

RNA Trans-splicing in *Fasciola hepatica*

IDENTIFICATION OF A SPLICED LEADER (SL) RNA AND SL SEQUENCES ON mRNAS*

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We have identified a new spliced leader (SL) in *Fasciola hepatica* by characterizing the 5'-terminal sequences of its enolase mRNA, an mRNA also trans-spliced in the flatworm *Schistosoma mansoni* (Rajkovic, A., Davis, R. E., Simonsen, J. N., and Rottman, F. M. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 8879–8883). This 37-nucleotide spliced leader is at the 5' ends of multiple *Fasciola* mRNAs and is likely to be derived from the 5' terminus of a nonpolyadenylated, 108-nucleotide RNA with a trimethylguanosine cap. The SL RNA gene is present in ~100 copies within a 1.1-kilobase genomic tandem repeat. Secondary structure predictions indicate that the *Fasciola* SL RNA contains three stem loops in contrast to two previously observed in *S. mansoni*. *Fasciola* and *S. mansoni* SLs are likely to be evolutionarily related although their sequence identity is only 65%. In contrast with nematodes, absolute conservation of SL sequences and secondary structure does not occur in trematodes. A spliced leader in *Fasciola* indicates that trans-splicing is likely to be a common feature in other trematodes and perhaps other flatworms.

Trans-splicing is an RNA processing event that accurately joins sequences derived from distinctly transcribed RNAs. In one form of trans-splicing, a leader sequence (the spliced leader, SL)¹ is donated from the 5' end of a small, non-polyadenylated RNA (the spliced leader RNA, SL RNA) to pre-mRNAs to form the 5'-terminal exon of mature mRNAs. This form of RNA maturation was first described in trypanosomes (1, 2) and subsequently in other kinetoplastida, the flagellated protozoan *Euglena* (3), and nematodes (4) (for reviews, see Refs. 5–7). Although once considered an anomaly of the kinetoplastida and then nematodes, our identification of trans-splicing in schistosomes, members of another metazoan phylum (Class Trematoda, Phylum Platyhelminthes), suggested that this particular form of RNA processing might be an evolutionarily important

and common form of gene expression in early metazoa (8). Knowledge of the distribution of trans-splicing and a comparison of spliced leader RNAs in flatworms and other early metazoa may help address the origin(s) and importance of trans-splicing in early metazoa, as well as our understanding of the significance and conservation of secondary structure, SL sequences, and other elements in metazoan trans-splicing.

The sequence of the spliced leader in multicellular nematodes is absolutely conserved in all members of the phylum examined. We previously observed from Northern blot hybridizations that the spliced leader from the flatworm *Schistosoma mansoni* was either absent or apparently not highly conserved in other trematodes or members of the flatworm phylum (8). Here we demonstrate that another trematode, *Fasciola hepatica*, has a spliced leader and uses trans-splicing as a mechanism of gene expression. We hypothesized a possible evolutionary conservation of enolase trans-splicing to search for and identify trans-splicing in *Fasciola*. By cloning and characterizing the 5'-terminal sequences of the *Fasciola* enolase mRNA, we have identified a new spliced leader. We describe and provide evidence for a *Fasciola* SL RNA and its secondary structure, the presence of the spliced leader on multiple mRNAs, and the SL RNA gene contained within its genomic repeat.

MATERIALS AND METHODS

Organisms—Frozen adult *F. hepatica* were kindly provided by Allison Rice-Ficht (Texas A&M University) and Tag Mansour (Stanford University).

Nucleic Acid Isolation and Blot Analyses—Genomic DNA was isolated from frozen worms powdered on dry ice as described (10). Total RNA was purified either by guanidinium-hot phenol (10) or acid-phenol extraction (11). Poly(A)⁺ mRNAs were selected either by oligo(dT)-cellulose (10) or biotinylated oligo(dT) and streptavidin-coated paramagnetic particles (PolyATract mRNA Isolation, Promega, Madison, WI). Agarose and PAGE Northern blots, genomic Southern blots, and hybridization and washing conditions were as described previously (8, 10). Immunoprecipitation of RNA with trimethylguanosine antibodies (OncoGene Sciences, Uniondale, NY) was as described previously (8).

Primer Extension Analysis and RNase Mapping—Primer extension and sequencing experiments were performed as described (10). RNase mapping was carried out using 5–10 µg of total or poly(A)⁺ RNA essentially as described (12) or using a ribonuclease protection assay kit (RPA II, Ambion, Austin, TX) with labeled RNA transcripts derived from subclones of the SL RNA genomic repeat.

Rapid Amplification of cDNA Ends (RACE)—5' RACE was performed by two methods: the 5' RACE System from Life Technologies, Inc. and a method we described previously (13). Both methods produced similar results. PCR was performed using the anchor primer provided with the 5' RACE kit (with C-tailed cDNA) or 5'-TCTAGAACTAGTG-GATCCCCCCCCCCC-3' (with G-tailed cDNA) and the *Fasciola* enolase-specific oligonucleotide, 5'-CAUCAUCAUATGCCTTTTGGT-CGATAGCTTT-3' (U = deoxyuridine) (Fas.Eno.1; see Fig. 1) as follows: 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2.5 min at 72 °C. The PCR product was agarose gel-purified (Magic PCR Prep, Promega, Madison, WI) and cloned into pT7Blue T-vector (14) (Novagen, Madison, WI) or pAMP vector using ligation-independent directional cloning with uracil glycosylase (Life Technologies, Inc.) (15).

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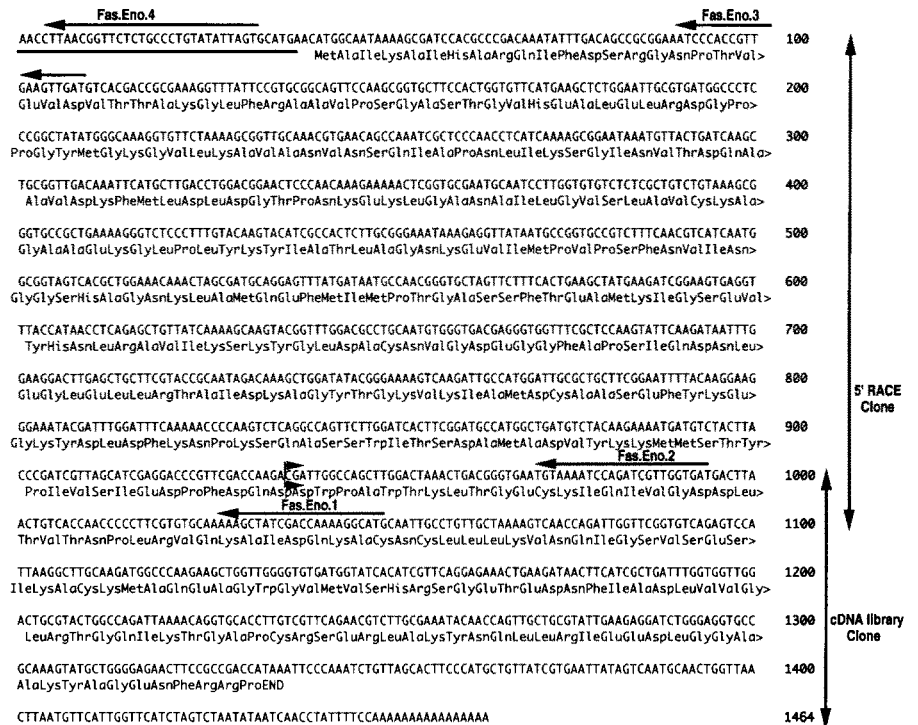
The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U10309–U10316, U10276, and U10286–U10297.

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¹ The abbreviations used are: SL, spliced leader; SL RNA, spliced leader RNA; TMG, trimethylguanosine; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s).

FIG. 1. Nucleotide and deduced amino acid sequence of *Fasciola* enolase mRNA. Overlined sequences represent antisense oligonucleotides used in this study, and the underlined sequence represents the 5'-terminal spliced leader. Vertical lines with two arrows indicate cDNA clones analyzed to generate the composite shown. The cDNA library clone (nucleotides 937–1464) was isolated by screening a *Fasciola* cDNA library with a schistosome enolase cDNA probe. The 5' RACE clone (nucleotides 1–1051) was obtained by carrying out 5' RACE PCR using the Fas.Eno.1 oligonucleotide primer.



5' RACE cDNA Library Construction and cDNA Library Screening—First strand cDNA was synthesized from 0.5–1.0 µg of poly(A)⁺ RNA using CAUCAUCAU-oligo(dT)₁₃ as primer and Superscript reverse transcriptase (Life Technologies, Inc.) using the conditions recommended. RNA and oligo(dT) were removed by RNase H treatment and GlassMax purification, and the cDNAs C-tailed as described in the Life Technologies 5' RACE kit. The cDNAs were amplified using the Life Technologies anchor primer and CAUCAUCAU-oligo(dT)₁₃ using the conditions described above for 5' RACE. Amplified cDNAs greater than 300 base pairs in size were cloned into pAMP vector (15). cDNAs with the *Fasciola* spliced leader sequence were identified by colony hybridization with an end-labeled oligonucleotide corresponding to the complement of nucleotides 4–27 of the spliced leader (Fas.Eno.4; Fig. 1). An adult *Fasciola* λ ZAP cDNA library (kindly provided by J. Ding and T. Mansour, Stanford University) was screened with a random primer-labeled schistosome enolase cDNA² as described previously (10). An adult λgt11 cDNA library (generously provided by Allison Rice-Ficht, Texas A&M University) was screened with an oligonucleotide corresponding to the spliced leader (Fas.Eno.4) (10). cDNA inserts were amplified from plaques corresponding to secondary screens as originally described (16) and cloned into either Bluescribe or Bluescript plasmid vectors (Stratagene Cloning Systems, La Jolla, CA).

SL RNA Gene Genomic Repeat PCR amplification—SL sense (nucleotides 4–22 of the SL: CGGGATCCGCTTAACGGTTCTCTGCCCT) and antisense (nucleotides 17–37 of the SL: GCTCTAGAGCCATGCACTAATATACAGGCA) primers (0.5 µM) with *Bam*HI or *Xba*I cloning sites, respectively, were used in a PCR reaction with 200 ng of *Fasciola* genomic DNA using the same amplification conditions as above. The PCR generated a 1.1-kb product that was cloned into Bluescript plasmid.

DNA Sequencing and RNA Secondary Structure Analysis—Plasmid DNA was prepared as described previously (8, 10) or by Magic Plasmid Prep (Promega). Clones were sequenced by the dideoxynucleotide method on alkali-denatured plasmids using the Sequenase Kit (U. S. Biochemical Corp.) as described (8, 10).

RESULTS

We previously observed that only ~10–20% of all schistosome mRNAs are trans-spliced. One of the mRNAs acquiring the spliced leader was strongly predicted to encode the glycolytic enzyme enolase.² Analysis of four additional glycolytic

enzyme mRNAs in *S. mansoni* indicated that only the enolase mRNA is trans-spliced (13).² To search for a spliced leader in another flatworm such as *Fasciola*, we hypothesized that mechanisms of gene expression including trans-splicing might remain conserved within homologous genes of evolutionarily related organisms. If true, characterization of the 5'-terminal nucleotides of the enolase mRNA might then lead to the identification and characterization of a SL in this genus.

To initiate this strategy, we screened a *Fasciola* cDNA library using the schistosome enolase cDNA² as a probe and isolated a cDNA corresponding to the 3'-terminal 500 bases of a putative *Fasciola* enolase mRNA (see Fig. 1). This *Fasciola* cDNA was determined to encode enolase based on alignment of the predicted amino acid sequences with other enolases (>70% amino acid identity over 132 residues with other eukaryotic enolases) (data not shown). Using sequences derived from the *Fasciola* enolase cDNA, we designed a *Fasciola* enolase-specific oligonucleotide primer (Fas.Eno.1, see Fig. 1) and performed 5' RACE on tailed first-strand cDNA synthesized from *Fasciola* poly(A)⁺ mRNA. A 5' RACE product of the expected size for an enolase mRNA (~1000 base pairs) was obtained, cloned, and sequenced. Analysis of the open reading frame at the 5' and 3' ends of the cloned RACE product (Fig. 1) indicated that the cDNA sequence encoded enolase (data not shown). Primer extension analysis on the *Fasciola* enolase mRNA confirmed that the terminal sequences obtained in the 5' RACE cloning corresponded to the 5' terminus of the mRNA demonstrating that the 5' RACE product was full-length (Fig. 2A, lane 2).

Based on the known examples of spliced leader trans-splicing, if the terminal sequence of the *Fasciola* enolase mRNA is a spliced leader sequence, one would predict that the sequence would be present at the 5' terminus of other *Fasciola* mRNAs and a small, nonpolyadenylated RNA, an SL RNA. Northern blots of *Fasciola* poly(A)⁺ RNA hybridized with an oligonucleotide probe corresponding to the complement of the 5'-terminal sequences of the *Fasciola* enolase mRNA (Fas.Eno.4, see Fig. 1) produced a smear of hybridization ranging from 300 bases to several kilobases, suggesting that multiple mRNAs carried se-

² R. E. Davis, C. Hardwick, P. Tavernier, J. Villanueva, S. Hodgson, and H. Singh, manuscript in preparation.

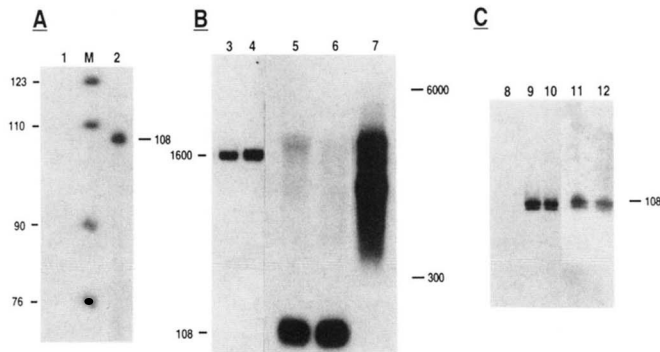


FIG. 2. Primer extension analysis of enolase and Northern blot analyses. A, primer extension of enolase mRNA. tRNA (lane 1, 15 μ g) and *Fasciola* total RNA (lane 2, 15 μ g) were annealed with end-labeled Fas.Eno.3 oligonucleotide and extended using reverse transcriptase in the presence of deoxynucleotides. The extension products were resolved by denaturing PAGE and the size of the extension products in bases determined by comparison with *Msp*I-labeled pBR322 (lane M) and DNA sequencing ladders (not shown). B, the 5'-terminal sequence of the *Fasciola* enolase mRNA hybridizes to a small poly(A)⁺ RNA and a smear of poly(A)⁺ RNAs ranging from 300 to 6000 bases. Total RNA (lane 5, 10 μ g), poly(A)⁺ RNA (lane 6, 10 μ g), and poly(A)⁺ RNA (lane 7, 2 μ g), were Northern blotted from 1.5% formaldehyde-agarose gels and hybridized with an end-labeled oligonucleotide complementary to the 5'-terminal sequence of *Fasciola* enolase (Fas.Eno.4). As a control, total (lane 3, 10 μ g) and poly(A)⁺ (lane 4, 0.5 μ g) RNA were hybridized with an oligonucleotide (Fas.Eno.2) corresponding to the coding region of the enolase mRNA. RNA size in bases was calculated using Life Technologies, Inc. RNA ladders. C, 5'-terminal sequence of the *Fasciola* enolase mRNA hybridizes to a small 108-nucleotide poly(A)⁺ RNA that is TMG-precipitable. *Fasciola* poly(A)⁺ (lane 8, 1 μ g), poly(A)⁺ RNA (lanes 9 and 12, 10 μ g), total RNA (lane 10, 10 μ g), and TMG-precipitated RNA (lane 11, corresponding to 15 μ g of poly(A)⁺ RNA) were Northern blotted from 8% denaturing PAGE gels and hybridized with an oligonucleotide complementary to the 5'-terminal sequence of *Fasciola* enolase (Fas.Eno.4). RNA size in bases was determined by comparison with DNA sequencing ladders.

quences homologous to the 5' terminus of the enolase mRNA (Fig. 2B, lane 7). Agarose and PAGE Northern blots of *Fasciola* RNA probed with the antisense oligonucleotide Fas.Eno.4 indicated that these sequences were also present in a nonpolyadenylated, trimethylguanosine (TMG)-precipitable RNA of approximately 108 nucleotides (Fig. 2, B and C). In contrast, *Fasciola* total and poly(A)⁺ RNA hybridized with an oligonucleotide probe corresponding to the coding region of enolase (Fas.Eno.2, Fig. 1) produced discrete hybridization to only an \approx 1.6-kb enolase mRNA (Fig. 2B, lanes 3 and 4).

To determine the nature and location of the homologous sequences in the 108-nucleotide RNA, we first cloned the gene encoding the small RNA. Initial Southern blot analysis of genomic DNA hybridized with the putative SL oligonucleotide indicated that sequences homologous to the putative SL were present in multiple copies in the *Fasciola* genome organized in direct tandem arrays of \approx 1.1 kb. Using the polymerase chain reaction with opposing oligonucleotide primers derived from the putative SL sequence (see "Materials and Methods"), we amplified the 1.1-kb genomic repeat containing the putative SL RNA gene from genomic DNA. Cloning and sequence analysis of the amplified genomic repeat indicated that the terminal 37 nucleotides of the enolase mRNA were present within the genomic repeat (Fig. 3A). Predicting that an RNA of 108 nucleotides was derived from this locus from the PAGE Northern blot hybridization data, we designed an antisense oligonucleotide (FasSLXT) corresponding to near the predicted 3' terminus of the RNA and performed primer extension sequencing of the small RNA in total and poly(A)⁺ RNA (Fig. 3B). The sequence of the small RNA derived from this analysis was identical to that of the cloned SL RNA gene. These data suggest that the sequence identified at the terminus of

the enolase mRNA is likely to be derived from the 5' terminus of the small TMG-precipitable RNA. This sequence was immediately followed by a consensus splice donor site in the RNA (Fig. 3). RNase mapping of the SL RNA confirmed the 5' and 3' terminus of the SL RNA (data not shown) as shown in Fig. 3 (A and C).

Northern blot hybridization of poly(A)⁺ mRNA suggested that multiple mRNAs in *Fasciola* contained the SL sequences. To confirm that this hybridization analysis indicated that the SL sequence was present at the termini of *Fasciola* mRNAs, we constructed a 5' RACE cDNA library in a plasmid vector and screened for cDNAs corresponding to mRNAs containing the SL sequence. Twelve clones hybridizing to the *Fasciola* spliced leader, each apparently representing a different gene, were characterized from the 5' RACE library and found to contain the SL sequence at their 5' termini (all 5'-terminal SL sequences were 35–36 nucleotides in length, i.e. within 1 or 2 nucleotides of the apparent full-length SL)(data not shown). These clones are likely to represent the 5' termini of their respective mRNAs based on our experience with 5' RACE products² (13). An additional 10 cDNAs with the SL sequence at their 5' termini were also isolated and examined from a λ gt11 cDNA library constructed by self-priming of second strand cDNA synthesis. These clones contained 5'-terminal SLs that were truncated averaging 25 out of 37 nucleotides of the full-length SL (data not shown). The truncation of the cDNA at the 5' end is not unexpected, as the use of S1 nuclease in the preparation of the cDNA for cloning often results in loss of 5'-terminal sequences. Not all *Fasciola* mRNAs acquire the spliced leader described here, since several apparently full-length mRNAs lacking the spliced leader have been characterized.^{3,4}

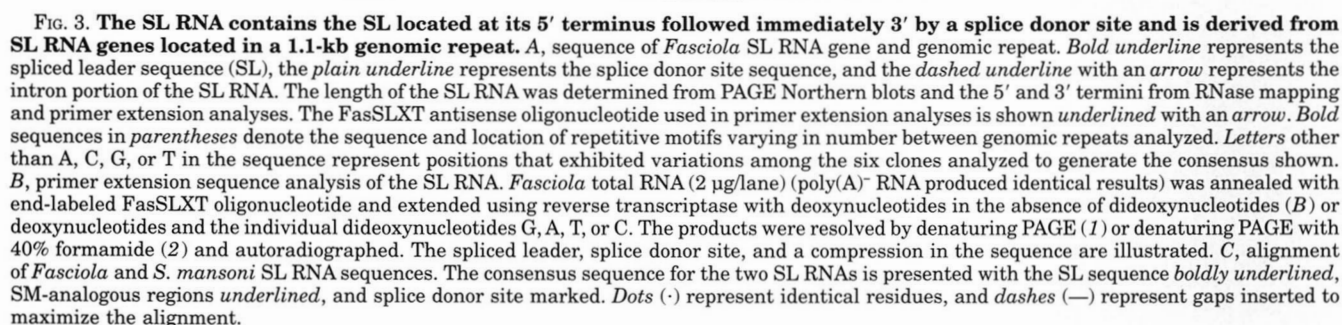
A comparison of the sequences of the *S. mansoni* and *Fasciola* spliced leader RNAs is shown in Fig. 3C, and the observed similarities suggest that the two trematode SL RNAs are likely to be evolutionarily related. Alignment of the SL RNAs showed that the sequence identity between the *S. mansoni* and *Fasciola* spliced leaders is approximately 65% and that both SLs were of similar length (36 and 37 nucleotides, respectively). The *Fasciola* SL RNA is 18 nucleotides longer than the *S. mansoni* SL RNA, and sequence identity 3' of the SL between the two SL RNAs is approximately 60% (including gaps to optimize the alignment).

A computer-generated energy minimization-derived secondary structure for the *Fasciola* SL RNA is shown in Fig. 4. The secondary structure shows the conserved features of other SL RNAs (3, 5, 7, 17) with the SL located in the first stem loop, base pairing across the splice donor site, and a sequence motif analogous to the SM-binding region of snRNAs present between two 3' stem loops. By contrast, although the secondary structure predicted for the *S. mansoni* SL RNA exhibits similar features, only two stem loops are present in schistosomes (8).

Southern blot analysis using partial genomic digests and genomic titration of the SL RNA gene confirmed that the SL RNA gene was organized as approximately 100 copies of a 1.1-kb direct tandem repeat (data not shown). The sequence for the SL RNA genomic repeat was determined (Fig. 3A), and although several regions 5' and 3' of the SL RNA genes are conserved between the *Fasciola* and *S. mansoni* SL RNA repeats (data not shown), phylogenetic analysis of potentially conserved elements upstream and downstream of the SL RNA will require characterization of additional flatworm SL RNA genomic repeats. In some of the kinetoplastida, *Euglena*, and nematoda, the genomic SL RNA repeat also contains the 5 S genes (5) (see Ref. 18, and references therein). No 5 S or other

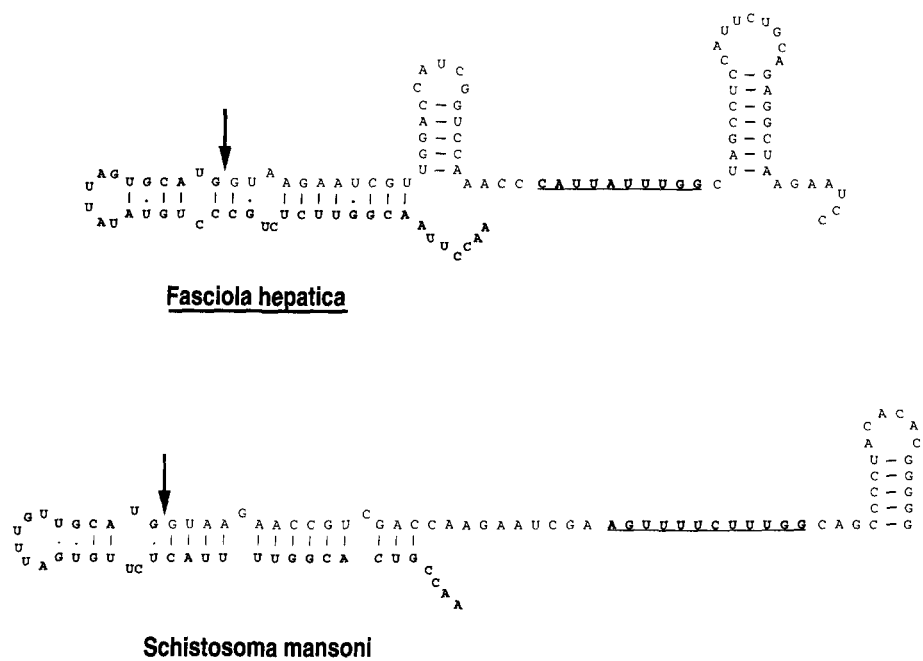
³ A. Rice-Ficht, personal communication.

⁴ R. E. Davis and H. Singh, unpublished observations.



In contrast with nematodes (5-7), the SL sequence is not absolutely conserved between the two trematodes *S. mansoni* and *Fasciola*. *In vitro* experiments, however, indicate that neither the sequence nor length of the SL is essential for nematode trans-splicing. Furthermore, *in vitro* transcription analysis suggests that at least one clear explanation for the conservation of the nematode SL sequence is associated with its demonstrated role as a promoter element in SL RNA transcription (20). The sequence identity between the two trematode SLs is only ~65%, significantly less than that observed even in divergent kinetoplastid flagellates. The presence of such divergent spliced leaders in these two trematodes raises the question of whether spliced leaders in trematodes, and perhaps in all flatworms, have quite variable sequences and what role, if any, the limited sequence conservation plays in trematode SLs, SL RNAs, and the trans-splicing reaction.

FIG. 4. Comparison of possible secondary structures of *Fasciola* and *S. mansoni* SL RNAs. Arrows indicate the 5' splice sites, the SL sequences are in bold letters, and the SM-analogous regions of the SL RNAs are in bold, underlined letters. The *S. mansoni* SL RNA sequence is redrawn from Ref. 8. The *Fasciola* SL RNA was folded using the algorithm of Zuker (24) with the constraint that SM-analogous sequence remain single-stranded. Structures represent the lowest energy structures calculated.



The identification of a spliced leader in *Fasciola* suggests that spliced leader trans-splicing is not restricted to schistosomes and is likely to be a common feature in other trematode flatworms. As flatworms (Phylum Platyhelminthes) are considered by some to be the first bilateral animals (9), their ancestral groups and trans-splicing may have been important in the evolution of early metazoa. As a whole, flatworms represent several divergent groups of organisms exhibiting both free-living and symbiotic forms. The various groups of parasitic flatworms are currently considered to be monophyletic (21–23). However, the phylogenetic relationships among free-living groups and the origin(s) and evolution of parasitism in flatworms is currently an area of significant interest that remains unresolved and controversial (22, 23). It remains to be determined if trans-splicing is present in other flatworm groups in general (planaria, tapeworms, and monogenetic trematodes) and particularly in flatworms considered to have been the likely ancestors of parasitic flatworms (22, 23). A phylogenetic comparison and distribution of trans-splicing in flatworms may prove useful in delineating the ancestor(s) and evolution of parasitic groups within the phylum. In addition, comparison of SL RNA sequences and secondary structures in different flatworm and metazoan groups might provide additional insight into essential elements and structural features of SL RNAs, the origin and distribution of spliced leader addition, and role of trans-splicing in the evolution of early metazoa.

The apparent lack of SL conservation in flatworms precludes hybridization and PCR analyses to identify the presence of SL RNAs and trans-splicing in other trematodes and flatworms. However, the described conservation of enolase trans-splicing between two distantly related trematodes suggests that characterization of the 5' ends of enolase mRNAs is a potential assay for trans-splicing at least in other trematodes and perhaps other flatworm groups. It will be of interest to see if this approach identifies spliced leaders in other trematodes and flatworms.

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