NOTE

SmD1 Is Required for Spliced Leader RNA Biogenesis

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Spliced leader RNA plays a central role in kinetoplastid gene expression, as it is trans-spliced onto every nucleus-derived mRNA. Spliced leader RNA maturation is a multistep process involving precursor 3′-end methylation (16, 18), 5′-end methylation (2), pseudouridylation of U229 (ψ229) (8), and trafficking to the cytoplasm mediated by exportin 1 (21). The details of spliced leader RNA nuclear import have not yet been described, and the order and intracellular locations of the various modifications have not been defined unambiguously.

A key structural element of spliced leader RNA is the Sm-binding site (11, 16). RAU 4-6GR, that is also present on many of the small nuclear RNAs (snRNAs) involved in splicing. The Sm-binding site interacts with a heptameric complex of Sm proteins (20). The mammalian U1 snRNP is assembled via SmD1 is necessary for accurate 3′-end formation. In L. tarentolae, spliced leader RNA Sm-binding site mutants lose 3′-end formation, accumulating in the form of primary transcription products with staggered 3′ ends (18). The Sm-protein complex has been implicated in nuclear import of trafficked snRNAs. Thus, the 3′-end phenotype and subcellular localization of spliced leader RNA in the SmD1 protein in the induced RNAi cell line were examined.

High-resolution RNA blots were hybridized initially with a stem-loop I probe (5′-TCATGGGAGCTTCTCATCA; Fig. 2A), which yielded a complex pattern of bands ranging from 3′-extended products to transcripts below mature spliced leader RNA size. In the uninduced RNA population, mature spliced leader RNA was identified as the band at 142 nucleotides. Based on 5′-end determination by primer extension (data not shown and Fig. 2C), higher-molecular-weight bands represent intermediates at various stages of 3′-end extension (21). In RNA from tetracycline-induced cells, 3′-extended forms of spliced leader RNA were detected, as were additional longer forms. A major spliced leader RNA band was present at 141 nucleotides with greater relative abundance than the uninduced RNA sample, and minor products were present at single-nucleotide intervals above and below the major band. Two lower-molecular-weight bands migrating at approximately 115 to 117 nucleotides were prominent in the induced RNA.
Hybridization of the blot for the splicedosomal U4 snRNA (21), which contains an Sm-binding site, showed a twofold reduction in induced cells, consistent with a stability defect in the absence of SmD1 protein; no 3’-extended forms of U4 snRNA were detected. Splicedosomal U2 snRNA, which also contains an Sm-binding site, was detected by the oligonucleotide 5’-TATCAGGAGTACTCTGATAAGAACA, revealing equivalent levels in both uninduced and induced cell lines and no longer forms. The unaltered U2 snRNA level is consistent with normal core U2 snRNP assembly in U2 Sm-binding site no longer forms. The unaltered U2 snRNA level is consistent with normal core U2 snRNP assembly in U2 Sm-binding site evident.

The accumulation of spliced leader RNA relative to U2 snRNA may be due to the hundredfold difference in gene copy number (4, 19) coupled with different rates of transcription (14), variable half-lives, and loss of consumption of spliced leader RNA in the trans-splicing reaction. As a substrate in trans-splicing, spliced leader RNA has a shorter half-life than the other splicedosomal components, estimated to be between 3 min (7) and 1.4 h (5) in T. brucei.

SmD1 depletion results in cytoplasmic accumulation of spliced leader RNA. The distribution of spliced leader RNA was analyzed by fluorescence in situ hybridization in the induced SmD1 RNAi cell line. In the absence of the SmD1 mRNA, spliced leader RNA signal became more intense throughout individual cells in an increasing percentage of the population over time (14% of cells at 24 h and 25% at 48 h), consistent with the increased abundance of spliced leader RNA observed in RNA blots. Higher magnification showed that spliced leader RNA was overaccumulating in the cytoplasm (Fig. 2B). The relative abundance and distribution of U3 snoRNA, which does not contain an Sm-binding site, were unchanged in the induced cells. All directly compared images were captured at the same shutter speed and processed in parallel. We have demonstrated previously that nuclear spliced leader RNA signal is not due to DNA or cytosolic signal to mRNA or autofluorescence (21).

These results demonstrate overaccumulation of spliced leader RNA in SmD1 T. brucei knockdown lines, consistent with the molecular phenotype of the Sm-binding site mutations in the L. tarentolae spliced leader RNA (16). Substrate spliced leader RNA overaccumulation in the cytoplasm indicated that the SmD1 protein is necessary for spliced leader RNA maturation prior to nuclear import; additional Sm-dependent spliced leader RNA modifications may be required as import signals.

Exon pseudouridylation occurs independent of Sm-binding site interaction. Although ψ28 of the spliced leader does not appear to be critical for trans-splicing (17) or association with polysomes (21), it may modulate or enhance mRNA interactions with the splicing or translation machinery. To determine whether pseudouridylation of spliced leader RNA is SmD1 dependent, total-cell RNA samples from induced and uninduced SmD1 RNAi lines were subjected to carboxymethyl cellulose treatment (1, 8). Irreversible carboxymethyl cellulose modification of ψ28 was visualized by primer extension with the oligonucleotide TBSL stem-loop 1 (Fig. 2C). As expected, RNA from uninduced cells yielded a termination at position 29 of the exon, indicative of ψ28 RNA from 48-h-induced cells also showed reverse transcriptase termination at this position, indicating that pseudouridylation was unimpaired in the absence of SmD1. An internal control for the integrity of RNA and efficacy of RNAi induction was apparent in the form of extension products terminating at the 5’ end of carboxymethyl cellulose-treated spliced leader RNA. In uninduced RNA, a fully modified cap 4 termination product was visible, whereas in induced RNA cap 0/cap 1 termination products were evident.

The localization of SLA1 RNA (8) combined with temporal and Sm-independent acquisition of the pseudouridylation of spliced leader RNA supports modification of ψ28 in the nucleus, most likely in the nucleolus, prior to cytosolic trafficking. However, the presence of SLA1 RNA in the nucleolus may be necessitated by its own processing requirements and does not preclude nucleoplasmic or cytosolic spliced leader RNA pseudouridylation. Furthermore, inhibition of cap 4 methylation in induced cell lines mirrored results obtained in Sm-binding site mutagenesis studies of spliced leader RNA in L. tarentolae and Leptomonas collosoma, in contrast to the cotranscriptional cap 4 acquisition model (10).

SmD1 defects define steps in the spliced leader RNA processing model. Our working model for biogenesis of kinetoplastid spliced leader RNA is as follows. Spliced leader RNA is transcribed as a 3’-extended precursor (18) and receives an m7G cap cotranscriptionally (10). The acquisition of ψ28 on spliced leader RNA is directed by SLA1 RNA (8) in the nucleus. The m7G cap of nascent spliced leader RNA is recognized by homologues of cap-binding complex and exported to the cytoplasm via a nuclear export complex containing exportin 1 (21). Sm-independent methylations to spliced leader RNA occur posttranscriptionally in the nucleus or cytoplasm. Spliced leader RNA is bound by Sm proteins in the cytosol, an interaction required for accurate 3’-end trimming and further Sm-dependent 5’-end methylation. Localization of these SmD1-dependent events has not been determined experimentally, but
they could occur in the cytoplasmic and/or nuclear compartments. Spliced leader RNA returns to the nucleus to participate in trans-splicing in an Sm complex-dependent manner. Several components of the nuclear import pathway have been described in eukaryotes. For snRNAs, binding of the Sm complex followed by acquisition of the m^2,2,7G cap, recognized by snurportin 1, creates the nuclear import signal (3). A similar pathway may function in the nuclear import of kinetoplastid U1, U2, and U4 snRNAs, which possess m^2,2,7G caps and Sm-binding sites. Spliced leader RNA may require an alternative nuclear import mechanism due to the absence of an m^2,2,7G cap. The location(s) of 5’ cap methylation acquisition has not been determined, and thus an intermediate cap structure may distinguish substrate spliced leader RNA that is destined to be imported into the nucleus from the fully mature cap 4 on trans-spliced mRNA in the cytoplasm. For translation, precursor spliced leader might not be recognized by cytosolic cap-binding proteins such as eIF4E, consist-

FIG. 2. Knockdown of SmD1 mRNA affects spliced leader RNA abundance, size, and subcellular distribution. (A) High-resolution 6% acrylamide–8 M urea gels were used to separate RNA transcripts to the single-nucleotide level. Blots were probed with spliced leader (SL) RNA, U2, and U4 snRNA probes. M, mature size (142 nucleotides). (B) Fluorescence in situ hybridization analysis for intracellular localization of spliced leader RNA with a fluorescein isothiocyanate-labeled probe was performed on uninduced (without tetracycline, –tet) and induced (+tet) cells at 48 h. Bar, 10 μm. U3 snoRNA was localized with a Tamara-labeled oligonucleotide probe; nuclear and kinetoplast DNAs were stained with DAPI. (C) ψ^28 occurs independent of SmD1. ψ^28 was assayed on RNA from induced (+tet) and uninduced (–tet) populations with carboxymethyl cellulose (CMC) modification (+ or –) and primer extension. Products extended with the 5’-end-labeled TbSL stem-loop I oligonucleotide were resolved alongside a corresponding DNA sequencing ladder through a 6% acrylamide–8 M urea gel. Additional primer extension stops at three AU and one AC dinucleotide were seen inconsistently in different experiments.
tent with the absence of polyribosome formation on mRNAs lacking mature cap 4 structures (22). The identification and experimental analysis of nuclear import factors, 5’ cap structure of nuclear import-blocked spliced leader RNA, and the cap 4 methyltransferases will clarify the roles of Sm proteins and subsequent modifications in spliced leader RNA nuclear import.

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