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A spliced leader is present on a subset of mRNAs from the human parasite *Schistosoma mansoni*

(trans-splicing/cis-splicing/spliced leader RNA/3-hydroxy-3-methylglutaryl-CoA reductase)

ALEKSANDAR RAJKOVIC, RICHARD E. DAVIS*, J. NEIL SIMONSEN†, AND FRITZ M. ROTTMAN

Department of Molecular Biology and Microbiology, Case Western Reserve University School of Medicine, Cleveland, OH 44106

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ABSTRACT **We present evidence that a subset of mRNAs in the human parasitic trematode *Schistosoma mansoni* contain an identical 36-nucleotide spliced leader (SL) sequence at their 5′ termini. The SL is derived from a 90-nucleotide nonpolyadenylylated RNA (SL RNA), presumably by trans-splicing. Neither the SL nor the SL RNA share significant sequence identity with previously described trans-spliced leaders and SL RNAs in trypanosomatid protozoans or nematodes. However, several features, such as predicted secondary structure, trimethylguanosine cap, and potential Sm binding site, suggest similarities among SL RNAs in widely divergent organisms. Our evidence also indicates that the exon 3 acceptor site of the 3-hydroxy-3-methylglutaryl-CoA reductase gene can be spliced either to the SL by trans-splicing or to an upstream exon, 2, by cis-splicing. The presence of a SL sequence in *S. mansoni*, a member of the phylum Platyhelminthes, suggests that trans-splicing may be a common feature of other lower invertebrates.**

Trans-splicing of pre-mRNA sequences was first described in trypanosomatid protozoans (1–3). In these organisms, small nonpolyadenylylated RNAs [spliced leader (SL) RNAs] of ≈100 nucleotides (nt) donate a 5′-terminal 39-nt SL sequence to all pre-mRNAs to form the 5′ termini of mature mRNAs. In metazoans, the description of trans-splicing has been confined to the phylum Nematoda. Trans-splicing in nematodes resembles the situation in trypanosomes as exemplified by the detection of Y-branched intermediates and the presence of consensus 5′ and 3′ splice sites flanking the SL and pre-mRNAs, respectively (4–6). There are, however, several notable differences. Unlike trypanosomatid protozoans, only a subset of nematode mRNAs (10–15%) mature via addition of a distinct 22-nt SL sequence (7). In addition, processing of nematode mRNA includes both cis- and trans-splicing (4). Analyses of these mRNAs and corresponding genomic clones have suggested that only the first exon serves as an acceptor for the 22-nt SL sequence (8).

The discovery of trans-splicing in nematodes raised the question of the prevalence of this reaction in other metazoans. Nucleotide sequence analysis of SL genes in different genera of nematodes has revealed perfectly conserved copies of the 22-nt SL sequence (7, 9, 10). The nematode SL, however, does not hybridize to RNA from other metazoans such as *Schistosoma mansoni, Dictyostelium, Drosophila, Xenopus,* and humans (7). If these organisms process their mRNAs by trans-splicing, it is likely to involve SL sequences that are not homologous to the nematode SL.

Here we present evidence that a subset of polyadenylylated transcripts in the human parasite *S. mansoni,* a trematode, are processed at their 5′ termini by addition of a distinct 36-nt SL sequence‡ that shares no sequence identity with the 22-nt SL in nematodes or the 39-nt SL in trypanosomatid protozoans. Trematodes, cestodes, turbellarians, and monogeneans are grouped together into the morphologically diverse phylum Platyhelminthes and are distinctly different from nematodes in both anatomy and physiology. The presence of a SL sequence in the phylum Platyhelminthes indicates that trans-splicing in metazoans is more widespread than heretofore suspected. We also present evidence that the 3′ splice site of an internal exon can be used in both cis- and trans-splicing.

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MATERIALS AND METHODS

Isolation of cDNA and Genomic Clones. HMG2 [nt 1–861 of the composite 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase cDNA sequence (11)] and HMG15 (nt 8–28 and 277–861 of the composite HMG-CoA reductase cDNA sequence (11)] cDNAs were isolated from an oligonucleotide-primed λgt10 cDNA library as described (11). An adult *S. mansoni* EMBL3 genomic library was screened with an HMG-CoA reductase cDNA fragment [nt 806–1167 of the composite cDNA (11)], radiolabeled by the random primer method (12), and with 5′ end-labeled oligonucleotide L (5′-ATGCAACAAATCACAAG-3′, complementary to nt 11–27 of HMG2 cDNA), as described (13) except that for oligonucleotide L hybridization and washing were at 42°C.

Genomic and cDNA fragments were subcloned into the pBS-m13(+) or pBluescript SK(+) vectors (Stratagene) and sequenced by the dideoxynucleotide method (14).

Blot Analyses and Primer Extension. RNA and genomic DNA were isolated from adult *S. mansoni* worms, and blot analyses were performed as described (13). Hybridization conditions for oligonucleotide L at 37°C included 1% bovine serum albumin (fraction V), 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, and 7% SDS. The blots were washed in 0.3 M NaCl/0.03 M sodium citrate, pH 7/0.1% SDS at 42°C.

For primer extension analyses, RNA was annealed to 10 ng of 5′ end-labeled synthetic oligonucleotides (as indicated in the figure legends) in 250 mM KCl/10 mM Tris·HCl, pH 8.0, at 50°C for 1 hr and the extension reactions were carried out as described (13). Products were resolved in 8% polyacrylamide/7 M urea gels and detected by autoradiography.

RNA Analyses by the Polymerase Chain Reaction (PCR). To detect and quantitate the two HMG-CoA reductase transcripts, an oligonucleotide (5′-ACTGCCTTTTATTCCTCAGC-3′) complementary to nt 657–676 of the composite HMG-CoA reductase cDNA sequence (11) was annealed to

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; PCR, polymerase chain reaction; SL, spliced leader; nt, nucleotide(s); snRNA, small nuclear RNA.

*Present address: Department of Biology, San Francisco State University, 1600 Holloway Avenue, San Francisco, CA 94132.
†Present address: Department of Medical Microbiology, University of Manitoba, 730 William Avenue, Winnipeg, R3E OW3, MB, Canada.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. M34074).

500 ng of polyadenylylated RNA isolated from adult *S. mansoni* worms and primer extension was performed as described (13). The first-strand cDNAs were then amplified in a PCR using 5′ and 3′ primers and a step cycle program (denaturation at 94°C for 20 sec, annealing at 55°C for 15 sec, and cDNA extension at 72°C for 1 min) for a total of 26 cycles as described (15). The 5′ primer corresponded to the first 24 nt of the *S. mansoni* SL sequence (5′-AACCGTCACGGTTTTACTCTTGTG-3′) and the 3′ primer (5′-GCCTACAGGTAAATTGTCAAGCAGC-3′) was complementary to nt 366–390 of the composite HMG-CoA reductase cDNA sequence (11).

RESULTS

Characterization of the 5′ Termini of HMG-CoA Reductase Transcripts. We previously isolated cDNAs encoding the schistosome HMG-CoA reductase (EC 1.1.1.34) (11). Characterization of cDNAs extending 5′ indicated that nt 8–28 in cDNA HMG2 and the first 21 nt in cDNA HMG15 were identical (Fig. 1*a*). This region of identity was immediately followed by sequences derived from either exon 2 (cDNA HMG2) or exon 3 (cDNA HMG15) as determined by sequence analysis and the presence of consensus splice sites in the corresponding genomic clone, 2A (Fig. 1*b*). This indicated that an additional sequence was joined to either exon 2 in cDNA HMG2 or exon 3 in cDNA HMG15. The 5′ end of the HMG-CoA reductase mRNA corresponding to cDNA HMG2 was mapped by primer extension using an oligonucleotide complementary to a portion of exon 2. A primer extension product of 65 nt indicated that the 5′ end of this HMG-CoA reductase mRNA extended 8 nt beyond the sequence of cDNA HMG2 (see Fig. 3*a*). Therefore, the length of the sequence upstream of exon 2 on the mRNA corresponding to cDNA HMG2 was 36 nt. Hybridization of oligonucleotide L (complementary to nt 11–27 in cDNA HMG2) to genomic clone 2A demonstrated that the 36-nt sequence was not present in at least 10 kb upstream of exon 2 (data not shown). Genomic titrations, genomic Southern blots, and analysis of additional HMG-CoA reductase genomic clones indicated that a single gene encodes HMG-CoA reductase (data not shown). Hence, it is likely that the two HMG-CoA reductase mRNAs are derived from the same single-copy gene.

Genomic Organization of the 36-nt Sequence. Surprisingly, genomic Southern blotting indicated that oligonucleotide L recognized a tandemly repeated 595-bp fragment (Fig. 2*a*) present in ≈200 copies per haploid genome (data not shown). Sequence analysis of the genomic repeat confirmed that the 36-nt sequence was encoded within the repeat. When hybridized to either total or poly(A)$^-$ RNA, oligonucleotide L detected a 90-nt RNA (Fig. 2*b*, lanes 1 and 2; Fig. 2*c*, lane 1) that was absent from poly(A)$^+$ RNA (Fig. 2*b*, lane 3). To determine whether the 90-nt RNA transcript was derived from the tandem repeat, primer extension sequencing was performed with an oligonucleotide 3′ of the 36-nt sequence in the genomic repeat (Fig. 3*d*). These data indicated that the 90-nt RNA transcript was encoded within the genomic repeat and that the 36-nt sequence was present at its 5′ terminus (Fig. 1*c*). Immediately following the 36-nt sequence in the small RNA was a consensus 5′ splice site (Fig. 1*c*). HMG-CoA reductase exons 2 and 3 were flanked at their 5′ ends with consensus 3′ splice sites (Fig. 1*b*).

The genomic organization of the 36-nt sequence and its presence at the 5′ terminus of both a small nonpolyadenylylated RNA transcript and HMG-CoA reductase transcripts were reminiscent of the 39- and 22-nt SLs of trypanosomes (18, 19) and nematodes (4, 20), respectively. In these organisms, SL RNAs (90–140 nt long) donate the 5′-terminal SL sequence to precursor mRNAs via a trans-splicing mecha-

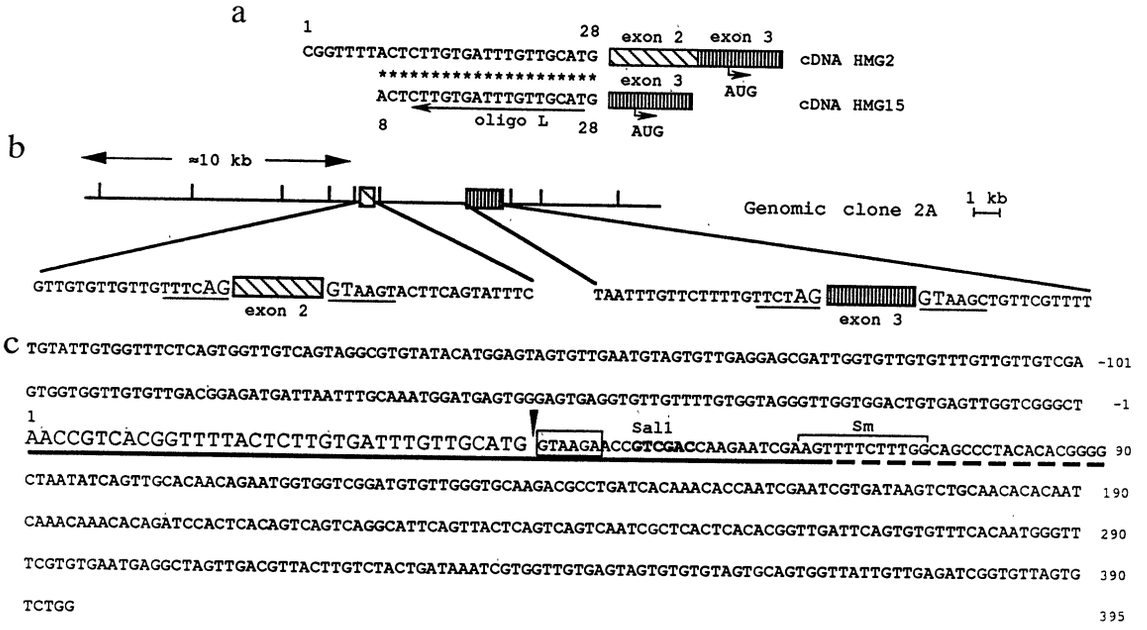

FIG. 1. (*a*) Schematic of HMG-CoA reductase cDNAs HMG2 and HMG15. The first 28 nt in cDNA HMG2 and the first 21 nt in cDNA HMG15 are shown and the region of sequence identity is indicated by asterisks. Sequences derived from exon 2 (nt 29–276) and exon 3 (nt 277–834) are represented by rectangles. The putative initiator methionine codon (AUG) is located within exon 3 (nt 412). The nucleotide sequence complementary to oligonucleotide L (oligo L) is underlined with an arrow. (*b*) Genomic organization of HMG-CoA reductase exons 2 and 3. Consensus splice sites flanking exons 2 and 3 are underlined and portions of the intron sequences are shown flanking the two exons. Invariant AG and GT consensus splice dinucleotides are indicated in large letters. kb, kilobase(s). (*c*) Complete nucleotide sequence of the 595-base-pair (bp) genomic repeat encoding the 90-nt SL RNA. The 36-nt SL sequence is shown in large letters. The first nucleotide of the 90-nt SL RNA gene is designated as 1. Solid line denotes the nucleotide sequence determined by primer extension sequencing (Fig. 3*d*). Broken line indicates the remainder of the SL RNA sequence based on its estimated size of 90 nt. The *Sal* I restriction enzyme cuts within the SL RNA gene and its recognition sequence is shown in bold type. The presumed Sm binding sequence is indicated. The consensus splice donor sequence is boxed, with an arrowhead indicating the splice donor site.

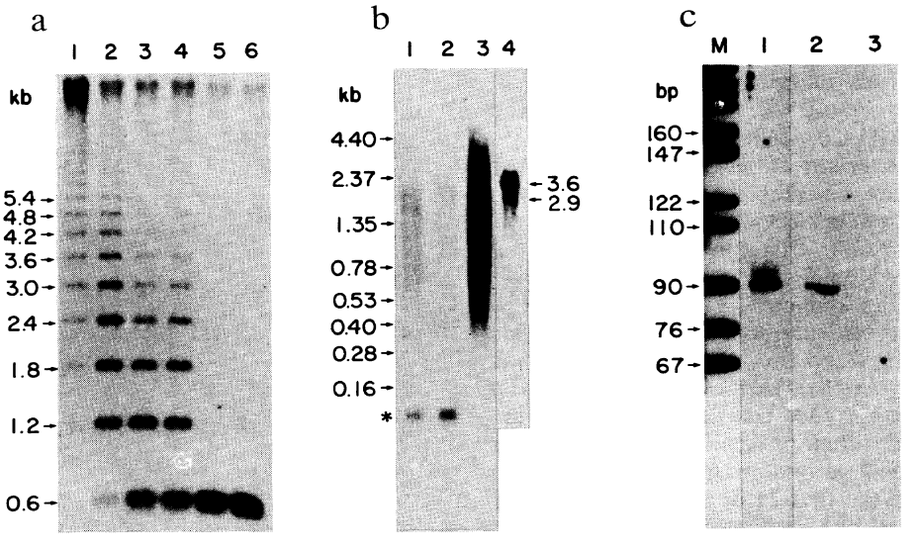

FIG. 2. (*a*) Genomic organization of the schistosome 36-nt SL sequence. Adult schistosome genomic DNA (1 μg) was digested with 0.1, 0.25, 0.5, 1.0, 5.0, and 20 units of *Sal* I and resolved in lanes 1–6, respectively. Blot was probed with 5′ end-labeled oligonucleotide L. Molecular sizes (kb) were calculated from DNA standards (BRL). (*b*) Samples (3 μg) of adult schistosome total RNA (lane 1), poly(A)$^-$ RNA (lane 2), and poly(A)$^+$ RNA (lanes 3 and 4) were electrophoresed in a 1.7% (lanes 1–3) or 0.7% (lane 4) agarose/formaldehyde gel, transferred to a Zeta-Probe membrane (Bio-Rad), and hybridized to 5′ end-labeled oligonucleotide L (lanes 1–3) or a random primer-labeled HMG-CoA reductase cDNA fragment [lane 4; nt 806–1167 of the composite cDNA (11)]. The two HMG-CoA reductase transcripts are indicated with their approximate sizes. Asterisk indicates the position of the 90-nt SL RNA. Molecular sizes were determined from RNA markers (BRL). (*c*) *S. mansoni* poly(A)$^-$ RNA (3 μg) was immunoprecipitated with K121 antibodies (17) directed against trimethylguanosine ($m_3^{2',2',7}G$) (6). Poly(A)$^-$ RNA alone (lane 1), the RNA immunoprecipitated with K121 (lane 2), and the RNA that was not bound to K121 (lane 3) were fractionated in a 6% polyacrylamide/7 M urea gel, transferred to GeneScreen (NEN), and hybridized to 5′ end-labeled oligonucleotide L. Molecular sizes of *Hpa* II-digested pBR322 fragments (lane M) are shown at left.

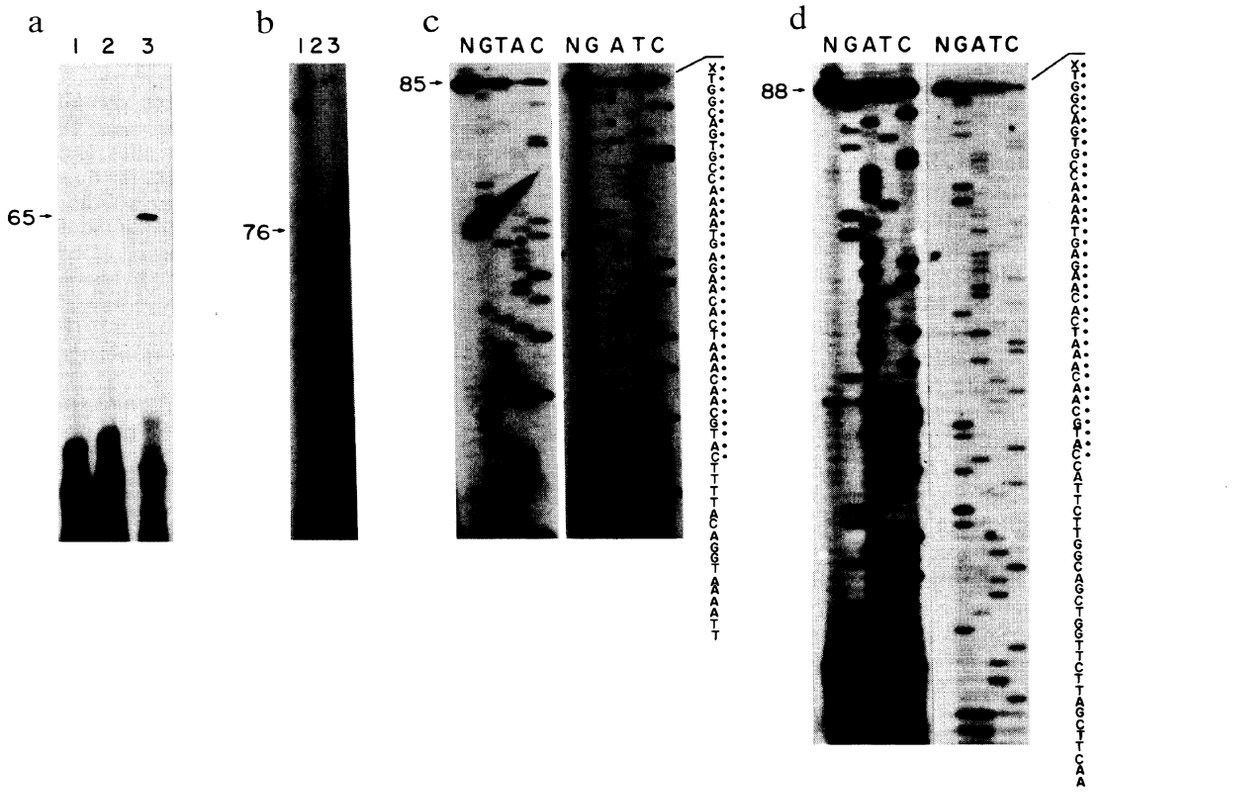

FIG. 3. Primer extension analysis of schistosome transcripts carrying the 36-nt SL sequence. Sizes of primer extension products (arrows) are given in nucleotides. Lanes N in *c* and *d* represent primer extension in the absence of dideoxynucleotides. The nucleotide sequence determined from primer extension sequencing is shown at right in *c* and *d*, and nucleotides complementary to the SL sequence are indicated by dots. (*a*) An oligonucleotide complementary to nt 38–57 in cDNA HMG2 was annealed to either no RNA (lane 1), 2.5 μg of poly(A)$^+$ RNA extracted from HeLa cells (lane 2), or 2.5 μg of adult schistosome poly(A)$^+$ RNA (lane 3) and primer extension was performed. (*b*) An oligonucleotide complementary to nt 55–76 in cDNA L11 was annealed to either no RNA (lane 1), 1.0 μg of poly(A)$^+$ RNA extracted from HeLa cells (lane 2), or 0.2 μg of adult schistosome poly(A)$^+$ RNA (lane 3) and primer extension was performed. (*c*). Primer extension sequencing of the L12 transcript with an oligonucleotide complementary to nt 64–85 in cDNA L12 [0.8 μg of adult schistosome poly(A)$^+$ RNA per lane]. (*d*) The 90-nt SL RNA was sequenced with an oligonucleotide complementary to nt 72–88 in the SL RNA gene [3 μg of adult schistosome poly(A)$^-$ RNA per lane].

nism (2, 3, 5, 6). Thus our data suggest that the 36-nt sequence, which we regarded as exon 1, is a SL derived from the 90-nt SL RNA and trans-spliced to either exon 2 or 3 of the HMG-CoA reductase pre-mRNA. Exon 2 is therefore the first true exon of the HMG-CoA reductase gene.

Analysis of the SL Sequence on Other *S. mansoni* Poly(A)$^+$ Transcripts. To determine whether the SL sequence was donated to other *S. mansoni* mRNAs, oligonucleotide L was hybridized to poly(A)$^+$ RNA. Oligonucleotide L detected transcripts ranging in size from 0.4 kb to 4.0 kb (Fig. 2*b*, lane 3), whereas the HMG-CoA reductase cDNA hybridized to only two discrete poly(A)$^+$ transcripts (Fig. 2*b*, lane 4). This hybridization pattern suggested that the SL sequence was present on transcripts other than HMG-CoA reductase. To determine whether these poly(A)$^+$ transcripts contained the same 36-nt SL sequence at their 5′ termini, a cDNA library was constructed by priming second-strand synthesis with an oligonucleotide spanning the first 24 nt of the SL sequence (7). Two cDNAs (L11 and L12) that contained the 36-nt SL sequence at their 5′ termini and hybridized to abundant poly(A)$^+$ transcripts were characterized further. Primer extension analysis of *S. mansoni* poly(A)$^+$ RNA with oligonucleotides complementary to sequences 3′ of the SL sequence in cDNAs L11 and L12 yielded products of a size consistent with the presence of the 36-nt SL sequence at the 5′ termini of these transcripts (Fig. 3 *b* and *c*). In addition, primer extension sequencing of the L12 transcript clearly demonstrated the presence of the 36-nt SL sequence at its 5′ terminus (Fig. 3*c*). On the basis of primer extensions and Northern analysis we conclude that a number of schistosome poly(A)$^+$ transcripts carry the identical 36-nt SL sequence at their 5′ termini. However, only a subset of *S. mansoni* poly(A)$^+$ transcripts carry the SL sequence at their 5′ termini, since primer extension analyses of transcripts encoding superoxide dismutase (21), egg shell protein (22), calcium-binding protein (23), and an antigen recognized by human sera from infected patients (13) failed to reveal the SL.

Structure of the *S. mansoni* SL RNA. Comparison of the SL RNAs of *S. mansoni*, nematodes, and trypanosomes reveals no significant sequence identity. However, the SL RNA of *S. mansoni* contains a sequence, AGUUUUCUUUGG (Fig. 1*c*), that resembles the consensus Sm binding sequence RAU$_n$GR (R is A or G) and a similar Sm-like binding sequence in sea urchin U7 small nuclear RNA (snRNA), AGUUUCUCUAG (24). Additionally, antibodies directed against $m_3^{2',2',7}$G precipitate *S. mansoni* SL RNA quantitatively (Fig. 2*c*, lane 2), suggesting the presence of a trimethylguanosine cap structure. This cap structure is characteristic of the nematode SL RNA (6, 25, 26) and most snRNAs (27) but is not a feature of the trypanosome SL RNA, which contains a highly modified m^{7}G cap (18, 19, 28).

Despite the absence of nucleotide sequence identity, the predicted secondary structures of SL RNAs in all of these organisms indicate that the SL sequence is part of the first stem–loop and that the Sm-like binding sequences are located between two stem–loops (29) (Fig. 4). However, unlike the nematode and trypanosome SL RNAs, *S. mansoni* SL RNA does not contain stem II.

Analysis of HMG-CoA Reductase Transcripts by PCR. Our data suggest that the exon 3 acceptor site of the HMG-CoA reductase gene can be spliced either to the upstream exon 2 by cis-splicing or to the SL by trans-splicing. The two transcripts that are generated by these reactions should encode proteins with identical amino acid sequences because the putative initiator methionine lies within exon 3 (Fig. 1*a*). We confirmed the existence of these two transcripts by amplifying HMG-CoA reductase cDNAs in a PCR with a 5′ primer corresponding to the first 24 nt of the SL sequence and a 3′ primer complementary to a portion of exon 3 (Fig. 5*a*). Using a quantitative PCR, we determined that 60% of HMG-CoA reductase transcripts contained the SL on exon 3 and 40% of transcripts contained the SL on exon 2 (Fig. 5*b*).

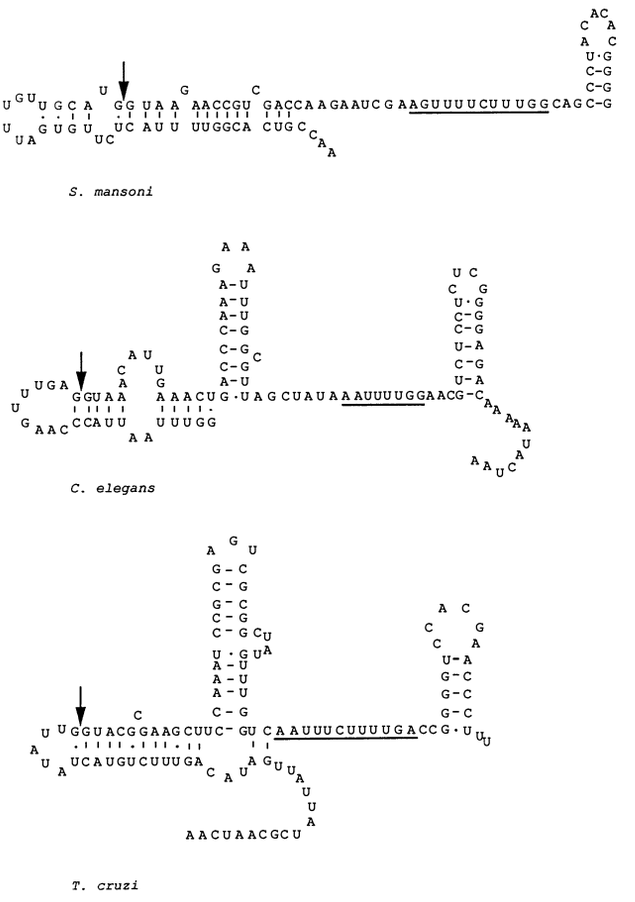

FIG. 4. Comparison of possible secondary structures for *S. mansoni*, *Caenorhabditis elegans*, and *Trypanosoma cruzi* SL RNAs. Arrows indicate the 5′ splice sites, and Sm binding sequences are underlined. Secondary structures for *C. elegans* and *T. cruzi* SL RNAs and their ΔG values were taken from Bruzik *et al.* (29). The *S. mansoni* SL RNA was folded using the algorithm of Zuker (30) with the constraint that the sequence resembling the Sm binding sequence (AGUUUUCUUUGG, underlined) is single-stranded. The ΔG (kcal/mol) values are −18.4 and −16.6 (*S. mansoni*), −17.1 and −16.0 (*C. elegans*), and −25.9 and −24.1 (*T. cruzi*) for the unconstrained and presented structures, respectively.

DISCUSSION

The presence of an identical 36-nt sequence at the 5′ termini of a number of *S. mansoni* poly(A)$^+$ transcripts, its genomic organization, and its presence at the 5′ terminus of a 90-nt nonpolyadenylylated RNA with snRNA-like features indicate the existence of trans-splicing in *S. mansoni*.

The use of trans-splicing for RNA processing in *S. mansoni* most closely parallels the situation in nematodes. For example, only a subset of *S. mansoni* mRNAs receive the SL, and pre-mRNAS contain introns. In addition, the *S. mansoni* SL RNA is immunoprecipitated by antibodies directed against trimethylguanosine and contains a potential Sm-like binding sequence. Unlike most nematode SL RNA genes, which are linked to 5S rRNA genes (7, 20), the *S. mansoni* SL genomic repeat (Fig. 1*c*) does not share sequence identity with any known RNA sequences, including 5S rRNA. Further, in separate studies we have cloned *S. mansoni* 5S rRNA genes and found that these sequences are not associated with the SL RNA genes (unpublished observations). We have also examined the degree to which the *S. mansoni* SL sequence

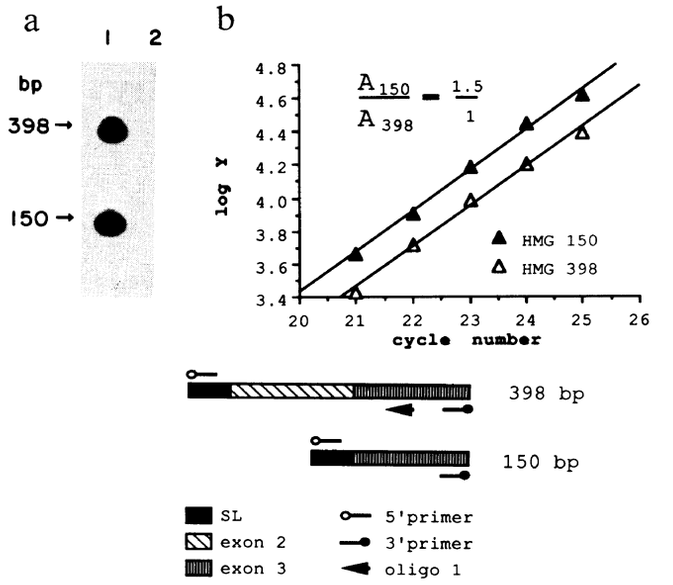

FIG. 5. PCR analysis. (*a*) Adult schistosome poly(A)$^+$ RNA was primed with an oligonucleotide complementary to HMG-CoA reductase mRNA in the presence (lane 1) or absence (lane 2) of avian myeloblastosis virus reverse transcriptase. The resulting cDNAs were amplified in a PCR with the 5′ and 3′ primers. The PCR products were resolved in a 2% agarose gel, transferred to hybridization membrane, and hybridized to 5′ end-labeled oligonucleotide 1 (oligo 1). The identity of the two PCR products was also verified by direct sequencing. (*b*) The relative amounts of the two HMG-CoA reductase transcripts in *a* were determined by quantitative PCR (16). The 3′ oligonucleotide was 5′ end-labeled and the logarithm of radioactivity incorporated into the two transcripts (log Y) was plotted against the number of PCR cycles. The ratio of the 150-bp fragment (A_{150}) to the 398 bp fragment (A_{398}) was determined from the formula $\log Y = \log A + [n \log(1 + R)]$, where Y is the extent of amplification, A is the amount of cDNA initially present after reverse transcription, R is the efficiency of amplification, and n is the number of cycles.

is conserved throughout the phylum Platyhelminthes. Although the *S. mansoni* SL hybridized to RNA from other schistosomes (*S. japonicum* and *S. haematobium*), it did not hybridize to RNA isolated from another trematode, *Fasciola hepatica,* or from the cestodes *Hymenolepis diminuta* and *Taenia crassiceps* (data not shown). If other members of the phylum Platyhelminthes process some of their mRNAs by trans-splicing, it is likely to involve SL sequences that are not homologous to that reported here. The first SL that was discovered in *Caenorhabditis elegans* is thought to be conserved throughout the phylum Nematoda (7, 20). Analyses of transcripts encoding glyceraldehyde-3-phosphate dehydrogenase in *C. elegans* has led to discovery of a second SL, which is not present in all nematodes (31). It is possible, therefore, that *S. mansoni* contains another, as yet unidentified SL that is shared with other trematodes and cestodes.

All of the trans-spliced mRNA transcripts analyzed in nematodes have the SL joined to the first exon (8). Our data show, however, that in *S. mansoni* the SL can be donated to an internal exon (exon 3) of the HMG-CoA reductase gene. In addition, the 3′ splice site of exon 3 is used almost equally in the cis- and trans-splicing reactions. Several possibilities can be proposed for the origin of these two transcripts. One possibility is that a single pre-mRNA is initiated upstream of exon 2 and processed by a splicing complex that can catalyze either the trans- or the cis-splicing reaction to generate the two HMG-CoA reductase transcripts. In this case the use of the 3′ splice site of exon 3 in both cis- and trans-splicing would represent a novel type of alternative splicing. We cannot rule out, however, that a separate promoter lies within the 3.0-kb intron that separates exon 2 and 3. Addition of the SL to exon 3 may then occur by default due to the lack of an upstream 5′ splice site. Further studies are necessary to address the origin and the mechanism by which the two HMG-CoA reductase transcripts are generated.

This report provides evidence for the presence of a SL sequence and probable trans-splicing in the phylum Platyhelminthes. Identification and characterization of spliced leaders in other invertebrates may offer insight into the conserved features, evolution, and function of the SL. The presence of SL sequences and trans-splicing in both protozoan and metazoan parasites offers a potential target for the development of specific chemotherapeutic agents capable of inhibiting a wide variety of parasitic pathogens.

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