* 7S L RNA (bottom) does not show any single consecutive block of 10 nt (or more) being identical to any of the three metazoan 7S L RNAs.

The observed high degree of conservation of 7S L RNA sequences contradicts an assumption of a sudden discontinuity in RNA primary structure in general, separating lamprey and hagfish. This conclusion was substantiated by a nucleotide sequence comparison of the respective U6 RNA genes. That RNA has been found to be somehow exceptional in that it shows an outstanding degree of sequence conservation among eukaryotes (Brow and Guthrie 1988). That is why we limited this comparison with the human sequence to the central 63 (of 106) residues of U6 RNA from lamprey and hagfish. The result is shown in Fig. 7B and confirms the extraordinary position of U6 RNA. A difference of only two nt is observed between the U6 RNA sequence of lamprey and that of hagfish. Furthermore, the lamprey sequence represents an exact match to its human homologue (100%). Therefore, aside from the results obtained for 7S K RNA before, small RNAs do not establish a basic difference between hagfish and lampreys, in general.

Discussion

Among the most abundant small RNA species of eukaryotic cells, the 7S K RNA holds a unique position. Up to now, no definite physiological role has been assigned to that particular RNA species. Some authors have suggested an involvement of 7S K RNA in the transcriptional control of oncogenes (Luo et al. 1997 and references therein), while others have proposed a role in splicing (Wassarman and Steitz 1991) or translation (Gunning et al. 1981) of mammalian mRNA. Together with the high copy number of these RNA molecules detected in mammalian cells, the extreme conservation in primary structure among rat, mouse, and human certainly points to a central function of 7S K RNA. In view of this assumption, we wanted to analyze 7S K (and other) RNA sequences in organisms that are separated from mammals by a larger phylogenetic distance, i.e., in lower vertebrates.

Up to now, the sequences of small eukaryotic RNA species have not been extensively used for phylogenetic purposes. This is surprising since, in particular, the RNA polymerase III transcription system, which is responsible for the synthesis of quite a number of these low molecular weight RNA species (reviewed by White 1994), in a number of aspects revealed considerable variation during evolution. Some of those features are directly related to the RNA polymerase III enzyme itself (White 1994). Furthermore, the promoter structure of pol III genes (genes transcribed by RNA polymerase III) has undergone considerable modifications, with four distinct classes of pol III promoters found in eukaryotic cells (Willis 1993). Finally, one pol III gene, coding for 7S L RNA, revealed several switches in promoter class among yeast [*S. pombe* (Rödicker et al. 1999)], trypanosomes (Nakaar et al. 1994), plants [*Arabidopsis thaliana* (Heard et al. 1995)], and mammals (Ullu and Weiner 1985; Bredow et al. 1990), with the *S. pombe* 7S L RNA gene promoter very likely representing the minimal promoter of a eukaryotic pol III gene, in general. It appears that the RNA polymerase III transcription system as a whole is a very ancient one and might be highly suitable for studying phylogenetic relationships.

It is not the aim of our study to present here some kind of a systematic quantification of phylogenetic distances between the hagfish and lampreys. Rather, the characteristics of small RNA species were taken to manifest a qualitative difference in RNA composition between these two extant agnathans. However, the described difference in 7S K RNA between hagfish and lamprey might be regarded as an additional argument against the Cyclostomata hypothesis. In terms of molecular evolution, the most intriguing question arising from our data on 7S K RNA is what new development in the area of the invertebrate/vertebrate boundary required the thorough redesigning of a preexisting (7S K) RNA into an essentially new nucleotide sequence. Alternatively, one might be tempted to speculate whether or not a small RNA, representing a 7S K analogue, exists in hagfishes. A systematic search for 7S K RNA-related sequences in vertebrate and invertebrate taxa is in progress and might help to approach the fundamental questions, Why 7S K RNA?

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