An in vivo genetic screen for genes involved in spliced leader trans-splicing indicates a crucial role for continuous de novo spliced leader RNP assembly

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ABSTRACT

Spliced leader (SL) trans-splicing is a critical element of gene expression in a number of eukaryotic groups. This process is arguably best understood in nematodes, where biochemical and molecular studies in Caenorhabditis elegans and Ascaris suum have identified key steps and factors involved. Despite this, the precise details of SL trans-splicing have yet to be elucidated. In part, this is because the systematic identification of the molecules involved has not previously been possible due to the lack of a specific phenotype associated with defects in this process. We present here a novel GFP-based reporter assay that can monitor SL1 trans-splicing in living C. elegans. Using this assay, we have identified mutants in sna-1 that are defective in SL trans-splicing, and demonstrate that reducing function of SNA-1, SNA-2 and SUT-1, proteins that associate with SL1 RNA and related SmY RNAs, impairs SL trans-splicing. We further demonstrate that the Sm proteins and pCln, SMN and Gemin5, which are involved in small nuclear ribonucleoprotein assembly, have an important role in SL trans-splicing. Taken together these results provide the first in vivo evidence for proteins involved in SL trans-splicing, and indicate that continuous replacement of SL ribonucleoproteins consumed during trans-splicing reactions is essential for effective trans-splicing.

INTRODUCTION

A wide range of eukaryotes engage in the trans-splicing of their pre-mRNAs, a process which results in the replacement of the 5′-end of the transcript with a short, ‘spliced leader’ (SL) (1,2). The essential mechanism of SL trans-splicing involves an intermolecular splicing event between the donor molecule, the SL RNA and the acceptor pre-mRNA, which possesses an intron-like 5′ untranslated region (5′ UTR), containing an unpaired 3′ splice site, termed the ‘outron’. However, most organisms that possess SL trans-splicing also contain a sub-set of genes organized into operons, consisting of several genes under the control of a shared promoter. Operons thus produce polycistronic transcripts, which are resolved into monocistronic mRNAs through SL trans-splicing coupled to 3′ end formation.

SL trans-splicing occurs on approximately 70% of mRNAs in C. elegans, including those that are derived from polycistronic transcripts (3). C. elegans uses two classes of SLs, SL1 and SL2, the former being trans-spliced to pre-mRNAs produced by monocistronic genes and to the pre-mRNAs derived from the first genes in operons (4). In contrast, SL2 is spliced exclusively to pre-mRNAs that are derived from downstream genes in operons (5). SL1 is essential, but lack of SL1 can at least in part be complemented by overexpression of SL2 RNA (6), presumably because they participate in fundamentally similar splicing events and the end products of these events are functionally similar. However, features that distinguish SL1 and SL2 trans-splicing have been defined, making it clear that these are distinct events (7–9).

SL RNAs form SL ribonucleoprotein particles (RNPs) and share some properties with the small nuclear RNPs (snRNPs) involved in cis-splicing reactions, in that they

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adopt similar secondary structures and are bound by proteins recognized by anti-Sm antibodies (10–13).

Additional proteins associated with SL RNAs were isolated from *Ascaris* splicing extracts, and homologs of these were identified in *C. elegans* (14,15). *C. elegans* SNA-2 was identified as the ortholog of the 95 kDa *Ascaris* protein, and was found to interact with SL1, but not SL2. The smaller, SL-30p *Ascaris* protein, was shown to have two *C. elegans* homologs, SNA-1 and SUT-1, with the former also interacting with SL1, while the latter was found to interact with a novel, nematode-specific class of snRNA termed SmY RNA (15,16). Subsequent work has shown that SNA-1 and SUT-1 are conserved throughout the Rhabditida (17). RNAi knockdown of sna-1 or sut-1 causes cold-sensitive sterility, and loss of both sna-1 and sut-1 function results in synthetic defects in viability, despite the fact that they show distinct biochemical properties (15).

To account for this MacMorris and colleagues have proposed that SNA-1 and SUT-1, together with SNA-2, associate with SL1 and SmY RNP complexes, respectively, and may function to recycle Sm proteins following trans-splicing (15). This hypothesis is based on the fact that SL trans-splicing consumes SL RNPs by transfer of the 5′ terminal spliced leader sequence onto mRNAs. According to this model, SNA-1 is involved in the recycling of Sm proteins following SL1 trans-splicing and thus loss of SNA-1 is compensated for by the presence of the other SUT-1/SmY-dependent Sm protein recycling pathway. Similarly, loss of SUT-1 would be tolerated because of the presence of the SNA-1/SL1 Sm recycling pathway. Loss of both SNA-1 and SUT-1 would compromise both pathways and thus results in a lethal loss of Sm protein recycling. Consistent with this model, loss of SNA-2, an essential component of both complexes, is lethal (15).

While this model explains the available molecular and genetic evidence, these novel proteins have not been directly shown to be required for SL trans-splicing in vivo. To better characterize SL trans-splicing we have designed a novel GFP reporter-based assay allowing us to genetically identify factors involved in this process in living *C. elegans*. We show that depletion of sna-1, sna-2 and sut-1 impairs SL trans-splicing in vivo and have identified new loss-of-function alleles of sna-1 from a genetic screen using our assay. We also show that depletion of Sm protein expression, and factors involved in snRNP assembly, leads to reduced SL trans-splicing. A role for these factors in SL trans-splicing was confirmed by analyzing SL trans-splicing of the GFP reporter gene transcripts, and of an endogenous trans-spliced RNA, by qPCR. To our knowledge, our molecular analysis demonstrates for the first time in a living system a role for these factors in SL trans-splicing. SL RNPs consumed during trans-splicing need to be replaced and our observations imply that the synthesis of new Sm proteins makes a significant and crucial contribution to the pool of Sm proteins available for the assembly of new SL RNPs.

**MATERIALS AND METHODS**

**Strains**

Nematodes were maintained using standard protocols at 20°C on *Escherichia coli* B strain OP50 unless otherwise stated (18,19). The following strains were used: N2 (Bristol); PE720 [feX308]; PE774 [sna-1(fe45) V; feEx308]; PE766 [sna-1(fe47) V; feEx308]; PE793 [feIs11 X]; FX3079 [sut-1(tm3079) II]. Transgenic genotypes are as follows: feEx308 is an extrachromosomal array consisting of [P*<em>vit-2</em>:outtron::gfpm10A P<em>myo-3</em>:<em>mCherry</em>]. ('gfpm10A' indicates that the initiator methionine of GFP has been changed to an alanine codon); feIs11 was generated by γ-ray-mediated integration of the feEx308 array into the X chromosome.

**Construction of SL trans-splicing reporter transgene and generation of transgenic strains**

We amplified the *vit-2* promoter (*P*<em>vit-2</em>) from wild type (wt) genomic DNA and inserted the resulting amplicon into BamHI-XbaI cut pPD95.75 (gift from A. Fire, Stanford School of Medicine; available from Addgene; plasmid #1494) to give pAWF29B. A synthetic ‘OU141’ outron based on previous studies (20) was assembled from oligonucleotides with overlaps filled in by polymerase chain reaction (PCR), and inserted into pGEM-T Easy (Promega). The resultant plasmid was digested with AgeI and BamHI and the OU141 outron insert was cloned into AgeI-BamHI cut pAWF29B to produce pAWF30. The GFP initiation codon was changed to an alanine (GCT) by site directed mutagenesis using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs), generating pLP6. The plasmid was sequenced to ensure no additional mutations were induced during the various cloning steps. This revealed that the OU141 outron sequence, which contained four copies of a 34 bp synthetic sequence (20) had undergone a truncation in pLP6 leaving only three copies (Supplementary Figure S2).

pLP6 was co-injected with pCFJ104, which expresses mCherry in body wall muscles (21). Five transgenic lines were generated, and all behaved similarly. One line, containing the extrachromosomal array feEx308, was selected for further study. The extrachromosomal array was integrated by subjecting transgenic animals to 3800 rad γ irradiation for 20 min (22). Four independent strains (feIs11-14) were recovered and backcrossed to wt six times. feIs11 was mapped to the X chromosome and chosen for use in this study.

**Spliced leader trans-splicing reporter construct genetic screen**

A population of PE720 worms were subject to ethyl methanesulfonate (EMS) mutagenesis (23) and allowed to become gravid adults. F1 progeny were picked singly to separate plates and their progeny screened using a stereomicroscope equipped with an epifluorescent light source for the presence of intestinal GFP expression. Individual fluorescent animals were then picked to fresh plates and their progeny scored to confirm the intestinal GFP expression phenotype.

Each mutant line was outcrossed with wt males and non-transgenic cross-progeny males crossed with unmutagenized PE720 hermaphrodites to ensure that the phenotype was not associated with changes to the transgenic array, and to determine the inheritance pattern of the mutation. Mutations in sna-1 were identified by PCR amplification of exons.
Epifluorescence microscopy

To determine the proportion of animals expressing GFP, ~50 eggs were seeded on 3 cm RNAi plates with E. coli K-12 derived HT115(DE3) transformed with the appropriate feeding plasmid and allowed to grow at 20°C for 3 days to L4/adult stage. The total number of hatched animals and the number of GFP fluorescent animals were determined using a Leica MZ16F fluorescent stereomicroscope, and used to calculate the proportion of GFP expressing animals.

To measure the intensity of GFP fluorescence, 10 animals were immobilized on slides with 1% agarose cushions, using 0.5% l-phenoxy-2-propanol as an anaesthetic, covered with a cover slip and sealed with Vaseline. A Zeiss Axioplan 2 fluorescence microscope was used to capture GFP and mCherry fluorescence for each animal. ImageJ software (24) was used to analyze the intensity of GFP and mCherry fluorescence. The GFP fluorescence intensity of each animal was normalized with respect to its mCherry fluorescence intensity. Statistical analysis by One-way ANOVA on ranks followed by Dunn’s post-test was done using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com.

RESULTS AND DISCUSSION

An in vivo assay to detect loss of SL1 trans-splicing

We generated a transgenic strain that would display GFP fluorescence dependent on suppression of SL trans-splicing (Figure 1A). The transgene consists of a modified GFP gene that is under the transcriptional control of the vit-2 promoter (20). The GFP gene was modified such that the original ATG initiation codon was changed into an alanine codon, GCT (GFPM1A). Upstream of this we engineered a synthetic outron sequence, which has previously been shown to confer on a pre-mRNA the ability to act as SL1 trans-splicing substrate (20). We placed a new ATG codon in-frame with the GFP coding region upstream of the 3’ splice site. Thus, in wild type worms, the removal of the synthetic outron from the pre-mRNA during SL trans-splicing would be expected to produce an mRNA lacking an AUG, and so no GFP fluorescence would be observed, whereas in animals with reduced SL trans-splicing, mRNA retaining the outron and its associated in-frame AUG would be predicted to accumulate, and thus allow GFP expression (Figure 1A). As predicted, transgenic lines established with this Pun-2::outron:GFPM1A construct displayed only sporadic, weak fluorescence (Figure 1B–D: unc-22(RNAi)).

To test the response of the transgenic strain to loss of SL trans-splicing, we carried out RNA interference (RNAi) targeted against sna-1 and sna-2, since these genes encode known components of the SL RNP, and sut-1, because of its genetic interaction with sna-1 and proposed role in Sm protein recycling (15). We observed a strong GFP expression response in animals subjected to sna-1(RNAi) and sna-2(RNAi), indicating that our assay is responding as expected (Figure 1B–D). We observed GFP expression in more than 60% of sut-1(RNAi) treated animals. While fluorescence was clearly detectable, it was weak and not significantly above the background values obtained in unc-22(RNAi) controls (Figure 1B–D), largely because typically relatively few intestinal cells displayed fluorescence in sut-1(RNAi) animals. To confirm that GFP expression correlates with retention of the outron in the GFP mRNA we used qRT-PCR to measure the levels of non-trans-spliced outron sequences, and of an internal part of the reporter gene transcript not involved in SL trans-splicing. Relative trans-splicing efficiencies were calculated by standardizing the levels of outron containing transcripts with respect to the internal fragments, which allows a direct comparison between different RNAi treatments (Figure 1E). This was complemented by the analysis of the SL trans-splicing of endogenous rps-3 transcripts, which are known to be trans-spliced by SL1 (3) (Figure 1F). In animals treated with sna-1(RNAi) and sna-2 (RNAi), the levels of non-trans-spliced GFP reporter transcripts (from here on referred to as outron-gfp RNA) were significantly higher than in the unc-22(RNAi) treated control animals, reflecting the increase in GFP expression observed (Figure 1B–D). Furthermore, the levels of non-trans-spliced rps-3 RNA (from here on referred to as outron-rps-3 RNA) were elevated in sna-1 and sna-2 (RNAi) treated animals (Figure 1F), indicating that the knockdown of these genes also interferes with the trans-splicing of endogenous transcripts. In animals sub-
Figure 1. A novel reporter gene assay for the in vivo detection of reduced SL1 trans-splicing. (A) The \(P_{\text{vit-2}}\)::\(\text{outron}::\text{gfp}\)^{M1A} transgene engineered to monitor SL1 trans-splicing. The original ATG of the GFP open reading frame (yellow shading) was changed to a GCT alanine codon. Note the \(\text{gfp}\) gene contains three introns. The two mRNA products produced in the presence, or absence of SL1 trans-splicing are shown below, and a cartoon of the predicted GFP expression in wild-type (wt) worms (green shading indicates intestinal GFP fluorescence). (B) Detection of SL1 trans-splicing inhibition by fluorescence microscopy. Micrographs of GFP expression in animals carrying the \(P_{\text{vit-2}}\)::\(\text{outron}::\text{GFPM1A}\) transgene subjected to \(\text{sna-1, sna-2, sut-1 or unc-22\,(RNAi)}\). Exposure times were identical for all micrographs (5 ms). The scale bar corresponds to 100 \(\mu\)m. (C and D) Quantitation of GFP expression in animals carrying the \(P_{\text{vit-2}}\)::\(\text{outron}::\text{GFPM1A}\) transgene subjected to \(\text{sna-1, sna-2, sut-1 or unc-22\,(RNAi)}\). (C) The proportion of GFP fluorescent animals was de-
jected to sut-1 (RNAi) we were not able to detect a significant increase of outron-gfp transcripts, reflecting the weak activation of the reporter observed by microscopy (Figure 1B-D). However, the level of outron-rps-3 transcripts was elevated, consistent with a role of sut-1 in SL trans-spooling.

As further support for the role of SNA-1 in SL trans-spooling, we identified two loss-of-function sna-1 mutations from a genetic screen for PE720 worms that displayed elevated levels of GFP fluorescence. The fe45 allele is a C to T transition on chromosome V at position 6,710,203, creating a premature stop codon at codon 34 (CAA > TAA, Q34STOP) and fe47 is a C to T transition on chromosome V at position 6,710,203, creating a premature stop codon at codon 50 (GGA > TGA; W50STOP) (Figure 2). Detailed analysis of GFP fluorescence was carried out for sna-1 (fe47) homozygotes (Figure 2B). In addition, the levels of outron-gfp and outron-rps-3 transcripts were more than 15-fold higher than in wild type animals (Figure 2C and D), confirming the inhibition of SL trans-spooling in these animals.

Although the two sna-1 alleles that we isolated are viable, previous studies have shown that sna-1 (RNAi) results in cold-sensitive sterility defects and that combined loss of sna-1 and sut-1 function results in embryonic lethality (15). We replicated these observations with the sna-1 (fe47) allele, demonstrating that it confers cold-sensitive defects in viability, similar to the sut-1 (tm3079) allele (Figure 2E). Furthermore, sut-1; sna-1 double mutants are sickly, with the majority (92.5%; n = 40) being completely sterile.

In conclusion, these experiments clearly demonstrate that reduced expression of sna-1 or sna-2 interferes with SL trans-spooling. The genetic interaction of sna-1 with sut-1, combined with the weak disturbance of SL trans-spooling by sut-1 depletion supports a function of sut-1 in SL trans-spooling. It has been demonstrated that SUT-1 interacts with SmY RNAs (15), and this provides further support for a functional role of SmY RNAs in SL trans-spooling. Taken together, our data demonstrate a role for these molecules in SL trans-spooling, as proposed in earlier molecular studies.

Previous biochemical analyses of SL RNPs using anti-Sm antisera imply that SL RNA is associated with Sm proteins (10,13,15). However, since the post-translational modification of Sm proteins, symmetrical dimethylation of arginines, is the major epitope for anti-Sm antibodies (28), it is possible that other proteins containing this epitope, such as Lsm4 or piRNA associated Mili protein, might explain the ability of anti-Sm antisera to interact with SL RNPs (29,30). It was therefore important to directly test the role of Sm proteins in SL trans-spooling.

Sm proteins form heptameric rings that bind to a variety of snRNAs and are found in snRNPs acting in, for example, mRNA splicing. The major U1, U2, U4 and U5 snRNAs associate with a ring formed by the Sm proteins B, D1, D2, D3, F, E and G. In C. elegans, these proteins are encoded by snr-1, snr-2, snr-3, snr-4, snr-5, snr-6 and snr-7, respectively (31). In addition, the related LSm proteins form heptameric rings that interact with other RNAs (32). U6 snRNA, involved in cis-splicing is bound by a ring formed from LSm2, 3, 4, 5, 6, 7, 8 proteins, which in C. elegans are encoded by the gut-2, lsm-3, lsm-4, lsm-5, lsm-6, lsm-7 and lsm-8 genes, respectively. A similar ring where mammalian LSm8 is replaced by LSm1 (encoded by lsm-1) is involved in the degradation of mRNA (32). Using similarity searches we also identified the genes K07A1.15, C49H3.4 and M142.5 as encoding proteins with Sm-like domains (33). M142.5 was identified by others as an orthologue of human LSm12 (34), while K07A1.15 is annotated in Wormbase as the orthologue of human LSm10. LSm10 protein associates with LSm11 and Sm proteins B, D3, G, E and F to form the U7 snRNP particle involved in histone RNA processing (35,36). Drosophila LSm10 and LSm11 mutants die as pupae (37), indicating that these functions are essential. Surprisingly, no phenotype has been attributed to K07A1.15 loss-of-function (33,38,39). We thus wanted to determine the effect of knocking down the expression of Sm and LSm protein function on GFP fluorescence in our reporter strain, as well as on the levels of outron-gfp and outron-rps-3 transcripts.

Figure 3 shows that the knockdown of snr gene transcripts led to GFP fluorescence in all, or nearly all animals tested, with a more than seven-fold increase of fluorescence compared to the unc-22 (RNAi) treated control animals (Figure 3A and B). This was accompanied by five-fold or higher increase of outron-gfp transcripts in animals subjected to snr (RNAi) (Figure 3C). Importantly, snr (RNAi) also impaired trans-splicing of rps-3 transcripts (Figure 3D). The effect on these transcripts appeared more severe than the effect on the reporter gene transcripts, with up to more than 100-fold higher levels of non-trans-spliced transcripts detected in snr-5 and snr-6 (RNAi) treated animals compared to the control treatment.

In contrast to the knockdown of Sm protein transcripts, knockdown of each of the eleven LSm protein transcripts failed to cause notable GFP fluorescence (Figure 3A and B). A low proportion of animals, not exceeding 10%, expressed GFP at a detectable level. This is distinct from the effect of sna-1 and sna-2 (RNAi), and the knockdown of snr RNAs, which results in clearly detectable GFP fluorescence in all

terminated by fluorescence microscopy. Error bars represent the standard deviation based on between two and five independent experiments (unc-22/RNAi): 2; sna-1/RNAi, sna-2/RNAi; 3; sut-1/RNAi; 5). The total number of animals analyzed is indicated at the top of the graph. (D) The intensity of GFP fluorescence (in arbitrary units) was determined from micrographs shown in (B), and standardized with respect to mCherry fluorescence. The graph plots the GFP fluorescence of 10 animals analyzed for each RNAi treatment; shown are the median and the first and third quartile. ‘ns’ indicates values not significantly higher than in unc-22 (RNAi) animals (P > 0.05; ANOVA). (E and F) Detection of SL1 trans-splicing inhibition by RT-qPCR. Animals carrying the Pvt-2::outron::GFP M1A transgene were subjected to sna-1, sna-2, sut-1 or unc-22 (RNAi), and trans-splicing of gfp reporter gene and rps-3 transcripts was analyzed by reverse transcription followed by qPCR. The schematic diagrams indicate the position of the primers used (drawings are not to scale). Outron sequences are shown in blue and the SL1 acceptor sites in purple. Outron-gfp and outron-rps-3 RNA levels were standardized with respect to an internal part of the mRNA (internal) and levels in unc-22 (RNAi) animals were defined as 1. Note that elevated outron-gfp or outron-rps-3 levels indicate inhibition of SL trans-splicing. ‘ns’ indicates values not significantly higher than in unc-22 (RNAi) animals (P > 0.05, t-test). Error bars show standard deviation from three technical replicates. Similar results were obtained in two independent experiments.
Figure 2. Loss-of-function mutations in sna-1 recovered from a mutagenesis screen for genes involved in SL1 trans-splicing. (A) Schematic representations of the location of the residues affected by the mutations in the SNA-1 protein. The regions in dark gray are conserved in nematode SNA-1 orthologues (Supplementary Figure S1). (B) Representative images of wild type (wt) and sna-1(fe47) animals transgenic for Pvit-2::outron::gfpM1A, showing intestinal GFP (green) expression and constitutive mCherry (red) expression in body wall muscle cells. Scale bar represents 100 μm. The graph plots the GFP fluorescence of 10 animals analyzed; shown are the median and the first and third quartile. (C and D) RT-qPCR analysis of SL1 trans-splicing in wt and sna-1(fe47) animals carrying the Pvit-2::outron::gfpM1A transgene. Trans-splicing of gfp reporter gene (C) and rps-3 transcripts (D) was analyzed as described in the legend of Figure 1, with levels in wt animals set as 1. Error bars indicate standard deviation from three technical replicates. (E) Viability of wt, sna-1(fe47) and sut-1(tm3079) animals at 20 and 15°C, as expressed by the proportion of eggs that develop to L4/adult stage in 5–10 independent experiments. (***P ≤ 0.001; ‘ns’ not significant; ANOVA). Note that while at 20°C there is a slight reduction in viability in sut-1(tm3079) animals, this is not significantly different from the viability of sna-1(fe47) animals.

Our findings indicate that SL1 trans-splicing is highly sensitive to the knockdown of the genes encoding Sm pro-
Figure 3. SL1 spliced leader trans-splicing is sensitive to the knockdown of snr, but not lsm, gene transcripts. Detection of SL1 trans-splicing inhibition by fluorescence microscopy. Quantitation of GFP expression in animals carrying the P_{vit-2}:outron::GFP^M1A transgene subjected to RNAi was done as described for Figure 1. (A) The proportion of GFP fluorescent animals. The number of animals analyzed are indicated above each dataset. (B) GFP fluorescence intensity. The graph summarizes individual measurements (n = 30) from three independent experiments. The distribution of measurements for each of the experiments is similar to the distribution of the pooled data. Median and the first and third quartile are indicated. ‘ns’ indicates values not significantly higher than in unc-22(RNAi) animals (P ≥ 0.05; ANOVA). (C) and (D) Detection of SL1 trans-splicing inhibition by RT-qPCR. Animals carrying the P_{vit-2}:outron::GFP^M1A transgene were subjected to RNAi, and trans-splicing of the gfp reporter gene (C) and rps-3 transcripts (D) was analyzed by reverse transcription followed by qPCR. Outron-gfp and outron-rps-3 transcript levels were standardized as described in the Figure 1 legend, and levels in unc-22(RNAi) animals were defined as 1. Note that snr-7(RNAi) was not analyzed by RT-qPCR. ‘ns’ indicates values not significantly higher than in unc-22(RNAi) animals (P ≥ 0.05; t-test). Error bars show standard deviation from three technical replicates.

proteins: the knockdown of snr genes, similar to the knockdown of sna-1 and sna-2, results in strong GFP fluorescence in the majority or all animals subjected to the RNAi treatments. This is supported by the significant increase of non-processed outron-gfp and outron-rps3 RNA in these animals, which also indicates that trans-splicing is impaired. In contrast, lsm gene knockdown only results in weak GFP fluorescence in a small number of the treated animals. The molecular analysis of trans-splicing confirms that any GFP fluorescence detected is due to a defect in trans-splicing. Together these data indicate that SL trans-splicing is highly sensitive to the knockdown of the genes encoding Sm proteins, while the knockdown of genes encoding LSm proteins has no consistent detectable effect.

Since the Sm proteins are also required for assembly of cis-splicing U snRNPs, one possible interpretation is that the loss of these factors, all of which apart from U1 are required for SL trans-splicing (40,41), could contribute to the impaired SL trans-splicing that we observe. However, two observations suggest that the depletion of Sm proteins primarily impacts upon SL RNP function in our experiments. First, the removal, by cis-splicing, of the three GFP transgene introns, two of which contain stop codons, is required for fluorescence, implying that cis-splicing factors are not significantly impaired by Sm protein depletion. Second, depletion of multiple LSm proteins, which are required for U6 snRNP formation, has minimal effects on SL trans-splicing, despite the fact that U6 is required for trans-splicing (42). Thus, the most likely explanation for the impaired SL trans-splicing in animals depleted for Sm proteins is due to the effect of this loss on the formation of the SL RNP. This raises the question: why would cis-splicing U snRNPs be less sensitive to Sm protein depletion than SL RNPs? One explanation may be that the latter are consumed by the splicing reaction in which they participate, and thus SL trans-splicing is dependent on the continuous formation of new SL RNPs. As described earlier, it has been proposed that Sm protein recycling contributes to the formation of new SL
Figure 4. Knockdown of snRNP assembly factors inhibits SL1 spliced leader trans-splicing. Detection of SL1 trans-splicing inhibition by fluorescence microscopy. Quantitation of GFP expression in animals with the Pvit-2::outron::GFP<sup>M1A</sup> transgene subjected to RNAi was done as described for Figure 1. (A) The proportion of GFP fluorescent animals. The graph summarizes results from two or five independent experiments (unc-22(RNAi): 2; smn-1(RNAi), smi-1(RNAi), ieh-1(RNAi): 5). The total number of animals analyzed is indicated. (B) GFP fluorescence intensity. The graph shows measurements of fluorescence intensity of 10 animals analyzed for each RNAi treatment. ns indicates values not significantly higher than in unc-22(RNAi) animals (P ≥ 0.05, ANOVA). (C and D) Detection of SL1 trans-splicing inhibition by RT-qPCR. Animals carrying the Pvit-2::outron::GFP<sup>M1A</sup> transgene were subjected to RNAi, and trans-splicing of the gfp reporter gene (C) and rps-3 transcripts (D) was analyzed by reverse transcription followed by qPCR. Outron-gfp and outron-rps-3 transcript levels were standardized as described in the Figure 1 legend, and levels in unc-22(RNAi) animals were defined as 1. "ns" indicates values not significantly higher than in unc-22(RNAi) animals (P ≥ 0.05, t-test). Error bars show standard deviation from three technical replicates.
RNPs (15). Our findings indicate that this is not sufficient, and that the formation of new SL RNPs is heavily reliant on the synthesis of new Sm proteins. An alternative, but not mutually exclusive, explanation is that SL RNA is stabilized by the integration into RNPs and is therefore sensitive to a reduction in RNP components.

Recycling of the snRNPs after completion of cis-splicing is an important, but poorly understood process. Investigations have mainly focused on the U6 snRNP, which undergoes major changes during the cis-splicing reaction and is associated with LSm protein (43,44). Our findings are compatible with a model where U6 snRNA and associated LSm proteins participating in SL trans-splicing are recycled, and do not require continuous replacement.

To determine whether factors involved in snRNP assembly also play a role in SL trans-splicing, we knocked down the expression of smn-1, smi-1, icln-1 by RNAi. smn-1 encodes a homolog of human SMN and smi-1 an ortholog of human Smn2 that interacts with SMN, and, together with SMN, is a component of the SMN complex (45). This complex mediates the specific assembly of the heptameric Sm protein ring onto snRNAs and interacts with both Sm proteins and snRNAs (46). icln-1 encodes a homolog of the human methylosome subunit, pICln. Sm proteins, B/B’, D1 and D3 are modified by symmetrical dimethylation of arginine residues by a complex containing pICln, which promotes the interaction with the SMN complex for snRNP assembly (29,47,48).

Knockdown of smn-1, smi-1 and icln-1 led to GFP fluorescence in most of the animals subjected to RNAi, similar to the knockdown of sna-1, sna-2 and the sn genes described above (Figures 1C and D, 3A and B), with icln-1 (RNAi) giving the strongest effect (Figure 4A and B). The inhibition of SL trans-splicing by smi-1 and icln-1 knockdown was confirmed by the increased levels of outron-gfp and outron-rps-3 transcripts (Figure 4C and D), indicating that the knockdown of these genes inhibits SL trans-splicing. smn-1 knockdown caused a weaker effect: while reporter gene activation was detected in the majority of animals, the fluorescence was not strong enough to produce a significant difference in the quantitative comparison to unc22 (RNAi) animals (Figure 4A and B). However, the increase of non-processed outron-gfp RNA in smn-1 (RNAi) animals indicates that trans-splicing is impaired. These data suggest that the same biogenesis factors involved in snRNP assembly are required for these processes during the lifecycle of SL RNPs, further reinforcing the similarities between the U snRNPs and SL RNPs.

In conclusion, we have developed an in vivo assay that allows us to monitor the levels of SL trans-splicing in C. elegans, and have confirmed that there is a strong correlation between GFP fluorescence and outron-retention in SL trans-spliced mRNAs. This has allowed us to demonstrate physiological roles in SL trans-splicing for SNA-1, SNA-2 and SUT-1, and for Sm proteins and snRNP assembly factors. Our findings indicate that Sm protein recycling is not sufficient to replace the SL RNPs used up during trans-splicing, but that the assembly of new SL RNPs is heavily reliant on the synthesis of new Sm proteins. Furthermore, we have shown that this assay system can be employed to identify mutations in genes specifically involved in SL trans-splicing. Further application of such screens, including searches for lethal mutations, should allow the comprehensive identification of molecules involved in this fundamental nematode process. Finally, we envisage that this will be useful as a bioassay in the search for compounds that inhibit SL trans-splicing.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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