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A 22-nucleotide spliced leader sequence in the human parasitic nematode *Brugia malayi* is identical to the trans-spliced leader exon in *Caenorhabditis elegans*

(spliced leader/*Brugia malayi*/trans-splicing)

ADRIENNE M. TAKACS, JOHN A. DENKER, KIMBERLY G. PERRINE, PATRICIA A. MARONEY, AND TIMOTHY W. NILSEN*

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

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ABSTRACT **The mRNAs encoding a 63-kDa antigen in the human parasitic nematode *Brugia Malayi* contain a spliced leader sequence of 22 nucleotides (nt) that is identical to the trans-spliced leader found on certain actin mRNAs in the distantly related nematode *Caenorhabditis elegans*. The 22-nt sequence does not appear to be encoded near the 63-kDa genes but is present in multiple copies in several locations within the parasite genome, including the 5S rRNA gene repeat. The 5S-linked copies of the 22-nt sequence are transcribed to yield a 109-nt nonpolyadenylylated RNA with the 22-nt leader sequence at its 5′ end. We suggest that the 22-nt leader is acquired by 63-kDa antigen mRNAs through trans-splicing. These results indicate that trans-splicing is widespread in nematodes and argue for the functional significance of the 22-nt spliced leader exon in nematode mRNA metabolism.**

Evidence suggests that intermolecular (trans) splicing is used in a variety of organisms during the maturation of some mRNAs. This is particularly clear for trypanosomatid protozoans, where all mRNAs contain a common leader derived from a small nonpolyadenylylated miniexon transcript (for review, see ref. 1). A trans-splicing mechanism of leader addition is supported by the primary structure of the miniexon transcript and the existence of appropriate branched intermediates (2, 3). Recent observations indicate that trans-splicing might also be used in the formation of mRNA for chloroplast ribosomal protein S12 (4) and in the maturation of certain actin mRNAs in *Caenorhabditis elegans* (5).

In *C. elegans*, mRNAs derived from three of four actin genes contain a 22-nucleotide (nt) leader sequence that is not encoded within 15 kilobases (kb) of the actin genes. This leader sequence is found as the first 22 nt of an abundant 100-base RNA transcribed from within the 5S rRNA gene cluster (5). Several lines of evidence, including the demonstration of branched intermediates containing a portion of the 100-nt RNA, suggest that the 22-nt leader is acquired by trans-splicing (5, 16). In contrast to the situation in trypanosomes, only a subset of *C. elegans* mRNAs appear to contain the trans-spliced leader. Furthermore, because *C. elegans* actin genes contain multiple introns, trans-splicing apparently occurs in conjunction with conventional cis-splicing. As discussed by Krause and Hirsh (5) the use of trans-splicing in *C. elegans* raises the possibility that this mechanism could be widespread in eukaryotes and may be a regulatory mechanism in gene expression.

We have recently described the isolation and characterization of cDNA and genomic clones encoding a 63-kDa protective antigen in the human parasitic nematode *Brugia malayi*, the causative agent of lymphatic filariasis (6, 7). Nuclease protection and primer-extension experiments indicated that the mRNAs encoding the 63-kDa antigen contained a short spliced leader sequence (7). We report here that this leader sequence is identical to the 22-nt trans-spliced leader sequence in *C. elegans*. The 22-nt exon does not appear to be encoded near the structural genes for the 63-kDa antigen but is present in multiple copies at various locations within the parasite genome including the 5S rRNA gene repeat. Nucleotide sequence analysis of four distinct genomic clones indicates that the 22-nt sequence is invariably adjacent to the dinucleotide GT characteristic of potential splice donor sites. There is little conservation in the 5′ or 3′ DNA flanking the 22-nt sequence in copies unlinked to 5S rRNA genes. The copies of the 22-nt sequences within the 5S locus are found tandemly reiterated within highly conserved spacer regions between the 5S rRNA structural genes. Although we do not detect transcription of the 22-nt sequences that lie outside the 5S rRNA gene repeat, Northern (RNA) blot, primer-extension, and S1 nuclease protection experiments show that the 5S-linked copies of the 22-nt sequence are transcribed to yield a 109-base nonpolyadenylylated RNA with the 22-nt sequence at its 5′ end. In contrast to *C. elegans*, this RNA is transcribed in the same orientation as 5S rRNA. We suggest that the 109-base RNA serves as a donor of the 22-nt spliced leader exon through a trans-splicing reaction. These results suggest that trans-splicing is used in widely diverged nematodes. Furthermore, whereas the 5S rRNA coding sequences of *C. elegans* and *B. malayi* are only ≈80% identical, the 22-nt leader sequence is completely conserved in these two nematodes.

MATERIALS AND METHODS

***B. malayi* Nucleic Acids.** *B. malayi* microfilariae were obtained by lavage of peritoneal cavities of jirds (*Meriones unguiculatus*; Mongolian gerbil) infected 12 to 20 weeks earlier with *B. malayi* larvae (8). Microfilarial RNA was isolated using guanidinium thiocyanate and hot phenol as described (9). DNA from *B. malayi* and *C. elegans* was extracted via solubilization in guanidinium thiocyanate, followed by phenol extraction and ethanol precipitation. Resuspended nucleic acids were digested with pancreatic RNase and proteinase K, extracted with phenol, and reprecipitated with ethanol.

Oligodeoxynucleotides and Hybridizations. Oligodeoxynucleotides complementary to the 22-nt leader (CTCAAACTTGGGTAATTAAACC), complementary to bases 25–47 of *C. elegans* 5S rRNA (10) (CCAGATCGGACGGGATGGCGTGC), complementary to bases 18–39 of *B. malayi* 5S rRNA (GACGAGATGTCGTGCTTTCAAC), or comple-

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Abbreviation: nt, nucleotide(s).

*To whom reprint requests should be addressed.

mentary to regions 3′ of putative transcripts from various copies of the *B. malayi* 22-nt sequence (see text and Fig. 5) (8D, bases 41–62, GCCACAGTAGCCTAAACTCTGG); 4F, bases 29–45, GAATTCACCGGCTGTCG; 4A, bases 46–67, ACCTTACCTCACCTCGGGCACC; 6G, bases 46–67, CACATATGTGATTTTATATGTG; and 3E, bases 62–83, CTGGTTCCGTTCTGTGTATCT) were synthesized on a 381A DNA synthesizer (Applied Biosystems, Foster City, CA), purified, and labeled with [γ-^{32}P]ATP by polynucleotide kinase. *B. malayi* RNA or DNA samples were fractionated by gel electrophoresis as described in figure legends and transferred to GeneScreen (New England Nuclear). Blot hybridizations were done at 37°C in 6× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7)/0.1% sodium dodecyl sulfate/10× Denhardt's solution (1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin)/50 mM sodium phosphate, pH 7.0, containing salmon sperm DNA at 100 μg/ml. Washes were done at 37°C in 6× SSC/0.1% sodium dodecyl sulfate.

Primer Extension. Labeled oligodeoxynucleotides as indicated in the figure legends were hybridized with *B. malayi* RNA at 37°C in 250 mM KCl/10 mM Tris, pH 8.3. Extensions were performed for 60 min at 37°C with 0.5 mM deoxynucleotide triphosphates/16 mM $MgCl_2$/8 mM dithiothreitol/20 mM Tris, pH 8.3, containing 15 units of avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL). Extension products were fractionated on 7 M urea/6% polyacrylamide gels and autoradiographed.

RESULTS

The 63-kDa Antigen mRNA Contains a 22-nt Spliced Leader Sequence. We have previously used S1 nuclease mapping and primer-extension analyses to demonstrate that the mRNA for the 63-kDa antigen contains a short spliced leader sequence (7). We have now determined the sequence of the leader by partial chemical degradation of 5′ end-labeled primer-extension products (Fig. 1). Remarkably, the sequence of this spliced leader exon was identical to the spliced leader found on some *C. elegans* actin mRNAs (5). To determine whether the 22-nt exon was closely linked to one or more of the 63-kDa antigen genes, a synthetic oligodeoxynucleotide complementary to the leader was used to probe restriction digests of several genomic clones corresponding to different copies of 63-kDa antigen genes (7). The probe did not hybridize to any of these clones that contain from 1 to 7 kb of 5′-flanking sequence (data not shown). Because the evidence suggests that, in *C. elegans*, multiple mRNAs in addition to actin contain the 22-nt spliced leader, a Northern (RNA) blot of *B. malayi* RNA was probed with the synthetic oligodeoxynucleotide complementary to the 22-nt sequence. A diffuse pattern of hybridization was observed, suggestive of multiple hybridizing species (see Fig. 4). In contrast, the mRNAs for the 63-kDa antigen migrate as a discrete 1.8-kb band (6).

The 22-nt Leader Exon Is Multicopy Within the *B. malayi* Genome. To address the genomic organization of the 22-nt sequence in *B. malayi*, we performed Southern blot analysis (12) of genomic DNA digested with various restriction enzymes using the synthetic 22-mer as a probe. We identified multiple hybridizing bands, indicating that the 22-nt sequence was highly reiterated within the *B. malayi* genome (data not shown). Krause and Hirsh (5) had previously found that the 22-nt leader sequence was reiterated within the 5S rRNA gene repeat in *C. elegans*. To determine whether the 22-nt sequence might also be associated with the *B. malayi* 5S gene family, we digested genomic DNA from *B. malayi* and *C. elegans* with the restriction endonuclease *Sca* I, which cleaves within the 5S rRNA gene-coding sequence of *C. elegans* (10). Because 5S sequences are conserved between related organisms, we anticipated that *Sca* I would also cleave within *B. malayi* 5S

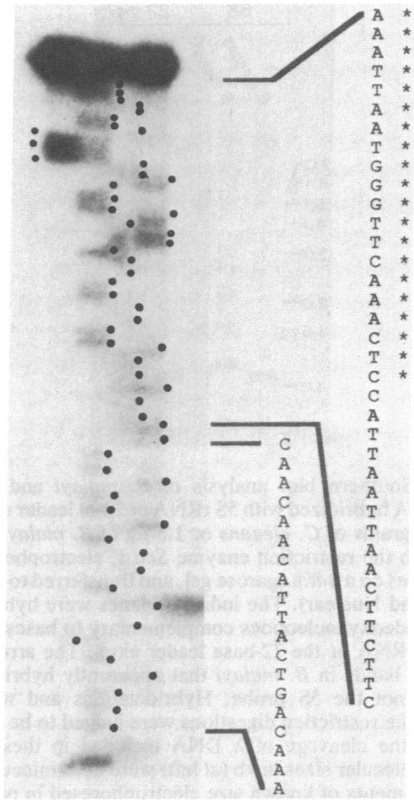

FIG. 1. Nucleotide-sequence analysis of the spliced leader exon present on 63-kDa-antigen mRNAs. An oligodeoxynucleotide complementary to 63-kDa-antigen mRNA (bases 41–60 of cDNA clone λW6) (6) was end-labeled, hybridized to 85 μg of total microfilarial RNA, and extended with reverse transcriptase as described. Extension products were resolved on a 7 M urea/8% polyacrylamide gel and recovered from the gel after autoradiography. Purified primer-extension products were sequenced by partial chemical degradation (11). Starred nucleotides correspond to bases complementary to the 22-nt leader region determined from this gel.

rRNA genes. Southern blots of these DNAs were done by using either the 22-mer or a synthetic oligodeoxynucleotide complementary to *C. elegans* 5S RNA as probes (Fig. 2). Both probes identified an ≈1-kb hybridizing band in *C. elegans*, reflecting the 1-kb 5S rRNA gene repeat in that organism. In contrast, both probes hybridized to multiple bands in *B. malayi* DNA (Fig. 2). Although comigration of many of these hybridizing bands suggested that the 22-nt sequences could be linked to 5S sequence in *B. malayi*, this analysis indicated that the genomic organization of 5S rRNA genes and the 22-nt sequence was more complex in *B. malayi* than in *C. elegans* (Fig. 2). Furthermore, some bands that hybridized to the 22-nt leader probe apparently did not hybridize to the 5S probe (Fig. 2), suggesting that in *B. malayi* the 22-nt sequence was also encoded in regions of DNA unlinked to the 5S rRNA gene family (see below).

Nucleotide-Sequence Analysis of the 22-nt Spliced Exon in *B. malayi* DNA. To perform a detailed characterization of the 22-nt sequence within *B. malayi* DNA, a *B. malayi* genomic library (7) was screened by hybridization with the leader oligodeoxynucleotide. Duplicate screens were carried out with the 5S-specific oligodeoxynucleotide as probe. Several plaques hybridized to both probes, and two were purified. These clones had identical restriction maps and one (designated clone 8) was chosen for further analysis. In addition, we purified phage from six plaques that hybridized to the 22-nt sequence, but not to the 5S probe. Restriction mapping of these clones indicated that they were derived from three

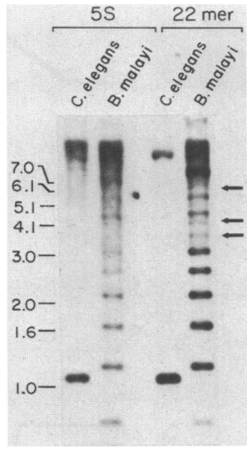

FIG. 2. Southern blot analysis of *B. malayi* and *C. elegans* genomic DNA hybridized with 5S rRNA or 22-nt leader exon probes. Three micrograms of *C. elegans* or 1.5 μg of *B. malayi* DNA were digested with the restriction enzyme *Sca* I, electrophoresed in the indicated lanes on a 0.8% agarose gel, and transferred to GeneScreen (New England Nuclear). The indicated lanes were hybridized with labeled oligodeoxynucleotides complementary to bases 25–47 of *C. elegans* 5S rRNA or the 22-base leader exon. The arrows indicate examples of bands in *B. malayi* that apparently hybridized to the 22-mer but not the 5S probe. Hybridizations and washings are described. The restriction digestions were judged to be complete by analysis of the cleavage of λ DNA included in these reactions. Indicated molecular sizes in kb (at left) were determined by mobility of DNA fragments of known size electrophoresed in parallel lanes.

separate genomic loci, and clones designated 3, 4, and 6 were analyzed further. To facilitate nucleotide-sequence analysis, we performed Southern blot analysis of genomic inserts digested with various restriction enzymes using the 22-mer as a probe. Fragments that hybridized to the probe were purified and subcloned into plasmid vectors.

We determined the nucleotide sequence of several fragments derived from clone 8, the genomic clone identified by hybridization to both the 22-nt and 5S probes (Fig. 3, 8K1,8C2 and 8K2,8D). This analysis revealed that perfect copies of the 22-nt sequence were present in highly conserved reiterated "spacer" regions between potential 5S rRNA-coding regions. During these determinations, we encountered unexpected heterogeneity in these potential 5S rRNA-coding sequences, including base substitutions in the *Sca* I recognition sequence. These substitutions, in part, account for the ladder of bands seen when *B. malayi* DNA was digested with *Sca* I and hybridized with the 5S probe (see Fig. 2). The heterogeneity in 5S rRNA-coding regions contrasted to the almost perfect conservation of "intergenic" regions containing the 22-nt sequence (Fig. 3). While the interspersion of 22-nt sequences with 5S rRNA genes is similar in *B. malayi* to the organization of these elements in *C. elegans*, the orientation of the 22-nt sequence with respect to 5S genes is opposite in the two organisms. Furthermore, there is no conservation of sequence 5′ of the 22-nt leader between *B. malayi* and *C. elegans* (see Fig. 3). a comparison of sequences 3′ of the 5S-associated 22-nt sequence in *B. malayi* with analogous sequence in *C. elegans* revealed three interesting similarities (Fig. 3): (*i*) The first four nucleotides immediately flanking the 22-nt sequence are identical and conform to a potential splice donor site; (*ii*) a block of 15 nt corresponding to bases 50–64 3′ of the 22-nt sequence in *B. malayi* is identical to bases 42–56 downstream of the 22-nt sequence in *C. elegans*; and (*iii*) a block of 10 nt corresponding to bases 78–87 3′ of the *B. malayi* 22-nt sequence are 90% identical to bases 69–78 3′ of the 22-nt sequence in *C. elegans* (Fig. 3). This last block of identity corresponds to the

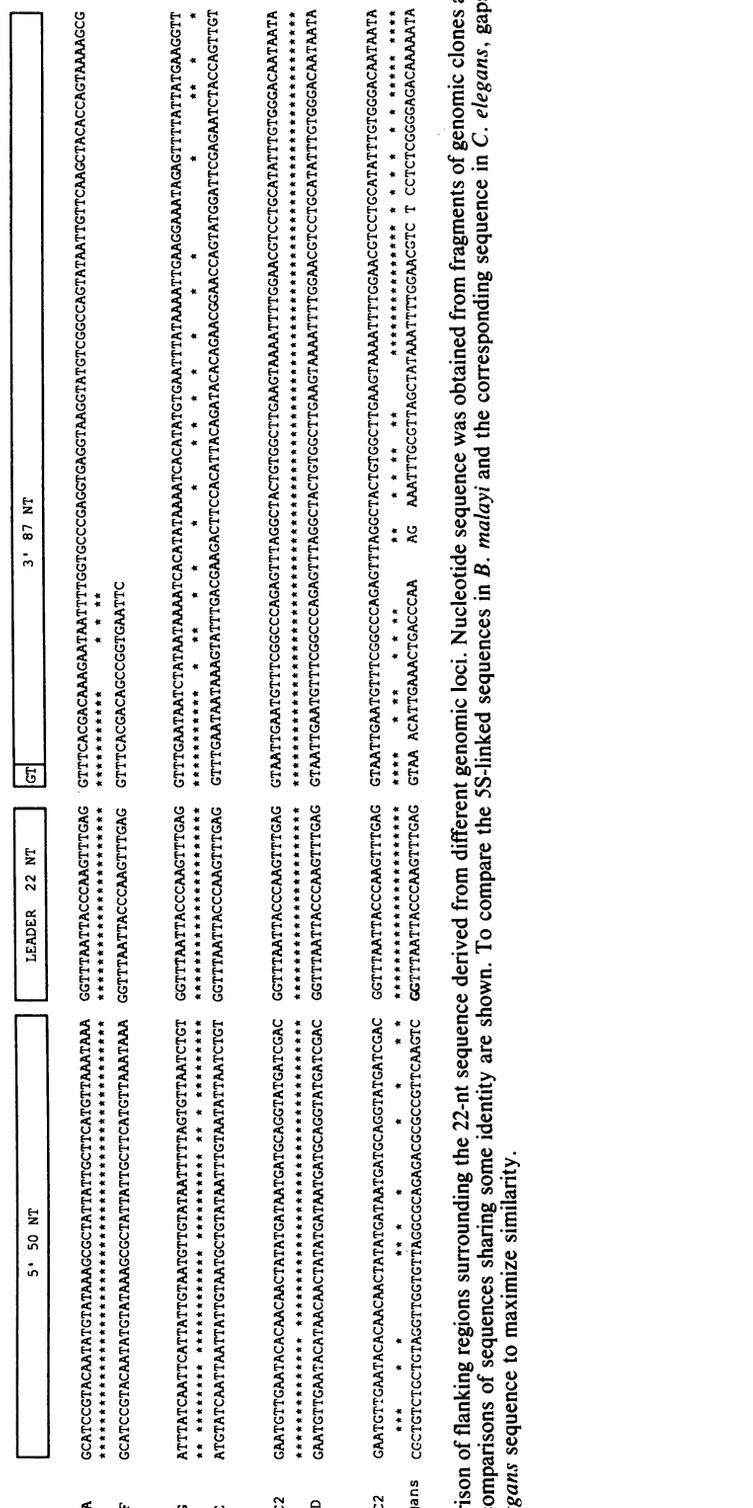

FIG. 3. Comparison of flanking regions surrounding the 22-nt sequence derived from different genomic loci. Nucleotide sequence was obtained from fragments of genomic clones as described (11, 15). Pairwise comparisons of sequences sharing some identity are shown. To compare the 5S-linked sequences in *B. malayi* and the corresponding sequence in *C. elegans*, gaps were intro duced in the *C. elegans* sequence to maximize similarity.

respective 3′ termini of RNAs containing the 22-nt sequence transcribed from within the 5S rRNA gene repeats of *B. malayi* and *C. elegans* (see below).

We also determined the nucleotide sequence of four restriction fragments derived from genomic clones that hybridized to the 22-nt sequence but not the 5S probe (Fig. 3; 4A, 4F, 6G, and 3E). We found perfect copies of the 22-nt sequence in each fragment. Furthermore, all of these copies of the leader sequence were immediately flanked by the dinucleotide GT (Fig. 3). However, beyond these similarities, a comparison of flanking sequences indicated that the 22-nt sequence was present in remarkably different sequence contexts. In fragments derived from clone 4 (4A and 4F), 5′-flanking sequences were identical for 50 nt before diverging; 3′ flanking sequences shared nine identical residues and then diverged. The 5′-flanking 50 nt in fragments from clone 6 (6G) and clone 3 (3E) were 88% identical to each other, but shared no significant identity with the 5′-flanking sequence found on fragments 4A and 4F. The 3′-flanking sequences in fragments 6G and 3E also shared nine identical residues (different than the nine in fragments 4A and 4F) before diverging completely (Fig. 3). None of these flanking regions share any significant identity with sequences 5′ or 3′ of the 22-nt sequences that lie within the 5S rRNA gene repeats of either *B. malayi* or *C. elegans* (Fig. 3).

In summary, the 22-nt sequence is encoded by multiple genomic loci in *B. malayi*, including the 5S rRNA gene repeat. Beyond the 22-nt sequence and the conserved GT, only the 5S-linked copies share sequence identity with analogous sequence in *C. elegans*, and this identity is confined to short blocks within the 3′-flanking region.

The 5S-Linked 22-nt Sequence Is Transcribed in *B. malayi*. Krause and Hirsh (5) have demonstrated that the 22-nt leader sequence in *C. elegans* is transcribed to yield a small nonpolyadenylylated RNA with the 22-nt leader at its 5′ end. To determine whether a similar RNA was present in *B. malayi*, a Northern (RNA) blot of nonadenylylated *B. malayi* RNA was probed with a synthetic oligodeoxynucleotide complementary to the 22-nt leader sequence. A single 109-nt RNA hybridized to the probe (Fig. 4). To determine which genomic locus yielded this RNA, we took three approaches: Northern blot analysis, primer-extension, and S1 nuclease mapping. To discriminate among the various genomic copies, we synthesized diagnostic oligodeoxynucleotides complementary to regions 3′ of each 22-nt sequence. When these oligodeoxynucleotides were used to probe Northern blots, only the probe complementary to sequences downstream of the 5S-linked 22-nt sequence detected the transcript initially identified using the 22-mer probe (Fig. 4). Similarly, when the oligodeoxynucleotides were labeled and hybridized with *B. malayi* RNA and extended with reverse transcriptase, only the oligonucleotide complementary to the 5S-associated 22-nt sequence gave rise to a primer-extension product (Fig. 4). The size of this product (62 nt) was consistent with a transcript the 5′ terminus of which was the 22-nt leader sequence. Partial chemical degradation sequencing of the extension product confirmed this interpretation (data not shown). Finally, results consistent with both the Northern blot and primer-extension experiments were obtained using diagnostic fragments in S1 nuclease-protection assays (data not shown).

We conclude from these results that the 22-nt sequence encoded within the 5S rRNA gene repeat of *B. malayi* is transcribed to yield a small, nonpolyadenylylated transcript, which could serve as a donor of the 22-nt spliced leader exon in a trans-splicing reaction.

DISCUSSION

We have shown that the mRNAs encoding a 63-kDa antigen in *B. malayi* contain a 22-nt spliced leader identical in sequence to the trans-spliced leader affixed to certain actin mRNAs in *C. elegans*. There are several similarities and some striking differences between our findings and those reported in *C. elegans* (5). In both organisms the 22-nt spliced leader could be derived by trans-splicing from a small miniexon-like RNA transcribed from within the 5S rRNA gene repeat. The case for trans-splicing is especially clear in *C. elegans*, where the 22-nt sequence is encoded almost

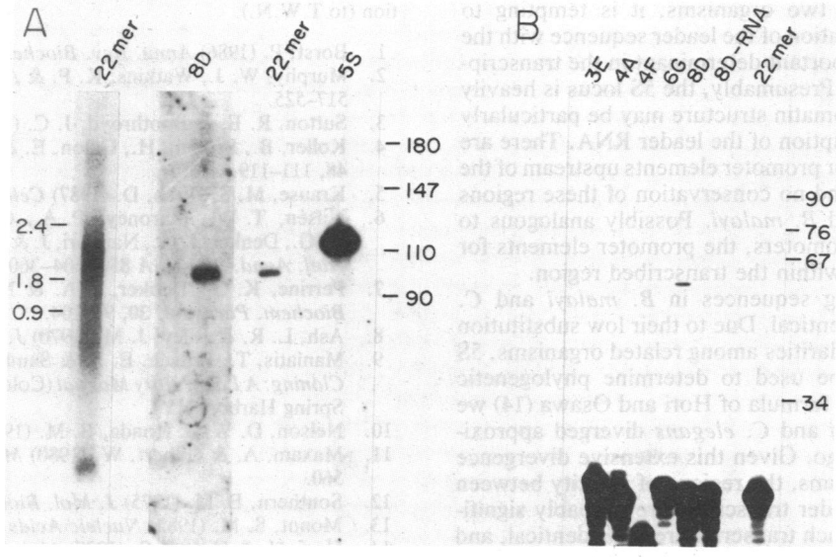

FIG. 4. Detection of transcripts containing the 5S-linked 22-nt sequence and presence of the 22-nt sequence on multiple RNAs in *B. malayi*. (*A*) Northern blot analysis. In the leftmost lane, 10 μg of adenylylated *B. malayi* RNA, extracted as described, was fractionated on a 1% denaturing formaldehyde agarose gel and transferred to GeneScreen. In the three adjacent lanes, 2 μg of nonadenylylated *B. malayi* RNA was electrophoresed on 7 M urea/5% polyacrylamide gel and transferred to GeneScreen. Blots were hybridized with the indicated oligodeoxynucleotides (8D, 22-mer, and 5S) and washed as described. Indicated sizes were determined by electrophoresis of labeled *Hpa* II restriction fragments derived from pBR322. (*B*) Primer-extension analysis. The indicated oligodeoxynucleotides were labeled, hybridized with nonadenylylated *B. malayi* RNA, and extended with reverse transcriptase as described and analyzed on a 7 M urea/6% polyacrylamide gel. Indicated sizes were determined by migration of labeled *Hpa* II restriction fragments as described above. Oligodeoxynucleotide 8D is complementary to bases 41–62 of the RNA containing the 22-nt sequence transcribed from within the 5S rRNA gene locus in *B. malayi*.

exclusively within the 5S rRNA gene repeat, and branched intermediates predicted by a trans-splicing mechanism have been observed (16).

The argument for trans-splicing in *B. malayi* is less rigorous. In addition to the 22-nt sequence residing within the 5S rRNA gene repeat, we characterized several additional genomic regions that encode this sequence. In each case the dinucleotide GT was found immediately 3′ of the 22-nt sequence, raising the possibility that all the 22-nt sequences could be competent to donate the 22-nt leader via cis- or trans-splicing reactions.

The 63-kDa antigen in *B. malayi* is encoded by a multigene family (7), and we have characterized several members of this family by restriction mapping and sequence analysis of genomic clones. Although none of the 5′-flanking sequences found in these clones contain the 22-nt sequence, we have not exhaustively characterized the 63-kDa-antigen genes and their 5′-flanking regions. Moreover, because we have not assessed which of these genes are transcriptionally active, we cannot exclude the possibility that the 63-kDa-antigen mRNAs are derived from genomic loci where the 22-nt sequence is encoded upstream of and contiguous with the remainder of the coding sequence. In this case, the 22-nt leader sequence could be derived by conventional cis-splicing. Although cis-splicing remains a possibility, we believe that our results are most consistent with a trans-splicing mechanism of leader addition.

Were the leader acquired by trans-splicing, it would most likely be derived from the 22-nt sequence transcribed from within the *B. malayi* 5S gene repeat. Primer-extension, S1 nuclease, and Northern blot analysis indicate that this is the only 22-nt locus that gives rise to a detectable transcript. Furthermore, the potential splice donor sequence adjacent to this 22-nt sequence is much closer to consensus (13) than those adjacent to the 22-nt sequences encoded in other genomic loci (Fig. 3). This transcript has several interesting similarities with the analogous transcript in *C. elegans*. In both organisms, the RNAs are nonpolyadenylylated, are of similar size, and are transcribed from within the 5S rRNA gene locus. However, because the orientation of transcription is opposite in the two organisms, it is tempting to speculate that the association of the leader sequence with the 5S locus *per se* is an important determinant in the transcription of the leader RNA. Presumably, the 5S locus is heavily transcribed, and its chromatin structure may be particularly suited for active transcription of the leader RNA. There are no obvious candidates for promoter elements upstream of the 22-nt leader sequence and no conservation of these regions between *C. elegans* and *B. malayi*. Possibly analogous to RNA polymerase III promoters, the promoter elements for the leader RNA reside within the transcribed region.

The 5S rRNA coding sequences in *B. malayi* and *C. elegans* are only 80% identical. Due to their low substitution rates and structural similarities among related organisms, 5S rRNA sequences can be used to determine phylogenetic distance (14). Using the formula of Hori and Osawa (14) we calculate that *B. malayi* and *C. elegans* diverged approximately 5×10^8 years ago. Given this extensive divergence between the two organisms, the regions of identity between the 3′ regions of the leader transcripts are probably significant. The last 10 nt of each transcript are 90% identical, and it seems possible that these nucleotides may be important in terminating transcription. The other region of identity, the contiguous 15 nt, could be involved in recognition of the leader transcript in trans-splicing. Development of appropriate *in vitro* systems should allow assessment of these possibilities.

The role of the 22-nt sequences found outside the 5S rRNA locus is unclear. As discussed above, the potential splice donors adjacent to these sequences are considerably diverged from consensus. Thus, they may represent nonfunctional orphans or pseudogenes; however, we cannot exclude the possibility that these sequences are transcribed at a low rate and simply were not detected by primer extension or Northern analysis. Alternatively, they could serve as donors of leader sequences to as yet uncharacterized genes through cis-splicing. Finally, we note that all our analyses were done on RNA extracted from microfilaria, a larval stage of the parasite. *B. malayi* has a complex life cycle including developmental stages within a mosquito vector. The 22-nt sequences that are not associated with 5S rRNA genes could be transcribed and used during different stages of parasite development.

The functional significance of the 22-nt sequence remains obscure. Krause and Hirsh (5) have discussed several potential roles of the leader, including the mediation of mRNA stability or translational efficiency. However, it remains possible that the 22-nt sequence could participate in and influence any aspect of mRNA metabolism. Perhaps the strongest argument for its importance comes from the perfect conservation of this sequence between widely divergent nematodes. This conservation becomes striking in comparison to the divergence of the 5S rRNA coding sequences (≈80% identity) between the two organisms.

Definitive determination of the role of the 22-nt sequence awaits more thorough characterization of the genes encoding mRNAs that contain the leader sequence and the development of appropriate assay systems to evaluate the biochemical significance of the leader sequence.

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