Spliced leader *trans*-splicing in the nematode *Trichinella spiralis* uses highly polymorphic, noncanonical spliced leaders

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ABSTRACT

The *trans*-splicing of short spliced leader (SL) RNAs onto the 5' ends of mRNAs occurs in a diverse range of taxa. In nematodes, all species so far characterized utilize a characteristic, conserved spliced leader, SL1, as well as variants that are employed in the resolution of operons. Here we report the identification of spliced leader *trans*-splicing in the basal nematode *Trichinella spiralis*, and show that this nematode does not possess a canonical SL1, but rather has at least 15 distinct spliced leaders, encoded by at least 19 SL RNA genes. The individual spliced leaders vary in both size and primary sequence, showing a much higher degree of diversity compared to other known *trans*-spliced leaders. In a survey of *T. spiralis* mRNAs, individual mRNAs were found to be *trans*-spliced to a number of different spliced leader sequences. These data provide the first indication that the last common ancestor of the phylum Nematoda utilized spliced leader *trans*-splicing and that the canonical spliced leader, SL1, found in *Caenorhabditis elegans*, evolved after the divergence of the major nematode clades. This discovery sheds important light on the nature and evolution of mRNA processing in the Nematoda.

Keywords: nematode; *Trichinella*; spliced leader; *trans*-splicing

INTRODUCTION

Trans-splicing involves the intermolecular ligation of exons derived from two independent RNA molecules. The *trans*-splicing of short RNA-leader, or spliced leader (SL), sequences onto the 5' ends of pre-mRNAs is found in a variety of eukaryotic taxa, including protists (Sutton and Boothroyd 1986; Tessier et al. 1991; Zhang et al. 2007), cnidarians (Stover and Steele 2001), platyhelminths (Davis 1997; Zayas et al. 2005), nematodes (Krause and Hirsh 1987; Spieth et al. 1993; Guiliano and Blaxter 2006), rotifers (Pouchkina-Stantcheva and Tunnacliffe 2005), and tunicates (Vandenberghe et al. 2001; Ganot et al. 2004; Satou et al. 2006). These spliced leaders are donated by longer, precursor SL RNAs through a splicing reaction that is mechanistically similar to the *cis*-splicing used to remove

introns from precursor mRNAs. The primary sequences of the respective SL RNAs are not conserved between these groups, but share common structural features. In all cases the SL is located at the 5' end of the SL RNA and is followed by an intron that is removed during the ligation of the SL onto the splice acceptor site present on the *trans*-spliced pre-mRNA. All SL RNAs form a stem-loop structure such that the donor splice site is base-paired to the SL sequence. This structure is analogous to the base pairing between U1 snRNA and the donor splice site in *cis*-splicing. The intron also contains a binding site for Sm proteins, and in many SL RNAs this site is flanked by stem-loop structures. The conservation of these features has led to the proposal that *trans*-splicing is an ancestral eukaryotic trait; however the alternative explanation, that SL *trans*-splicing has arisen independently multiple times, cannot be excluded (Nilsen 2001; Hastings 2005).

In nematodes, spliced leader *trans*-splicing was first described in *Caenorhabditis elegans* (Krause and Hirsh 1987), whereby a common 22-nucleotide (nt) RNA molecule (SL1) is donated by a 100-nt SL RNA onto the 5' ends of ~70% of mRNAs. A related spliced leader, SL2, is added to the mRNAs derived from genes that lie downstream of the first gene in operons, as part of the processing of the

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polycistronic RNAs produced by such gene clusters (Spieth et al. 1993). *C. elegans* has 110 copies of the SL1 RNA gene, arranged in tandem with the 5S rRNA gene. In contrast, SL2 is encoded by 18 genes that are dispersed throughout the genome. These show a limited degree of sequence variability, but there does not appear to be a bias in their participation in *trans*-splicing (Blumenthal 2005). SL1 has been identified in all nematodes studied to date, suggesting that it may have arisen in the ancestor of all modern nematodes, whereas SL2 seems likely to have evolved as a variant of SL1, in the ancestor of the Rhabditina (the nematode clade, which includes *C. elegans*). Operons are more widespread than SL2, being found in the more distantly related nematodes of the Tylenchina and Spirurina clades, where polycistronic RNAs produced from operons are resolved by SL1 and SL1-like variants (Guiliano and Blaxter 2006).

An important question is whether spliced leader *trans*-splicing is a common feature of all modern nematodes. The available data on *trans*-splicing in the phylum Nematoda is derived from nematodes that fall within the same subclass, Chromadorea (Guiliano and Blaxter 2006). Evidence for the existence of *trans*-splicing in the more basal nematode subclasses of Enoplia and Dorylaimia has been missing or inconclusive (Lindh et al. 1998). Serendipitous observations of discrepancies in the comparison between 5' rapid amplification of cDNA ends (5'RACE) cDNA and genomic sequences derived from two genes of the Dorylaimid *Trichinella spiralis* suggested to us the possibility that these genes were *trans*-spliced and provided the impetus to investigate this likelihood further. Here we show that *T. spiralis* engages in spliced leader *trans*-splicing, but that it does not possess SLs that resemble SL1, SL2, or any other nematode SLs. Uniquely, it has at least 19 active SL RNA genes, which are highly polymorphic in their primary sequences, all of which are located within the same region of the genome. The diversity of SL sequences is unprecedented and raises the possibility that the use of a limited number of highly conserved SLs, such as is found in the Chromadorea, does not represent the ancestral state for the nematode phylum. However, our definitive identification of *trans*-splicing in a Dorylaimid nematode confirms that *trans*-splicing is an ancestral nematode trait.

RESULTS

mRNAs in *T. spiralis* are *trans*-spliced using multiple distinct spliced leaders

During the characterization of 5'RACE cDNA sequences derived from two *T. spiralis* genes, *blgs-1* (Bcl9/legless homolog 1) and *tll-2* (TGF-beta-like ligand 2) (GenBank accession numbers EF682094 and EF681125, respectively), we noticed that the first 21–22 nt did not derive from genomic DNA found upstream or anywhere within 50 kb of

the two genes (data not shown). A draft *Trichinella spiralis* genome sequence, which has been produced by the Genome Sequencing Center at Washington University School of Medicine in St. Louis, allowed a comparison of cDNA and genomic sequences. Inspection of the genomic sequence for the two genes revealed that the points at which the genomic and cDNA sequences no longer matched corresponded to consensus splice acceptor sites (Table 1). In addition, no cDNA suggested that there were any exons upstream of the acceptor site for either of the genes. Such “orphan” splice acceptor sites have been shown in *C. elegans* to be predictive of genes whose mRNAs are *trans*-spliced to spliced leaders, leading to replacement of the 5' UTR, or “outtron,” with the spliced leader (Conrad et al. 1991). Given that the *trans*-splicing of 22-nt spliced leaders to mRNAs is a common feature found in all characterized nematodes, it seemed likely that these additional nucleotides corresponded to *T. spiralis* spliced leaders. We obtained in total 25 5'RACE cDNA sequences for *blgs-1* and four for *tll-2*, all but two of which had putative spliced leader sequences at their 5' ends (Table 1). This revealed a total of 10 distinct putative spliced leader sequences, none of which showed sequence identity with the canonical nematode spliced leader SL1 (5'-GGTTTAATTACCCAAGTTT GAG-3'), or to any of its derivatives. This survey demonstrated that pre-mRNAs from the same gene can utilize multiple spliced leaders and furthermore revealed instances of the same spliced leaders being found in cDNAs from both *blgs-1* and *tll-2*. Since these genes map to different supercontigs, it is unlikely that the leader sequences are added via long-range *cis*-splicing.

We named the spliced leaders *trans*-spliced leaders 1–10 (TSL1–10) to distinguish them from the SL1 and SL2 of other nematodes. To determine whether these spliced leaders were found at the 5' ends of other mRNAs we carried out sequence similarity searches against the publicly available *T. spiralis* ESTs (~22,000) for cDNAs whose 5' ends matched all or part of the TSLs (Supplemental Table 1). All matches were screened to confirm that the position of the spliced leader in the cDNA sequence correlated with a consensus *T. spiralis* splice acceptor site (5'-TTT(T/C)AG-3') (B. Connolly, unpubl.) in the corresponding genomic sequence. For four of these genes whose mRNAs were identified as SL *trans*-splicing targets (DQ132801, GM2 activator protein GM2-AP; BG353788, cytoskeleton associated protein CKAP1; BG731798, RNA helicase; BG732141, Bcl-X homolog) we independently confirmed that their mRNAs received spliced leaders by using 5'RACE (Table 1). This led to the identification of two new TSLs (TSL11, TSL12), bringing the total of distinct spliced leaders being actively used in *trans*-splicing to 12. Three additional putative spliced leaders (TSL13–15) were later defined by further computational identification of TSL RNA genes (see below). We also carried out similarity searches of the EST database with SL1 and SL2 but failed to

TABLE 1. 5' UTR nucleotide sequence of *T. spiralis* trans-spliced cDNA clones

5'-End sequence of 5'RACE cDNA clones ^a	TSL	No
<i>blgs-1</i> ^b		
Genomic DNA sequence: <u>TTTTAGCTGCTGTTTTTCGGAAACAGCTTCTGTGTATG</u>	—	—
AGGTATTACCAGATCTAAAAGCTGCTGTTTTTCGGAAACAGCTTCTGTGTATG	1	9
AGGTATTACCAGgTCTAAAAGCTGCTGTTTTTCGGAAACAGCTTCTGTGTATG	1*	1
AGGTATTACCAGATtTAAAAGCTGCTGTTTTTCGGAAACAGCTTCTGTGTATG	1*	1
AGGTATTACCGAATTAAAAAGCTGCTGTTTTTCGGAAACAGCTTCTGTGTATG	2	2
AGGTATTACCGAATcAAAAAGCTGCTGTTTTTCGGAAACAGCTTCTGTGTATG	2*	1
cGGTAcTTACCGAATTAAAAAGCTGCTGTTTTTCGGAAACAGCTTCTGTGTATG	2*	1
GGTtATTACCGAACTTAAAAAGCTGCTGTTTTTCGGAAACAGCTTCTGTGTATG	3	1
GCGATTGTTCGAATTACTGAAGCTGCTGTTTTTCGGAAACAGCTTCTGTGTATG	4	1
AAATACCTTTCAATTTGTTGAAGCTGCTGTTTTTCGGAAACAGCTTCTGTGTATG	5	1
AACCTGCACGACTTGTTTCAAGCTGCTGTTTTTCGGAAACAGCTTCTGTGTATG	7	1
AACCTGCACGACTTGTTTCGgAGCTGCTGTTTTTCGGAAACAGCTTCTGTGTATG	7*	1
ATCTGTCGGTATTCCTGAAAGCTGCTGTTTTTCGGAAACAGCTTCTGTGTATG	8	1
AcACGTGGTtATTtATTGAAGCTGCTGTTTTTCGGAAACAGCTTCTGTGTATG	9*	1
GGTAATATTACTGAATTCAAGCTGCTGTTTTTCGGAAACAGCTTCTGTGTATG	10	1
AATGTTGCTATACATTTAGCTGCTGTTTTTCGGAAACAGCTTCTGTGTATG	NS	1
ATTTAGCTGCTGTTTTTCGGAAACAGCTTCTGTGTATG	NS	1
<i>tll-2</i> ^c		
Genomic DNA sequence: <u>TTTAAAGGAAAACCCCTATG</u>	—	—
AAATACCTTTCAATTTGTTTGAAGGAAAACCCCTATG	5	1
AACCTTTGCGCATCGTTTAAAGGAAAACCCCTATG	6	1
AACCTGCACGACTTGTTTCAAGGAAAACCCCTATG	7	1
ATCTGTCGGTATTCCTGAAAGGAAAACCCCTATG	8	1
GM2 activator protein ^d		
Genomic DNA sequence: <u>TTCAGTGTGCTGAAAAATG</u>	—	—
AGGTATTACCAGATCTAAAAGTGTGCTGAAAAATG	1	1
AGGTATTACCGAATTAAAAAGTGTGCTGAAAAATG	2	3
GGTtATTACCGAACTTAAAAAGTGTGCTGAAAAATG	3	1
AACCTTTGCGCATCGTTTAAAGTGTGCTGAAAAATG	6	3
AACCTGCACGACTTGTTTCAAGTGTGCTGAAAAATG	7	1
ATCTGTCGGTATTCCTGAAAGTGTGCTGAAAAATG	8	4
AACCTTTGAACCACTTCAAGTGTGCTGAAAAATG	11	8
Cytoskeleton associated protein ^e		
Genomic DNA sequence: <u>TTTTAGCTAATAAAATG</u>	—	—
ATCTGTCGGTATTCCTGAAAGCTAATAAAATG	8	2
AACCTGCACGACTTGTTTCAAGCTAATAAAATG	7	1
RNA helicase ^f		
Genomic DNA sequence: <u>TTACAGCGTATTGAGAAGAATG</u>	—	—
CAGACGTGGTtATTtATTGAAGCGTATTGAGAAGAATG	9	1
AAATCAGTATATTGTGTTGTACCACCGGAAGTTTGGCTTTGTTGTAATTCTTATTTACGTATTGAGAAGAATG	CS	2
TATATTGTGTTGTACCACCGGAAGTTTGGCTTTGTTGTAATTCTTATTTACGTATTGAGAAGAATG	CS	1
ATATTGTGTTTATATCACCAGGAAGTTTGGCTTTGTTGTAATTCTTATTTACGTATTGAGAAGAATG	CS	2
TTGT-CACCGGAAGTTTGGCTTTGTTGTAATTCTTATTTACGTATTGAGAAGAATG	CS	1
Ts-bcl-X _L ^g		
Genomic DNA sequence: <u>TTTTAGCTTGTGAATTTATATATTCACGATG</u>	—	—
AACCTTTGCGCATCGTTTAAAGGTTGTAATTTATATATTCACGATG	6	1
ACGAATTACCGTATTtGTCAAGGTTGTAATTTATATATTCACGATG	12	1
AACCTACAGTATGTCGTTGTTCAAAGTGTGTTGCTGTAATTCGGTTGTAATTTATATATTCACGATG	CS	1
GTTGCTGACTGCTTTGCTGAATTCGGTTGTAATTTATATATTCACGATG	CS	1
CTTTCAAAGTTCCATTTCTTCGAATGTGTGTTGTAATTTATATATTCACGATG	CS	1

(NS) not spliced; (CS) *cis*-spliced; (*) TSL variants.^aTSL sequence shown in bold; initiator ATG codon of protein coding sequence is shaded; splice acceptor site in genomic DNA is underlined.^bGenBank accession number EF682094.^cGenBank accession number EF681125.^dGenBank accession number DQ132801.^eGenBank accession number BG353788.^fGenBank accession number BG731798.^gGenBank accession number BQ732141.

identify any ESTs that were *trans*-spliced with either spliced leader sequence. Among the SL *trans*-spliced ESTs there was a clear preference for utilization of TSL1–4 (Fig. 1A), but whether this reflects a real bias, or a bias in the selection of clones for sequencing and/or library construction remains to be determined. This analysis further showed that TSL addition is not confined to muscle L1 larva, but also occurs in the immature L1 larva and in the adult worm. The relatively few numbers of immature L1 or adult ESTs identified probably reflects the fact that ESTs in the database are predominantly derived from the muscle L1 larva.

The 5'RACE sequence analysis revealed that three of the six genes produced mRNAs that did not always receive a spliced leader, as has been observed in other organisms (Zayas et al. 2005; Satou et al. 2006). Two of the 25 *blgs-1* 5'RACE clones did not show addition of a spliced leader, instead beginning with sequences derived from the outtron (Fig. 1B). The RNA helicase and Bcl-X homolog genes fell into a different category where we recovered products of both *trans*-splicing and *cis*-splicing: six of the seven RNA helicase cDNAs were *cis*-spliced to an upstream noncoding exon whereas only one was *trans*-spliced (Fig. 1C). This could represent competition between *cis*- and *trans*-splicing or could be due to the presence of an alternative promoter

located in the intronic sequence. This alternative promoter use has been observed for *C. elegans* genes. The final example is that of the Bcl-X gene, representing a third category in which two of the five 5'RACE cDNAs are *trans*-spliced and the remaining three are *cis*-spliced but to two alternative upstream noncoding exons (Fig. 1D). Interestingly, we found that among the seven Bcl-X ESTs available from the public database that included 5'-end cDNA sequence only two are *trans*-spliced; the remaining five are *cis*-spliced to an upstream exon (Fig. 1D, Ia). In addition, all of the twenty 5'RACE clones we sequenced for the GM2 activator protein were found to be *trans*-spliced; this compares well with the 13 full-length GM2-activator protein ESTs present in the database, of which 12 are *trans*-spliced (Supplemental Table 1) and one is not spliced. We also noticed an apparent gene-specific bias in TSL *trans*-splicing. TSL-1 is the most frequently added to *blgs-1* mRNAs (11/23), whereas for the GM2 activator protein mRNA it is TSL-11 (8/20) that is most commonly added. One possible explanation for this observed bias is tissue-specific expression of the respective TSLs.

Identification of *T. spiralis* SL RNA genes

Taken together the cDNA data suggested that we had identified spliced leader sequences added to the mRNAs by *trans*-splicing. If these additional sequences represent genuine *T. spiralis* spliced leaders then we expected that we should be able to identify the genes that encode them by searching through the complete *T. spiralis* genome for regions that exactly matched the spliced leader sequence. As predicted, using this strategy we were able to identify matches to all of the putative spliced leaders (Fig. 2A; Table 2). A number of TSLs identified from cDNA sequences showed single nucleotide mismatches when aligned to the *T. spiralis* genome sequence. These mismatches might have arisen from errors introduced during PCR amplification or could indicate allelic variants. Strikingly, all the matches were confined to supercontig 0 (Fig. 2A), suggesting that as in other organisms the *trans*-spliced leader RNA (*tslr*) genes in *T. spiralis* are located together. Notably, and in contrast to a number of other species including *C. elegans*, the *tslr* genes are not organized in clusters with the 5S rRNA genes, although these latter rRNA genes are also found on supercontig 0. The *tslr* genes are found over an 8-Mb region interspersed with putative protein coding genes. Some of the *tslr* genes appear to be single transcription units (e.g., *tslr-10*, *tslr-8*), whereas others appear to be grouped (*tslr-4.1*, *tslr-4.2*, *tslr-5.1*, *tslr-5.2*, *tslr-5.3*, *tslr-14.1*, *tslr-14.2*), although they are not necessarily transcribed in the same direction.

All known nematode spliced leader RNAs are produced as ~100-nt precursor RNAs consisting of the 22-nt spliced leader followed by a splice donor site used to *trans*-splice the SL onto the target mRNA. The remainder of the SL

FIGURE 1. Patterns of TSL addition to *Trichinella spiralis* mRNAs. (A) Graph showing the number of times that each TSL was detected at splice acceptor sites, identified via 5'RACE (gray bars) or in a survey of publicly available EST sequences (~22,000) (black bars). (B–D) Instances of TSL *trans*-splicing detected in 5'RACE analysis of *blgs-1* and genes represented by BG731798 (RNA helicase) and BQ732141 (*bcl-X_L*). (B) An example of a gene that utilizes a classic outtron splice acceptor site: a splice site that is involved in spliced leader *trans*-splicing, but not in *cis*-splicing. (C,D) Examples of *trans*-splice acceptor sites that can also participate in *cis*-splicing to upstream donor splice sites. Exons are denoted by boxes, with shading used to indicate open reading frames; unshaded regions denote untranslated regions. Introns and intergenic regions are represented by horizontal lines. The spliced leader is represented by a black box. In each case the number of representative clones as a fraction of the total number analyzed is given. The scale bar indicates 100 nt.

FIGURE 2. Identification and characterization of spliced leader RNA genes and transcripts. (A) Schematic showing the distribution of the 19 SL RNA genes identified on supercontig 0. Each SL RNA gene is represented by a numbered arrow head that indicates the direction in which it is transcribed, the number indicating the *tslr* gene number. The *tslr* genes that encode identical TSLS are indicated by numbered suffixes. The position of the 5S RNA gene cluster is shown by diverging gray arrowheads. The coordinates show the position of the region along supercontig 0, given in base pairs. (B) Predicted secondary structures for SL RNAs derived from *tslr-1* and *tslr-14*. The spliced leader sequences are depicted in white with dark countershading. Arrows indicate the spliced leader-intron boundary. (C) Northern blot of muscle larvae RNA probed with TSL-1 and TSL-14 radiolabeled oligonucleotides. Arrow indicates expected size of the two SL RNAs.

TABLE 2. Genomic sequence of *T. spiralis* *tslr* genes

Gene	TSL	<i>tslr</i> gene DNA sequence (5'–3')
<i>tslr-1</i>	1	AGGATTTACCGATCTAAAAGGTAAATATTTCAAATCTGACACTTTTAGTGTCTGACAAATTTTGTCTTGGACTTTGGGTTCCCATGGTCCCTTT
<i>tslr-2</i>	2	AGGATTTACCGAATTAAGGCTAAATATTTCATCTGACACGTGAGTGTCTGACAAATTTTGTCTTAGGATTTGGGCAATCCGAATCCGTTATCTTT
<i>tslr-3</i>	3	GGTATTTACCGAACTTAAAGGTAAATATTCTAACTACCTCCGATTGTTTAATCGGCACCAAATTTTGTCTCTGGGTTCTTGGTCTCCAAGATCCTATATTT
<i>tslr-4.1</i>	4	GCGATTGTTCGAATTTACTTTGAAGGTAAATATTCTAAATAATAACCCCTTGGGTTATTCAAAATTTTGTCTTGGAGACTTTCAGTCTCCTGTATTT
<i>tslr-4.2</i>	4	GCGATTGTTCGAATTTACTTTGAAGGTAAATATTCTAAATAATAACCCCTTGGGTTATTCAAAATTTTGTCTTGGAGACTTTCAGTCTCCTGTATTT
<i>tslr-5.1</i>	5	AAATACCTTTCAATTTGTTTGAAGGTAAATATTCTGTAACAATAATTTGTTGACAAAAATTTTGTCTTGGAAATCATAATGATTTCCTTT
<i>tslr-5.2</i>	5	AAATACCTTTCAATTTGTTTGAAGGTAAATATTCTGTAACAATAATTTGTTGACAAAAATTTTGTCTTGGAAATCATAATGATTTCCTTT
<i>tslr-5.3</i>	5	AAATACCTTTCAATTTGTTTGAAGGTAAATATTCTGTAACAATAATTTGTTGACAAAAATTTTGTCTTGGAAATCATAATGATTTCCTTT
<i>tslr-6</i>	6	AACCTTTGCGCATCTTTTGAAGGTAAATATTCTTAAACCTTTTGTGTTAGTCAAAATTTTGTCTTGGAAATCCTTTCCGGATTTCCTGTATTTCT
<i>tslr-7</i>	7	AACCTGCAGCACTTTGTCGAAGGTAAATATTCTAATACCACTTTGGGTTGTGTGAAATTTTGTCTTGGAAATCCTTTCCGGATTTCCTTT
<i>tslr-8</i>	8	ATCTGTCGGTATTTCTGAAAGGTAAATCTTAAATACCACTTTGAGTTGTATTGAAATTTTGTCTTGGAAATCCTTTGCGGATTTCCTGTATTT
<i>tslr-9</i>	9	AGACGTGGTTATTTTGAAGGTAAATATTCTAATACAGGCAATTTTGTGAAATTTTGTCTTGGAAATCCTTTGAAATCCTTT
<i>tslr-10</i>	10	GGTAATATTACTGAATTTCAAGGTAAATATTCTAATACCACTTTAGTATTGAAATTTTGTCTTAGGATTTGGGATCCATAATCCGTAAATTT
<i>tslr-11</i>	11	AACCTTTGAACCCACTTCAAGGTAAATATTCTAATACCACTTTTGGTGTGTTGAAATTTTGTCTTGGAAATCCTTTCCGATTCTT
<i>tslr-12</i>	12	ACGAATTTACCGTATTTTGAAGGTAAATAACTAACTGACACTTAAGTGTGTCAAAATTTTGTCTTGAAGGACTTTGGGATATCCATCGTCGATATTT
<i>tslr-13</i>	13	TACCATTTCAATTTTGAAGGTAACTAAACCTGTACAGCAATAATTTGTGTACAAATTTTGTCTTGAAGGACTTTGGGATATCCATCGTCGATATTT
<i>tslr-14.1</i>	14	TACCGTTCAATTTAATTTTGAAGGTAACTGAAACGTGTACAGCAATAATTTGTGTACAGAAATTTTGTCTTGGAAATCATAATGATTTCCTTT
<i>tslr-14.2</i>	14	TACCGTTCAATTTAATTTTGAAGGTAACTGAAACGTGTACAGCAATAATTTGTGTACAGAAATTTTGTCTTGGAAATCATAATGATTTCCTTT
<i>tslr-15^a</i>	15	TACCGTTCAATTTCAATTTTGAAGGTAACTGAAACGTGTACAGCAATAATTTGTGTACAGAAATTTTGTCTTGGAAATCATAATGATTTCCTTT

Spliced leader is in bold, the putative Sm-binding site is highlighted, and T-rich sequence is underlined.
^a*tslr-15* is included because of its similarity to *tslr-14.1* and *tslr-14*.

RNA, usually referred to as the SL intron, is removed during *trans*-splicing. The 100-nt SL RNAs adopt a characteristic secondary structure whereby the spliced leader base pairs with parts of the intron, forming a hairpin. Two additional hairpins in the intron flank the Sm binding site. We thus examined the DNA downstream of the matches to the putative splice leaders for evidence of these structures. We found that all contained the consensus splice donor site (5'-GTAA-3') immediately 3' of the predicted splice leader that in RNA folding predictions was contained within a hairpin. The spliced leader was followed by two regions that were again predicted to be able to form hairpins, and between them was a stretch of nucleotides (5'-ATTTTGTG-3') that closely matched the consensus Sm binding site in *C. elegans* (5'-purine-AU₄₋₆G-purine-3') (Mattaj 1988; Thomas et al. 1990).

In order to carry out a more exhaustive search for SL RNA genes we used an algorithm to search for combinations of conserved features diagnostic for the SL RNA genes that we had already identified on the basis of TSL sequence identity. Using this approach we were able to identify an additional 29 predicted *tslr* genes (Table 2; Supplemental Table 2), although at present we have no experimental evidence that any of these are actively used. Thus, in total we were able to find potentially 48 individual *tslr* genes, 28 of which are located in the cluster on supercontig 0 and the remaining 20 distributed over a number of other supercontigs. Large gaps in the genomic sequence of supercontig 0 still exist, and it is likely that as these are filled in additional *tslr* genes will be uncovered. Indeed our analysis of the EST database has identified putative orphan TSLs (Supplementary Table 1), not found in the currently available genomic sequence. Furthermore, in our searches we failed to find any evidence for the presence of SL RNA genes encoding the "canonical" nematode SL1 or SL2 or any of the known variants (Guiliano and Blaxter 2006) in the assembled genome sequence or the raw genomic sequencing traces.

To determine the structure of the *T. spiralis* SL RNA we mapped the 3' end of the RNAs derived from *tslr-1* and *tslr-14* using an approach involving in vitro polyadenylation of *T. spiralis* total RNA with yeast poly(A) polymerase. This enzyme adds non-genome-encoded adenosine residues to the 3' ends of RNA molecules. RNA treated in this way was then reverse transcribed using an oligo dT-anchor primer. We used *tslr-1*- and *tslr-14*-specific primers together with the anchor primer to PCR amplify fragments of the introns of *tslr-1* and *tslr-14*, which were subsequently cloned and sequenced (for each gene we sequenced four cDNAs). By comparing the resultant cDNA sequences to genomic sequence and noting at which point nontemplate adenosine residues were added, we were able to map the 3' ends of the SL RNAs (Fig. 2B). Assuming that there are no nucleotides upstream of the spliced leader, the mapping of the 3' ends of TSL1 gives a size of between 97 and 99 nt for

the precursor TSL RNA, very close to that of the canonical nematode SL1 RNA. We noted that the 3' ends of both RNAs mapped to U-rich sequences, which in several organisms have been associated with transcription termination (Maroney et al. 1990a; Huang and Maraia 2001). We could subsequently identify T-rich sequences toward the 3' ends of the other *tslr* genes (Table 2). The structures of the *tslr-1* and *tslr-14* RNAs were modeled using MFOLD version 2.3 (Zuker 2003), with the only constraint that the Sm-like binding site was required to be single stranded. Both TSL-RNAs adopted the same structure with the splice donor site in a stem-loop and two additional stem-loops flanking the Sm-binding site (Fig. 2B). As was found for other SL RNAs, the putative splice donor site is located in the hairpin formed between the SL and the intron.

Finally, we performed Northern analysis with total RNA from muscle L1 larvae to confirm that *tslr-1* and *tslr-14* genes are transcribed as small RNAs. As expected each gene-specific anti-sense probe identified an RNA species of ~100 nt (Fig. 2C) representing the SL-RNA; the smear of larger transcripts detected representing *trans*-spliced mRNAs. The hybridization signal for *tslr-1* was much stronger (>20-fold) than that for *tslr-14* when we corrected for the specific activity of the probe and exposure times. Most likely this is because *tslr-14* is expressed at lower levels, but it may also indicate that it is less efficiently used in *trans*-splicing events, at least in the muscle L1 larva, than *tslr-1*. In fact, to date we have not identified any cDNA or EST which utilizes TSL-14 (Fig. 1A).

DISCUSSION

Recent work from several groups, based upon small-subunit rDNA sequences, has demonstrated that the lineage leading to *T. spiralis* (and the other members of the subclass Dorylaimia) branches close to the base of the nematode phylogenetic tree (Aleshin et al. 1998; Blaxter et al. 1998; Holterman et al. 2006). All other nematodes for which data on *trans*-splicing is available group together in a single subclass termed Rhabditida (Fig. 3). Our data thus indicate that SL *trans*-splicing is likely to be an ancestral nematode feature, since it is found in two subclasses whose last common ancestor lies at the origin of the nematode phylum. However, the absence of spliced leaders that are related to the "universal" SL1 found in all other nematodes studied raises questions about whether SL1 and SL1-like spliced leaders are an ancestral nematode feature, lost in the lineage leading to *T. spiralis*, or whether the diverse SL RNA complement found in *T. spiralis* is the ancestral state, from which SL1-like spliced leaders evolved. Recently we found that SL *trans*-splicing also occurs in another *Trichinella* species, *T. pseudospiralis*, and that at least some of the TSLs are conserved between species (B. Connolly, unpubl.). An important area for future work will be determining the

FIGURE 3. Nematode phylogeny showing two possible scenarios to explain extant spliced leader complements. The phylogeny shown is based upon SSU rDNA-based studies (Aleshin et al. 1998; Blaxter et al. 1998; Holterman et al. 2006). All recent studies are agreed on the relative position of *C. elegans* with respect to *T. spiralis*, though the exact topologies differ. Boxes indicate SL RNA genes, with the shading used to indicate distinct genes. In model 1, the ancestor of all extant nematodes possessed multiple, different SL RNA genes, which were maintained in the lineage leading to *T. spiralis*. In this model, all but one of these were lost in Rhabditida lineage, the surviving one becoming the ancestor of the SL1 and SL2 RNA genes. In model 2, the ancestral nematode possessed only one (possibly multicopy) SL1-like gene, that in the Dorylaimia lineage underwent multiple rounds of duplication and diversification, but was maintained in the Rhabditida lineage. Information on *trans*-splicing in Enoplid nematodes should shed light on which of these models is correct.

distribution of spliced leaders in other members of the Dorylaimia, as well as in the other major nematode subclass, the Enoplia.

The most striking feature of the family of SL RNA genes that we have identified in *T. spiralis* is their diversity. Although other nematodes have families of SL RNA genes, these show limited diversity. The *T. spiralis* spliced leaders show variability at almost all positions, with the exception of the last three residues, and share none of the diagnostic features of SL1-like or SL2-like spliced leaders identified by Guiliano and Blaxter (2006).

We have not been able to show that there are functional differences between the SL RNA genes, although the existence of polymorphic TSLs raises the prospect that each may confer unique translational efficiencies on the recipient mRNA. Beyond this, it was nevertheless clear in our study that some SL RNAs are more frequently used than others. Although we observe a bias in the use of certain TSLs, it is unclear whether this reflects real differences in the efficiency with which each TSL participates in *trans*-splicing reactions. Furthermore, it remains to be determined whether a bias in TSL utilization persists throughout all life-cycle stages or whether particular TSLs exhibit stage-specific or tissue-specific usage. The preponderance of *blgs-1* mRNAs receiving TSL1 contrasts with the observation that GM2 activator protein mRNAs more frequently are *trans*-spliced to TSL11. If this is due to tissue-specific expression, then one would expect, for

instance, that TSL1 would be more strongly expressed in tissues that express *blgs-1*, compared to other TSL genes. No such tissue-specific expression has been reported for SL RNAs in other nematodes. Furthermore, we recently found that *trans*-splicing onto the *T. pseudospiralis* GM2-AP mRNAs also exhibits a strong bias, with TSL7 being *trans*-spliced onto 10/11 of the 5'RACE clones examined (B. Connolly, unpubl.).

In a survey of *T. spiralis* ESTs, only about 1% of mRNAs were identified with a spliced leader. However, without knowing how many of the ESTs are derived from full-length transcripts, it is unclear if this low number results from most ESTs being 5' truncated, and thus fail to include the spliced leader, or whether this actually represents the true proportion of *trans*-spliced mRNAs. Additionally, given that the *T. spiralis* genome sequence is incomplete, it remains possible that we have not identified all of the TSLs. Thus, our data may underestimate both the number of TSLs and the proportion of mRNAs that are *trans*-spliced.

The majority of what we know about the mechanism of *trans*-splicing in nematodes has largely come from studies in just two nematodes: *C. elegans* and *Ascaris spp.* Although not closely related, these nematodes both add the canonical SL1 spliced leader to the majority of their mRNAs (Maroney et al. 1995). Biochemical and molecular genetic investigations have built up a detailed picture of the mechanism and biological significance of spliced leader addition to mRNA in nematodes (Bruzik et al. 1988; Thomas et al. 1988; Maroney et al. 1990a,b; Van Doren and Hirsh 1990; Denker et al. 1996, 2002; Ferguson et al. 1996; Ferguson and Rothman 1999; Lall et al. 2004; Cheng et al. 2007; MacMorris et al. 2007). The conservation of the SL1 primary sequence between *C. elegans* and *A. suum* suggests a functional constraint. In addition, all previously identified nematode SLs are clearly variants of SL1. This is in contrast to comparisons between nematode SLs and those from organisms from other phyla, which show no evidence of conserved primary sequence. It is known that one reason for this constraint is in part because the SL1 sequence encodes a transcriptional control element. However, it may also be that SL1 and SL1-like RNAs are constrained by the RNA-protein interactions involved in the formation of the snRNP complexes that form during *trans*-splicing. In both *Ascaris* and *C. elegans*, SL1 snRNPs contain a conserved pair of proteins (Denker et al. 2002; MacMorris et al. 2007). Database searches show that these proteins are also present in *Brugia malayi*. We have not been able to identify genes encoding homologs of these proteins in the *T. spiralis* genome sequence assembly, suggesting the possibility that they have coevolved with SL1-type spliced leaders. An important question for future studies to address will be the extent to which the mechanisms of *trans*-splicing differ between *C. elegans* and *T. spiralis*.

Operons appear to have coevolved with spliced leader *trans*-splicing as they provide a means for resolving polycistronic

pre-mRNAs (Blumenthal 2005). The choice of spliced leader that is added to downstream mRNAs processed from such polycistronic RNAs is variable, with some nematodes using the universal SL1, while others have evolved specialized variants apparently derived from SL1 (Guiliano and Blaxter 2006). However, as stated above, the fact that all such nematodes share a common ancestor does not allow any inferences to be made about the use of operons in more distantly related nematodes. None of the genes that we have shown to be *trans*-spliced in *T. spiralis* appear to be part of operons, and there is no evidence to suggest that they exist in this nematode. However, the analysis of the *T. spiralis* genome is at an early stage and further work will be required to address the existence of operons in this nematode. The findings described here nevertheless provide an important and revealing insight into the evolution and complexity of mRNA processing in a wide range of taxa.

MATERIALS AND METHODS

Nematode isolation and RNA preparation

Trichinella spiralis parasites were maintained in female ICR mice and muscle L1 larvae recovered as previously described (Connolly et al. 1995). Total RNA from *T. spiralis* muscle L1 larvae was prepared as described previously (Kuratli et al. 1999).

Northern analysis

Ten micrograms of total RNA were fractionated by 2% glyoxal/DMSO agarose gel electrophoresis and transferred to Hybond N membranes using standard techniques. TSL-1 and TSL-14 RNAs were detected using the ³²P labeled oligonucleotide probes anti-TSL-1 and anti-TSL-14 (Supplemental Table 3), respectively. Probes are complementary to the respective splice leader sequences and were ³²P end-labeled using γ -³²P ATP and T4 polynucleotide kinase. Hybridization was performed according to a standard protocol (Church and Gilbert 1984) except that hybridization was at 42°C and wash steps were at room temperature, 36°C, and 42°C. Blots were analyzed by autoradiography or using a Fuji FLA3000 Phosphorimager and Aida 2.0 software (Raytest GmbH).

Determination of cDNA 5' ends

The 5' ends of cDNAs representing full-length mRNAs were obtained through 5'RACE using the GeneRacer kit (Invitrogen), according to the manufacturer's instructions. Gene-specific primers used are given in the Supplemental Table 3, and the cDNAs, amplified by PCR using the Expand High Fidelity polymerase (Roche), were cloned into pGEM T-Easy (Promega) or pCR4-TOPO (Invitrogen) and the resulting plasmid inserts sequenced by the University of Dundee Sequencing Service.

Identification of *trans*-spliced *T. spiralis* ESTs

Using each of the TSL sequences we carried out a megaBLAST search of the publicly available *T. spiralis* EST sequences, looking

for matches only at or within 13 nt of the 5' end of the sequence. We noted that the 12-nt sequence 5'-CCCACGCGTCCG-3' was found at the 5' end of virtually all ESTs with accession numbers of the form ESxxxx. Independent analysis 5'RACE clones of several of the genes (both *trans*-spliced and non-*trans*-spliced) represented by these ESTs failed to identify this sequence at the 5' ends of the mRNAs (data not shown). We concluded that this sequence is an artifact and we thus excluded it when screening the 5' end sequence of these ESTs. A BLAST search using the positively identified cDNA sequences was then carried out against the *T. spiralis*-1.0 supercontig assembly (<http://genome.wustl.edu/tools/blast/>) to confirm the presence of an outtron at the point at which the TSL was predicted to be added.

Identification of predicted TSL RNA genes

The *T. spiralis* genome draft release comprising 6261 contigs (downloaded March 23, 2006, from the University of Washington Genome Sequencing Center; ftp://genome.wustl.edu/pub/organism/invertebrate/Trichinella_spiralis) was surveyed for the presence of novel splice leader genes using a motif search algorithm (Trichinella-SL-scan.pl) written in the language Perl (Active State Perl version 5.005_3). The algorithm employs a sliding search window to look initially within each contig sequence for examples of the Sm-binding motif-like sequence (denoted element 1), with the sequence 5'-AATTTTTGTCCTGGG-3'. If such a sequence is found, the algorithm then searches in a region between 45 and 22 nt upstream (5') of the Sm-binding motif sequence for the splice donor sequence 5'-AAGGTA-3' (element 2). Having found this second element, the algorithm then searches the sequence between elements 1 and 2 for a third element, comprising any sequence capable of forming a stem-loop with stem length of at least 11 nt. The algorithm allows G-U wobble base pairing within the stem. During scanning, when an element 1 example is found at a given position, but there is no element 2 within the defined upstream window, the sliding window search automatically resumes for the next 3' example of element 1. The same process is triggered if juxtaposed elements 1 and 2 are found, but no intervening element 3. The algorithm carries out the search pattern on both DNA strands. The algorithm can operate at varying levels of stringency. Based upon known examples of TSL sequences identified at the 5' ends of *T. spiralis* mRNA sequences by this study, the search was carried out allowing three mismatches among 15 nt within the Sm-binding motif, and with three nucleotide mismatches within the first 11 nt of the element 3 stem permitted. Subsequently, identified sequences were manually examined, and only those with a more stringent match to the putative Sm-binding site (one mismatch allowed in 5'-AATTTTTGT-3') were retained as putative *tslr* genes. The Perl script is freely available on request from the authors.

TSL RNA 3' end mapping

TSL RNA 3' ends were determined essentially as described previously (Keall et al. 2007). *T. spiralis* total RNA (4 µg) was poly(A) tailed using yeast poly(A) polymerase, reverse transcribed using an oligo-dT-anchor primer, and then PCR amplified using TSL-specific primers and an anchor primer. PCR fragments were inserted into pGEM-T easy (Promega), and inserts were sequenced at the University of Dundee DNA Sequencing Facility.

The TSL-1-specific primer was SL1-F1 and the TSL-14-specific primers were SL14-F1 and SL14-F2 (Supplemental Table 3).

Secondary structure predictions

Secondary structure predictions of the TSL RNAs were performed using MFOLD Version 2.3 (Zuker 2003) using the default folding conditions (1 M NaCl, 37°C), and with the constraint that the Sm-binding site (5'-AAUUUUUGU-3') was required to be single stranded.

Accession numbers

Primary sequence data reported in this article are available in the GenBank database under the accession numbers EF681123 (*tslr-1*), EF681124 (*tslr-14*), EF681125 (*tll-1*), and EF682904 (*blgs-1*).

SUPPLEMENTAL DATA

Supplemental materials are available at <http://www.abdn.ac.uk/~bch182/PettittSupplTables.pdf>.

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