REPORT

SL2-like spliced leader RNAs in the basal nematode *Prionchulus punctatus*: New insight into the evolution of nematode SL2 RNAs

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ABSTRACT

Spliced-leader (SL) *trans*-splicing has been found in all molecularly characterized nematode species to date, and it is likely to be a nematode synapomorphy. Most information regarding SL *trans*-splicing has come from the study of nematodes from a single monophyletic group, the Rhabditida, all of which employ SL RNAs that are identical to, or variants of, the SL1 RNA first characterized in *Caenorhabditis elegans*. In contrast, the more distantly related *Trichinella spiralis*, belonging to the subclass Dorylaimia, utilizes a distinct set of SL RNAs that display considerable sequence diversity. To investigate whether this is true of other members of the Dorylaimia, we have characterized SL RNAs from *Prionchulus punctatus*. Surprisingly, this revealed the presence of a set of SLs that show clear sequence similarity to the SL2 family of spliced leaders, which have previously only been found within the rhabditine group (which includes *C. elegans*). Expression of one of the *P. punctatus* SL RNAs in *C. elegans* reveals that it can compete specifically with the endogenous *C. elegans* SL2 spliced leaders, being spliced to the pre-mRNAs derived from downstream genes in operons, but does not compete with the SL1 spliced leaders. This discovery raises the possibility that SL2-like spliced leaders were present in the last common ancestor of the nematode phylum.

Keywords: gene expression; nematode; phylogeny; spliced leader RNA; spliced leader *trans*-splicing

INTRODUCTION

Spliced leader *trans*-splicing involves the modification of the 5' ends of pre-mRNAs via an intermolecular splicing event, which results in the addition of a short RNA, the spliced leader (SL), to mRNAs (Hastings 2005). The SL is donated by the independently transcribed SL RNA and targets an unpaired splice acceptor site near the 5' end of the target pre-mRNA. SL RNAs contain two functionally distinct domains: an SL exon and an intron that includes the Sm sequence motif, a binding site for Sm proteins involved in *cis*-splicing (Mattaj 1988). They also generally

conform to a similar structure consisting of three stem-loops, two of which flank the Sm sequence motif. However, there is considerable sequence diversity, both in terms of length and content. There is no obvious conserved function for SL *trans*-splicing, although in some groups of organisms functions have been clearly assigned, including the regulation of mRNA stability and translation and the resolution of polycistronic RNAs (Davis and Hodgson 1997; Zeiner et al. 2003; Lall et al. 2004; Blumenthal 2005; Satou et al. 2006; Marletaz et al. 2008; Wallace et al. 2010).

The range of organisms in which SL *trans*-splicing has been identified is broad, extending from protists through to chordates (Sutton and Boothroyd 1986; Tessier et al. 1991; Davis 1997; Stover and Steele 2001; Blumenthal 2005; Pouchkina-Stantcheva and Tunnacliffe 2005; Zayas et al. 2005; Guiliano and Blaxter 2006; Zhang et al. 2007; Marletaz et al. 2008; Pettitt et al. 2008; Zhang and Lin 2009; Derelle et al. 2010; Douris et al. 2010; Matsumoto et al. 2010), although it appears to be absent from plants, fungi, and vertebrates. The phylogenetic distribution of SL *trans*-splicing is sporadic, however, and does not allow one to distinguish whether this distribution reflects a single origin

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of SL *trans*-splicing followed by multiple independent losses or whether the process has arisen through multiple, independent events in the different lineages in which it occurs. The similarity of SL RNA structure would seem to support a common, divergent origin for SL *trans*-splicing. It is also consistent with the hypothesis that snRNAs associated with splicing, in particular U1 snRNA, have the potential to mutate into SL RNAs, explaining both the conserved features of SL RNAs and their sporadic appearance throughout eukaryotic evolution.

From the perspective of the evolution of SL *trans*-splicing, the nematodes are an important group, since it seems likely that this process was present in the last common ancestor of the phylum. Most of what we know about nematode SL *trans*-splicing is derived from a single species, *Caenorhabditis elegans*, in which there are two SL RNAs that participate in distinct SL *trans*-splicing events: SL1 RNA is employed in all cases where a pre-mRNA possesses a 5' untranslated region (5'UTR) that includes an unpaired splice acceptor site. In contrast, SL2 RNA is spliced onto mRNAs whose 5' ends are formed during the processing of polycistronic operon transcripts into monocistronic mRNAs for translation (Spieth et al. 1993).

SL1 and closely related SL1-like molecules have been identified in other nematodes. However, all of these fall within the same monophyletic group as *C. elegans*, termed the Rhabditida (Fig. 1A; Guiliano and Blaxter 2006; Holterman et al. 2006; Meldal et al. 2007). In contrast, the distribution of SL2 RNAs appears to be more limited, being found only in the clade of nematodes that includes *C. elegans*, the Rhabditina (Guiliano and Blaxter 2006), suggesting that this class of SL RNAs evolved relatively late in nematode evolution.

An important question is the extent to which this view of SL *trans*-splicing can be applied to the less well-studied members of the phylum. This question has recently been addressed with the availability of the draft genome sequence from *Trichinella spiralis*. This nematode occupies a significant phylogenetic position, being a member of the Dorylaimia clade, which branches close to the base of the nematode phylum (Fig. 1A; Holterman et al. 2006; Meldal et al. 2007). Members of this basal clade display molecular, cellular, and embryological traits found in other animal groups that appear to have been lost in the Rhabditida (Lavrov and Brown 2001; Bürglin 2008; Heger et al. 2009; Schulze and Schierenberg 2009).

FIGURE 1. *P. punctatus* SL sequences are related to SL1 and SL2. (A) A simplified nematode phylogeny based on ribosomal RNA sequences showing clades I-V (Holterman et al. 2006; Meldal et al. 2007). Species indicated are *Caenorhabditis elegans*, *Aphelenchus avenae*, *Ascaris suum*, *Prionchulus punctatus*, and *Trichinella spiralis*. (B) Alignment of *P. punctatus* Ppu-SL1 to Ppu-SL6 spliced leader sequences with *C. elegans* SL1 and SL2 spliced leaders. Conserved sequence elements outside the splice junction are boxed in gray. (C) Proposed secondary structures of Ppu-SL2 and Ppu-SL3.1 RNAs (GenBank entries GU936306 and GU936307) produced using M-fold (Zuker 2003). The SL sequence is shown outlined, and the putative Sm sequence motif is underlined.

We have previously shown that the canonical SL1 *trans*-splicing found in members of the Rhabditida does not appear to be present in *T. spiralis* (Pettitt et al. 2008). Rather, the SL RNAs from this nematode showed unprecedented levels of sequence diversity and could not be readily placed in either of the SL1 or SL2 families previously described. We proposed two possible scenarios to explain these data. First, the ancestral nematode resembled *T. spiralis*, having a diverse array of distinct SL genes, which in the lineage leading the Rhabditida underwent homogenization events leading to the presence of SL1 and SL1-like RNA genes. Alternatively, a single ancestral SL RNA gene that resembled SL1 underwent successive rounds of gene duplication and diversification events in the lineage leading to *T. spiralis* (Pettitt et al. 2008).

To attempt to distinguish between these possibilities, we have investigated SL *trans*-splicing in another dorylaimid nematode, *Prionchulus punctatus*. Surprisingly the SLs that we identified from this nematode do not closely resemble those from *T. spiralis*. Rather they resemble the SL2 family that has previously only been identified in Rhabditina nematodes (Guiliano and Blaxter 2006). Consistent with this, we show that one of these SL2-like molecules, when expressed in *C. elegans*, can specifically participate in SL2-mediated SL *trans*-splicing but is not apparently added to genes whose pre-mRNAs are matured via SL1 *trans*-splicing. This discovery raises questions about the likely nature of SL *trans*-splicing in the last common ancestor of the nematode phylum.

RESULTS

From a random sample of *P. punctatus* cDNA clones, prepared by 5' RACE using oligo-dT as a primer, we identified a cDNA sequence corresponding to a gene encoding a vacuolar protein, hereafter designated *Ppu-vha-1*. The 5' end of the *Ppu-vha-1* cDNA was similar in terms of size and nucleotide content to the SL2 family of SLs found in *C. elegans* and other members of the Rhabditina (Guiliano and Blaxter 2006). We designated this putative SL *Ppu*-SL1 (Fig. 1B). However, in the absence of genomic sequence, we were unable to directly verify that this sequence was not simply a chance match to the SL sequences. We reasoned that if this sequence arose from the *trans*-splicing of a *P. punctatus* SL sequence, then we would expect to find it at the 5' ends of mRNAs derived from additional genes. To identify such genes, we used an oligonucleotide corresponding to the putative SL in 3' RACE reactions. In this way, we identified cDNAs corresponding to seven different *P. punctatus* genes (Table 1). The presence of this same sequence at the 5' ends of multiple different mRNAs made it highly likely that we had identified a bona fide SL in *P. punctatus*.

To attempt to identify additional SLs in *P. punctatus*, we carried out 5' RACE using a *Ppu-vha-1* gene-specific primer. This allowed us to sample the range of SLs *trans*-

TABLE 1. *Prionchulus punctatus* cDNA sequences with *Ppu*-SL1 at their 5'-ends

Gene	Predicted product	GenBank entries
<i>Ppu-vha-1</i>	Vacuolar protein	GU936298
<i>Ppu-rpl-36</i>	60S Ribosomal protein L36	GU936300
<i>Ppu-gst-1^a</i>	Glutathione-S-transferase	GU936301
<i>Ppu-ubc-2</i>	Ubiquitin-conjugating enzyme	GU936303
<i>Ppu-ger-1</i>	GDP-keto-6-deoxymannose 3,5-epimerase/4-reductase	GU936302
<i>Ppu-rps-15</i>	Ribosomal protein S15	GU936305
<i>Ppu-ubl-1</i>	Ubiquitin/ribosomal protein S27a	GU936304

These cDNA sequences were obtained by 3' RACE using the *Ppu*-SL1 primer in combination with 3' RACE primers.

^aIncomplete sequence.

spliced onto the pre-mRNA of this gene, and we were thus able to identify three additional putative SLs (*Ppu*-SL2, -SL3, and -SL4) that differed from the original sequence in only a few positions (Fig. 1B, Supplemental Table 1). The fact that the sequence variations were only observed in the putative SL sequences, and that most variations were sequenced in several independent 5' RACE products, suggested that these were not the result of PCR-induced mutations but represented different SL variants.

To rule out the possibility that the SL-carrying cDNAs were amplified from the *R. dolichura* prey worms that we use to culture *P. punctatus*, we showed that *Ppu-vha-1* could be PCR amplified from lysates of single *P. punctatus* animals but not from the prey worms (Supplemental Fig. 1). Similarly, phylogenetic analyses using the coding sequences derived from the SL-cDNA sequences reported here showed clear affinity between *P. punctatus* and other dorylaimid species to the exclusion of nematodes from the Rhabditida clade (Supplemental Fig. 2).

Since all known SLs are derived from longer SL RNAs, to provide further evidence that these moieties were the products of SL *trans*-splicing, we attempted to clone the predicted precursor SL RNAs via poly(A) tailing of total *P. punctatus* RNA. We chose two SLs (*Ppu*-SL2 and *Ppu*-SL3) to apply this technique to, and we identified cDNAs of ~100 nucleotides (nt) (Fig. 1C) for each one. While we identified one transcript with *Ppu*-SL2, we found two *Ppu*-SL3 containing RNAs (*Ppu*-SL3.1, -SL3.2) with subtle but significant sequence variations (Supplemental Table 1A). These sequences are predicted to form three stem-loops: one contains the SL splice site, and the other two flank an Sm sequence motif, as has previously been observed for all other nematode SL RNAs (Fig. 1C). Sequencing of the products of the 5' RACE experiments, using gene-specific primers located in the SL RNA introns for both SL RNA genes, confirmed the SL RNA sequences in each case.

In *C. elegans* the SL1 RNA genes are clustered in a tandem array (Blumenthal 2005). We reasoned that if this were the case in *P. punctatus*, it might be possible to detect such

a situation by amplifying from genomic DNA using combinations of primers derived from each SL RNA. Using this approach, we were able to identify two additional SLs (SL5 and SL6) (Fig. 1B) and four additional SL RNA genes (*Ppu-SL1*, *Ppu-SL5.1*, *Ppu-SL5.2*, and *Ppu-SL6*) (see Supplemental Table 1). One of the genomic PCR products that we isolated contained three tandem SL RNA genes, showing that at least some of the SL RNA genes are clustered. Unlike the situation in *C. elegans*, the *Ppu-SL* RNA genes that we identified did not appear to be interspersed with 5S RNA genes.

Previous studies of rhabditid nematodes have demonstrated the existence of SLs specifically associated with the SL *trans*-splicing to downstream genes in polycistronic operons (Spieth et al. 1993; Evans et al. 1997; Blumenthal et al. 2002). This SL2 family of SLs can be distinguished from SL1 and SL1-like SLs on the basis of specific sequence motifs present in the SL itself (Guiliano and Blaxter 2006). Comparing the *Ppu-SL*s with previously described SLs from other nematodes, it was clear that they could be classified as members of the SL2-like family based upon the spacing between the 5' GGTWW, the central CCCA, and the splice-donor site. In addition, previous studies have identified residues located in the third stem-loop of SL2 RNAs that are essential for SL2 *trans*-splicing in *C. elegans*; a GGGG stretch separated from UCCG by 2 or 3 nt (Evans and Blumenthal 2000). Of the *Ppu-SL* RNAs that we have identified, all but the *Ppu-SL3* RNAs have these residues conserved in the loop region of their predicted third stem-loops (Fig. 1C; Supplemental Table 1A), providing further support that they are SL2-like SL RNAs.

To provide experimental verification that the *Ppu-SL*s fall into the SL2-like family, we investigated whether they are capable of competing with the endogenous *C. elegans* SLs, and of being *trans*-spliced onto pre-mRNAs that normally received SLs. We also reasoned that if they are able to participate in SL *trans*-splicing in *C. elegans*, then they might show a bias for splicing onto pre-mRNAs derived from downstream genes in operons. To this end, we generated transgenic *C. elegans* strains expressing *Ppu-SL2* and *Ppu-SL3.1* RNAs under the control of a *C. elegans* U2 RNA gene promoter (Fig. 2A; Ferguson and Rothman 1999).

For both constructs, we tested several independent transgenic lines and assayed for the presence of the *Ppu-SL* at the 5' ends of previously reported *trans*-spliced *C. elegans* genes. We were unable to detect any messages that received *Ppu-SL3.1* in the three transgenic lines produced, and were not able to detect *Ppu-SL3.1* RNA using the sensitive method used for the cloning of this RNA from *P. punctatus* RNA preparations. In contrast, *trans*-splicing of *Ppu-SL2* was observed in the two transgenic lines produced, indicating that *Ppu-SL2* was capable of acting as a substrate for the SL *trans*-splice reaction in *C. elegans*.

We first tested a known SL2 *trans*-spliced gene, Y67H2A.5. Using a gene-specific primer combined with

FIGURE 2. *Ppu-SL2* RNA expressed in *C. elegans* is *trans*-spliced to SL2 accepting transcripts. (A) Schematic of the expression construct. *Ppu-SL2* is expressed under the control of the *C. elegans* U2.3 snRNA promoter, and *s/s-1* terminator in strains PE535/536. (B) Detection of *trans*-splicing of *Ppu-SL2* RNA. Total RNA prepared from strains PE535/536 was reverse transcribed. cDNA synthesis was tested by amplification of *act-1*; *trans*-splicing of *Ppu-SL2* to Y67H2A.5 transcripts was detected using a gene-specific primer in combination with the *Ppu-SL2* primer. In a control reaction (–RT), the reverse transcriptase was omitted from the cDNA synthesis reaction. PCR reactions were analyzed by agarose gel electrophoresis (shown in inverted colors), and PCR products were analyzed by sequencing. The 5' end of the sequence including the *Ppu-SL2* primer sequence is shown. Critical residues that allow the distinction between *trans*-splicing to *Ppu-SL2* and to SL1 or SL2 or *Ppu-SL2* RNAs are indicated with arrows, and the Y67H2A.5 transcript region is boxed black, with the beginning of the open reading frame in italics. The SL2 splice acceptor site is indicated (Δ). (C) *Trans*-splicing of *Ppu-SL2* to *tct-1*, *rla-1*, and *rps-3* transcripts in PE536. *Trans*-splicing to SL1 or SL2 or *Ppu-SL2* RNAs was detected using gene-specific primers in combination with SL1, SL2, or *Ppu-SL2* primers. Control PCR amplifications were performed with mock cDNA preparations performed without reverse transcriptase (–RT). Amplification products were identified by DNA sequencing. M indicates marker lane, in base pairs (bp). The acceptor sites for SL2 and *Ppu-SL2* are indicated in the genomic *tct-1* and *rla-1* sequences (▲).

one derived from *Ppu-SL2* we were able to amplify a product that when sequenced was shown to correspond to the addition of *Ppu-SL2* to the previously determined SL acceptor site of Y67H2A.5 (Fig. 2B). As critical *Ppu-SL2*-specific residues are not part of the primer, the sequence analysis reliably identifies the participation of *Ppu-SL2* in *trans*-splicing. Thus, *Ppu-SL2* was able to participate in SL

trans-splicing in *C. elegans* and compete with endogenous *C. elegans* SL2 RNAs.

To further investigate the specificity of *Ppu*-SL2 for SL acceptor sites, we applied the same assay to a set of genes known to show a very high degree of specificity for the addition of either SL1 or SL2 SLs at their 5' ends. The *tct-1* and *rla-1* genes show close to 100% specificity for *trans*-splicing to *C. elegans* SL2, whereas the *rps-3* gene shows a similar degree of specificity for SL1 (T Blumenthal, pers. comm.). We were able to detect PCR products corresponding to the addition of *Ppu*-SL2 to both *tct-1* and *rla-1*, but not to *rps-3*, indicating that *Ppu*-SL2 was specifically added only to the 5' ends of SL2 *trans*-spliced transcripts (Fig. 2C). We also tested the addition of *Ppu*-SL2 to a pair of genes that form the upstream and downstream genes of an operon: *kup-1* and *pkc-1*, respectively (Spieth et al. 1993). Like most upstream genes, *kup-1* is predominantly *trans*-spliced to SL1, and we were unable to detect splicing of *Ppu*-SL2 to *kup-1* (Fig. 3). *Trans*-splicing of *pkc-1* occurs at two alternative acceptor sites, one of which splices mainly to SL2, whereas the other occurs largely via SL1. Consistent with our previous results, we only found addition of *Ppu*-SL2 to the SL2 acceptor site of *pkc-1* (Fig. 3).

Taken together, our results demonstrate that *Ppu*-SL2 RNA is able to participate in SL *trans*-splicing reactions in *C. elegans* with a specificity that indicates it interacts with

the SL2 splicing machinery of *C. elegans*. Thus, it seems likely that the sequence similarity between *Ppu*-SLs and the *C. elegans* SL2s is reflective of functional conservation.

DISCUSSION

The identification of SL *trans*-splicing in another member of a basal nematode clade reinforces the hypothesis that this process was present in the last common ancestor of all extant nematodes, and is consistent with the fact that it has been identified in all members of the phylum that have been characterized in molecular detail.

Our previous study showed that SL RNAs in *T. spiralis* exhibited a high degree of molecular heterogeneity and did not share strong sequence similarity to previously identified SL RNAs from the Rhabditida clade (Pettitt et al. 2008). Somewhat surprisingly, given that it is located in the same basal clade as *T. spiralis* (Fig. 1A), the SL RNAs that we have identified from *P. punctatus* do not closely resemble any of those from *T. spiralis*. Rather they show clear similarity to the SL2 family derived from the much more distantly related Rhabditina nematodes. Given the biased nature of the strategies that we employed to identify SLs following the identification of *Ppu*-SL1, it is very likely that we have only sampled a subset of the complement of SL RNA genes in this nematode. It is possible that the

FIGURE 3. *Ppu*-SL2 is specifically *trans*-spliced to the SL2 acceptor site of *pkc-1*. (A) Schematic showing the first (*kup-1*) and second (*pkc-1*) gene in operon CEOP5312 with SL1 and SL2 splice acceptor sites. Note that the SL2 acceptor site and the two first exons of *pkc-1* are not currently annotated in WormBase. (B) *Trans*-splicing of *Ppu*-SL2, SL1, and SL2 RNAs to *kup-1* and *pkc-1* in PE536 was detected using the combination of gene-specific primers with SL1, SL2, or *Ppu*-SL2 primers indicated. Control PCR amplifications were performed with mock cDNA preparations performed without reverse transcriptase (–RT). Amplification products were identified by DNA sequencing. The SL1 and SL2 acceptor sites (Δ) are indicated in the sequence of the *Ppu*-SL2/*pkc-1* fragment shown. The first two exons are boxed in gray; the following exons, in black. The beginning of the open reading frame is italicized. M indicates marker lane, in base pairs (bp).

P. punctatus SL RNA gene family shows much greater diversity than that represented by the genes we report here. However, we have carried out exhaustive searches of the *T. spiralis* draft genome and failed to identify any SL RNA genes that resemble those from *P. punctatus*. Thus it seems likely that there are differences in the complements of SL RNAs between these two nematodes, underlining the importance of investigating this process in multiple representatives of a given taxon.

The identification of *P. punctatus* SL RNAs further emphasizes the diversity of SL RNAs in *T. spiralis*. Similarly diverse SL RNAs have recently been found in other groups of animals (Marletaz et al. 2008; Derelle et al. 2010; Douris et al. 2010), and it seems that the high degree of sequence conservation found between the majority of known nematode SLs is atypical. Recent work has shown that there are sequence constraints in the majority of nematode SLs associated with efficient translation of tri-methyl guanosine-capped RNAs generated by SL *trans*-splicing (Wallace et al. 2010). While the *P. punctatus* SLs conform to these sequence constraints, this is not the case for most *T. spiralis* SLs, reflecting their greater molecular heterogeneity. Those *T. spiralis* SLs that do conform also stimulate translation in vitro, although with lower efficiency than *C. elegans* SL1 (Wallace et al. 2010). The functional significance of these observations is unclear. However, since the SL RNA complements from many nonnematodes are characterized by high sequence diversity, a consensus picture is emerging whereby the acquisition of SL RNA genes (via mutation of snRNA genes) is followed by gene duplication and rapid sequence evolution. If this is how SL *trans*-splicing has arisen, the situation in *T. spiralis* would appear to represent the preservation of the ancestral nematode SL RNA state. It will thus be important to obtain a broader sampling of *P. punctatus* SL RNAs to determine whether this nematode has more diverse SL RNAs of the kind found in *T. spiralis*.

The assignment of the *P. punctatus* SL RNAs to the SL2 family was initially based upon sequence similarity; however, the outcome of expressing one of the *P. punctatus* SL RNAs in *C. elegans* provides further support for this classification. The fact that *Ppu*-SL2 could be detected at the 5' ends of *C. elegans* mRNAs showed that the RNA could be recognized by the *C. elegans* *trans*-splicing machinery. More importantly, this SL RNA was detected only at the 5' ends of mRNAs derived from downstream genes in operons, indicating that it was specifically recognized by the SL2 splicing machinery, as predicted on the basis of sequence similarity.

We were unable to detect SL *trans*-splicing of *Ppu*-SL3.1 in similar assays in *C. elegans* and were also unable to detect the expression of this RNA in *C. elegans*, presumably because it was unstable. This indicates that there are functional differences between the *P. punctatus* SL RNAs. It is noteworthy that *Ppu*-SL3.1 does not contain elements in the third stem-loop essential for SL2 *trans*-splicing in *C.*

elegans (Evans and Blumenthal 2000) and so may not be capable of forming a ribonucleoprotein complex important for stability in *C. elegans*. This sequence is also lacking in most *T. spiralis* SL RNAs (it is only present in *tslr*-2, and closely related sequences are found in *tslr*-6, -11, and -12) (Pettitt et al. 2008), but this is not indicative of significant similarity between *Ppu*-SL3 RNAs and *T. spiralis* SL RNAs. Since *Ppu*-SL3.1 can be detected at the 5' ends of *P. punctatus* mRNAs, it is clearly able to form a functional ribonucleoprotein complex in that nematode.

The fact that at least one of the *Ppu*-SL RNAs can participate in SL2 *trans*-splicing in *C. elegans* suggests the possibility that these SL RNAs might also be involved in SL *trans*-splicing onto mRNAs derived from downstream genes in operons in *P. punctatus*. Operons have not been identified in any nematode that lies outside the Rhabditida group, although this may be a reflection of the relative paucity of molecular information available. The identification of SL2-like SL RNAs in *P. punctatus* offers a potential experimental route to identify and characterize putative operons in this phylogenetically important nematode.

Previous surveys of SL *trans*-splicing have indicated that the SL2 family is confined to the Rhabditina clade (Guiliano and Blaxter 2006), where it is associated with the SL *trans*-splicing of mRNAs derived from downstream genes in operons. In nematodes that lie outside the Rhabditina, although operons exist, the mRNAs derived from downstream genes are *trans*-spliced to SL1 and SL1-like SLs, and SL2 RNAs appear to be absent from several species for which draft genome sequences are available (Guiliano and Blaxter 2006). This led to the hypothesis that SL2 RNAs are an innovation specific to the Rhabditina. Our identification of a group of SL2-like SL RNAs in *P. punctatus* throws into question the hypothesis that the SL2 family is a relatively recent evolutionary acquisition. Based on our current understanding of the phylogenetic position of *P. punctatus* and Rhabditina nematodes, their last common ancestor was the founder of the nematode phylum. Thus the presence of SL2-like molecules in these distantly related nematodes opens up the possibility for a more ancient origin for this family. If this is the case, then there must have been multiple independent losses of SL2-like molecules to explain the current distribution of SL2 family members. Recent surveys of EST data from the nonrhabditine *Aphelenchus avenae* have identified SL2-like SLs, providing further evidence that this family is more broadly distributed than previously determined (Reardon et al. 2010).

An alternative explanation for the presence of the SL2-like SL RNAs in *P. punctatus* is that they were acquired via horizontal transmission from the rhabditine nematodes that likely constitute their prey in the wild. This would also explain their absence from *T. spiralis*. However, we are not aware of any examples of horizontal gene transfer between nematodes, but transfer of genes for plant cell wall degrading enzymes between bacteria and plant parasitic

nematodes is now accepted (Bird et al. 2009). It is also possible that the similarities between the *P. punctatus* SL2-like and *C. elegans* SL2s are due to independent, convergent events, although the constraints that might lead to the acquisition of similar sequences are unknown. Ultimately, however, it will be necessary to obtain a more comprehensive picture of the SL RNA complement in *P. punctatus*, and in other dorylaimid nematodes, in order to distinguish between these possibilities and to obtain a more complete picture of the evolution of nematode SL *trans*-splicing.

MATERIALS AND METHODS

Nematode culture

Rhabditis dolichura were grown and maintained on NGM agar seeded with a lawn of the *Escherichia coli* strain OP 50 and incubated at 20°C. *P. punctatus* was a gift from Gaetan Borgonie (Ghent University). *P. punctatus* was grown on soil agar prepared as described by Small and Evans (1981), supplemented with nystatin (62.5 KU/mL) and cholesterol (5 mg/mL). One day prior to the addition of *P. punctatus*, the prey nematodes, *R. dolichura*, were added to the soil agar plates. Incubation was at 20°C. Cultures were incubated for about 4–6 wk, and the adult *P. punctatus* were harvested by washing plates with M9 medium.

RNA isolation

Isolation of total RNA was carried out using Tri Reagent (Sigma), following a protocol described elsewhere (Keall et al. 2007).

Cloning of cDNAs and determination of cDNA ends

cDNA synthesis from *C. elegans* total RNA was carried out using either random hexamers or an oligo-dT primer and M-MLV reverse transcriptase. Cloning of *P. punctatus* cDNAs and determination of the 5' and 3' cDNA ends were done using total RNA and the GeneRacer kit (Invitrogen). The gene-specific primers used are given in Supplemental Table 2. cDNAs were amplified by PCR and cloned into pGEM T-Easy (Promega) or pCR4-TOPO (Invitrogen) plasmid vectors, and the resulting DNA inserts sequenced by the University of Dundee Sequencing Service. *P. punctatus* sequence data linked to this study have been deposited in GenBank with accession nos. GU936298–GU936311.

SL RNA 3' end mapping.

The *P. punctatus* SL RNA 3' ends were determined essentially as described previously (Pettitt et al. 2008).

Amplification of *P. punctatus* genomic DNA.

Normally, 1–10 animals were picked from soil agar plates and transferred into lysis buffer (20 mM Tris [pH 7.5], 50 mM EDTA, 200 mM NaCl, 0.5% SDS). Proteinase K (10 mg/mL) was then added to a concentration of 100 µg/mL, and the mixtures were incubated for 1 h at 60°C, followed by 15 min at 95°C. PCR was carried out with the primers given in Supplemental Table 2 using the Expand Long Template PCR Kit (Roche). For DNA sequencing, fragments were excised from agarose gels, isolated using the

QIAquick gel extraction kit (Qiagen), and cloned into pGEM-T easy (Promega) or pCR4-TOPO (Invitrogen).

Expression of a *P. punctatus* SL RNA in *C. elegans*

The *C. elegans* U2-3 promoter was amplified from wild-type genomic DNA using the primers KF99 and KF101 (all primers are listed in Supplemental Table 2). *Ppu*SL2_U and *Ppu*SL2_D are partially overlapping and encode the *Ppu*-SL2 RNA sequence. *Ce*SL13_U and *Ce*SL13_D are also partially overlapping and encode the 3' untranslated region (3'UTR) of the *C. elegans* SL1 gene. The construct composed of the *C. elegans* U2-3 promoter, *Ppu*-SL2 RNA, and the 3'UTR of the *C. elegans* SL1 gene was created in a single PCR reaction utilizing the oligonucleotides KF99 and *Ce*SL13U-pr. The resulting product was cloned into pGEM-T easily and sequenced to verify that it contained no mutations. The plasmid was co-injected with pTG96 to generate transgenic *C. elegans* strains using standard procedures (Mello and Fire 1995). Multiple lines were obtained, and two (PE535 and PE536) were chosen for RT-PCR analysis. After RNA preparation and reverse transcription, *trans*-splicing was analyzed by PCR using combinations of SL-specific and gene-specific primers (Supplemental Table 2). PCR reactions were analyzed by agarose gel electrophoresis and the results were visualized by staining with ethidium bromide. The grayscale pictures in Figures 2 and 3 are inverted for increased clarity.

SUPPLEMENTAL MATERIAL

Supplemental material can be found at <http://www.rnajournal.org>.

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NOTE ADDED IN PROOF

We have identified additional upstream sequences of the *Ppu-vha-1* gene including a typical *trans*-splice acceptor site at the correct location (GenBank entry GU936299) (see Supplemental Table 3).

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