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Most mRNAs in the nematode *Ascaris lumbricoides* are *trans*-spliced: A role for spliced leader addition in translational efficiency

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ABSTRACT

Some pre-mRNAs in nematodes are processed by *trans*-splicing. In this reaction, a 22-nt 5′ terminal exon (the spliced leader, SL) and its associated 2,2,7-trimethylguanosine cap are acquired from a specialized Sm snRNP, the SL RNP. Although it has been evident for many years that not all nematode mRNAs contain the SL sequence, the prevalence of *trans*-spliced mRNAs has, with the exception of *Caenorhabditis elegans*, not been determined. To address this question in an organism amenable to biochemical analysis, we have prepared a message-dependent protein synthesis system from developing embryos of the parasitic nematode, *Ascaris lumbricoides*. Using this system, we have used both hybrid-arrest and hybrid-selection approaches to show that the vast majority (80–90%) of *A. lumbricoides* mRNAs contain the SL sequence and therefore are processed by *trans*-splicing. Furthermore, to examine the effect of SL addition on translation, we have measured levels of protein synthesis in extracts programmed with a variety of synthetic mRNAs. We find that the SL sequence itself and its associated hypermethylated cap functionally collaborate to enhance translational efficiency, presumably at the level of initiation of protein synthesis. These results indicate that *trans*-splicing plays a larger role in nematode gene expression than previously suspected.

Keywords: cap; mRNA processing; Sm snRNPs; translation; *trans*-splicing

INTRODUCTION

5′ End-maturation by spliced leader (SL) addition *trans*-splicing is used as a mechanism of pre-mRNA processing in a variety of lower eukaryotes including the unicellular trypanosomatid protozoans and euglenoid protists (see Nilsen, 1994, 1995 for recent review and references). Among metazoans, only nematodes (roundworms) and trematodes (flatworms) are currently known to *trans*-splice (reviewed in Nilsen, 1994; Blumenthal, 1995; Davis, 1995). In all nematodes studied to date, some, but not all, mRNAs acquire a common 22-nt SL sequence from a small (~100-nt) SL RNA. Nematode SL RNAs are a *trans*-splicing specific snRNA; they contain a 2,2,7-trimethylguanosine cap structure and must assemble into an Sm snRNP in order to function in *trans*-splicing (Bruzik et al., 1988; Thomas et al., 1988; van Doren & Hirsh, 1988; Maroney et al., 1990a). mRNAs matured via *trans*-splicing contain not only the SL sequence at their 5′ ends, but also a hypermethylated cap structure (Liou & Blumenthal, 1990; van Doren & Hirsh, 1990). With the exception of *Caenorhabditis elegans* (see below), the prevalence of *trans*-spliced mRNAs (i.e., the fraction of mRNAs containing the SL sequence) has not been determined. Such knowledge is important in assessing the impact of *trans*-splicing on nematode gene expression and is a necessary prerequisite to understanding the biological function(s) of this unusual RNA-processing reaction.

Several years ago, it was suggested that approximately 10–20% of mRNAs in *C. elegans* were processed by *trans*-splicing (Bektesh et al., 1988). This estimate was derived from hybrid-arrest translation experiments performed in a heterologous (rabbit reticulocyte) cell-free translation system (Bektesh et al., 1988). Retro-

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spectively, these experiments are difficult to interpret because, as noted above, *trans*-spliced mRNAs possess a hypermethylated cap structure and it has since been established that reticulocyte lysates translate mRNAs containing such caps very poorly (Darzynkiewicz et al., 1988). Furthermore, these experiments were conducted prior to the discovery of a second SL RNA (SL2) in *C. elegans* (Huang & Hirsh, 1989). (To date, SL2 RNAs have only been observed in *C. elegans* and *C. briggsae* [Lee et al., 1992]; variant SL RNAs have not been detected in other nematodes). Accordingly, it seems likely that these early experiments underestimated the prevalence of *trans*-splicing in *C. elegans*. Indeed, Blumenthal and colleagues have recently analyzed information garnered from the *C. elegans* genome project and have directly assessed the structure of many specific mRNAs (Zorio et al., 1994). Their results have led to a revision in the estimate of *trans*-spliced mRNAs in *C. elegans*; it is now thought that about 70% of mRNAs are processed by *trans*-splicing in this organism (reviewed in Blumenthal, 1995).

We have previously reported that cell-free extracts prepared from synchronously developing embryos of the parasitic nematode *Ascaris lumbricoides* can catalyze *cis* and *trans*-splicing as well as transcription by RNA polymerases II and III (Maroney et al., 1989, 1990b; Hannon et al., 1990b). We show here that similar extracts efficiently synthesize proteins in vitro and that translation can be made dependent upon the addition of exogenous mRNA. Using a variety of approaches, including hybrid-arrest and hybrid-selected translation, we show that most (80–90%) *A. lumbricoides* mRNAs contain the *trans*-spliced SL sequence. We also show that the presence of the SL sequence and its associated hypermethylated cap structure cooperate to enhance the translational efficiency of a specific mRNA. Implications regarding the role of *trans*-splicing in nematode gene expression are discussed.

RESULTS

Message-dependent cell free translation system from *A. lumbricoides*

In preliminary experiments, whole-cell homogenates of 32-cell *A. lumbricoides* embryos were prepared using varying concentrations of monovalent (KCl) and divalent ($MgCl_2$) salts in the homogenization buffers. Each extract was tested for protein synthesis activity by monitoring incorporation of labeled methionine into acid-insoluble material. When extracts were prepared under optimal conditions (see the Materials and methods) incorporation increased for 30 min and the level of incorporation was significantly above background (Fig. 1A, endogenous). Autoradiography of one-dimensional gels of translation products indicated that labeled methionine was incorporated into proteins encompassing a wide molecular weight range (Fig. 1B, lane 1).

In an effort to render protein synthesis dependent upon the addition of exogenous mRNA, extracts were subjected to mild digestion with micrococcal nuclease (Pelham & Jackson, 1976). The level of nuclease treatment required to abolish endogenous protein synthesis was determined empirically (see the Materials and methods). Following inactivation of the nuclease, increasing concentrations of polyadenylated *A. lumbricoides* RNA were added back to the extract. As shown in Figure 1A, addition of exogenous mRNA restored protein synthesis. At a concentration of 100 μg/mL of exogenous mRNA, the level of incorporation of labeled amino acids approached that of extracts that had not been treated with nuclease. Figure 1B shows that translation products synthesized in extracts programmed with exogenous mRNA are qualitatively similar to those synthesized from endogenous mRNA.

Prevalence of SL-containing mRNAs

As a preliminary assessment of the fraction of translation products that were synthesized from mRNAs containing the 22-nt SL sequence, hybrid-arrest translation experiments were performed. The design of these experiments paralleled that used by Donelson and colleagues (Walder et al., 1986) in their analysis of the prevalence of *trans*-splicing in trypanosomes. When *A. lumbricoides* mRNA was pre-annealed with an oligodeoxynucleotide identical to the SL sequence, very little inhibition of protein synthesis was observed. In contrast, the same treatment with an oligodeoxynucleotide complementary to the SL sequence reduced protein synthesis dramatically (Fig. 2A, *Ascaris*). This effect cannot be attributed to nonspecific inhibition of protein synthesis, because both the sense and antisense oligodeoxynucleotides did not inhibit protein synthesis when the *A. lumbricoides* extract was programmed with HeLa cell polyadenylated RNA (Fig. 2A, HeLa). Figure 2B shows that the antisense oligonucleotide inhibited the synthesis of most *A. lumbricoides* proteins without affecting the pattern of HeLa proteins. These results suggested that the bulk of incorporation of labeled amino acid was directed by mRNAs containing the 22-nt SL sequence and indicated that the specific effect of the antisense oligonucleotide on *A. lumbricoides* protein synthesis could not be explained by the inhibition of translation of a few highly abundant mRNAs.

Although one-dimensional gel electrophoresis was suggestive, we attempted to quantitate the percentage of *trans*-spliced mRNA more accurately using two-dimensional gels. When such experiments were attempted using the hybrid-arrest approach, it was determined that the antisense SL oligonucleotide did not completely inhibit the synthesis of any specific pro-

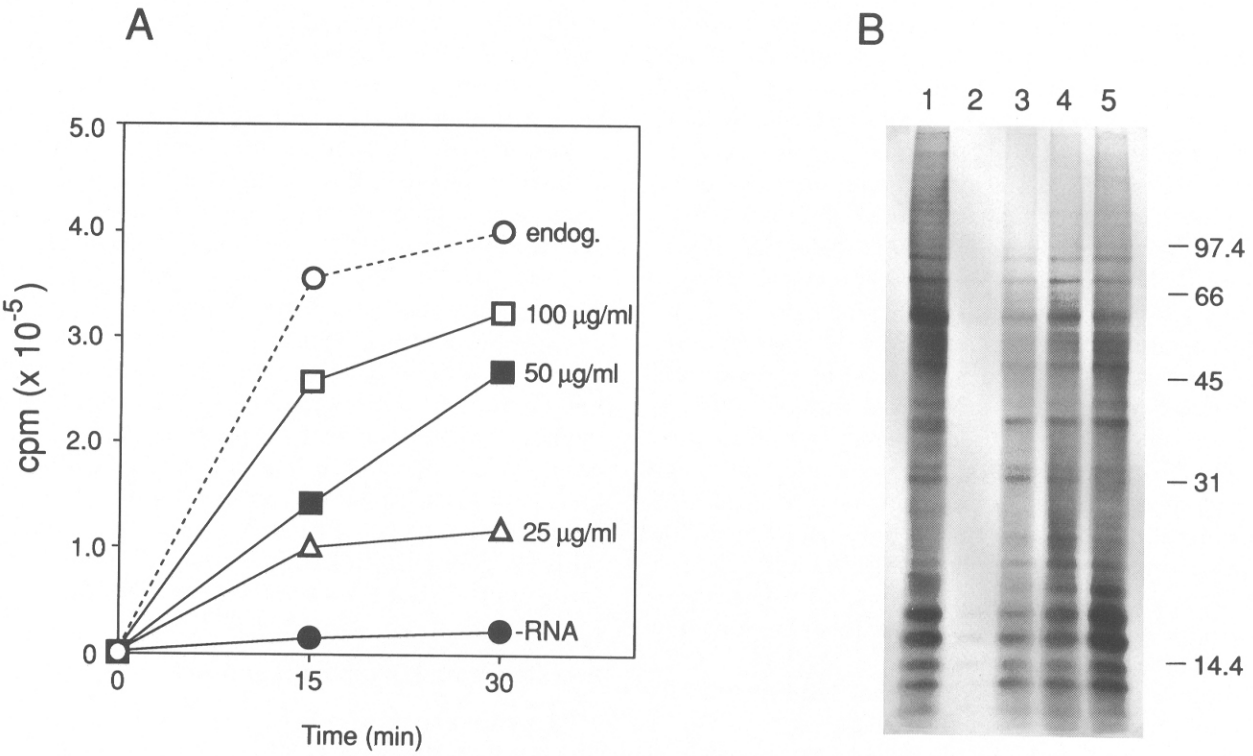


FIGURE 1. A message-dependent protein synthesis system derived from *A. lumbricoides* embryos. Cell-free extracts were prepared from 32-cell *A. lumbricoides* embryos and protein synthesis activity was monitored as described in the Materials and methods. **A:** Incorporation of [^{35}S]-methionine into acid-insoluble material was measured either in extracts not treated with micrococcal nuclease (endog.) or in nuclease-treated extracts supplemented with the indicated concentration of *A. lumbricoides* polyadenylated RNA. **B:** Equal aliquots of 30-min time points of the reactions in A were fractionated on 12.5% denaturing polyacrylamide gels. Following electrophoresis the gel was fixed, dried, and autoradiographed. Lane 1, endogenous; lane 2, no RNA; lane 3, 25 µg/mL; lane 4, 50 µg/mL; lane 5, 100 µg/mL. The positions of proteins of known molecular weight electrophoresed in parallel are indicated.

tein (data not shown). Thus, it was difficult to visualize or quantitate the number of proteins whose synthesis was not affected by the antisense oligonucleotide (those presumably synthesized from mRNAs lacking the SL sequence). As an alternative approach to estimate the fraction of mRNAs that contained the SL sequence, hybrid-selection experiments were performed. Briefly, a biotinylated oligodeoxynucleotide complementary to the SL sequence was used to affinity select those mRNAs that contained the 22-nt SL sequence. Control selections were performed using a biotinylated oligodeoxynucleotide identical in sequence to the SL. To monitor the selection procedure, we took advantage of the fact that we have previously characterized mRNAs that either contain a 5′ terminal SL sequence or do not (Hannon et al., 1990b). Figure 3 (lane 1) shows a northern blot of total polyadenylated RNA probed simultaneously for the mRNAs of gene 4 (*trans*-spliced) and gene 9 (non-*trans*-spliced). These mRNAs (which code for proteins of unknown function) appear to be of equivalent relative abundance. Blot analysis of selected RNAs (Fig. 3, lane 2) revealed that the *trans*-spliced mRNA was hybrid-selected quite efficiently; no detectable non-*trans*-spliced mRNA was present in this sample. The sense oligodeoxynucleotide did not select either mRNA (Fig. 3, lane 3). Similar results were obtained when labeled synthetic mRNAs (either containing or lacking the SL sequence) were mixed with total mRNA and then hybrid-selected (data not shown). We concluded from these experiments that hybrid-selection was a viable strategy for obtaining a population of mRNAs processed by *trans*-splicing. Nevertheless, it is important to note that this approach is subject to some inherent limitations. For example, we cannot exclude the possibility that some mRNAs are hybrid-selected due to fortuitous hybridization or because they contain internal SL sequences; mRNAs with internal SL sequences have been observed in two filarial nematodes, *Onchocerca volvulus* (Zeng et al., 1990) and *Brugia malayi* (our unpubl. obs.). The presence of such mRNAs (not yet documented in *A. lumbricoides*) would yield an overestimate of the prevalence of *trans*-splicing. Other factors might lead to an underestimate. For example, it is possible that longer mRNAs or mRNAs where the SL sequence is occluded in secondary structure would not be selected efficiently. In addition, our experimental strategy did not permit an assessment of those SL-containing mRNAs that are not

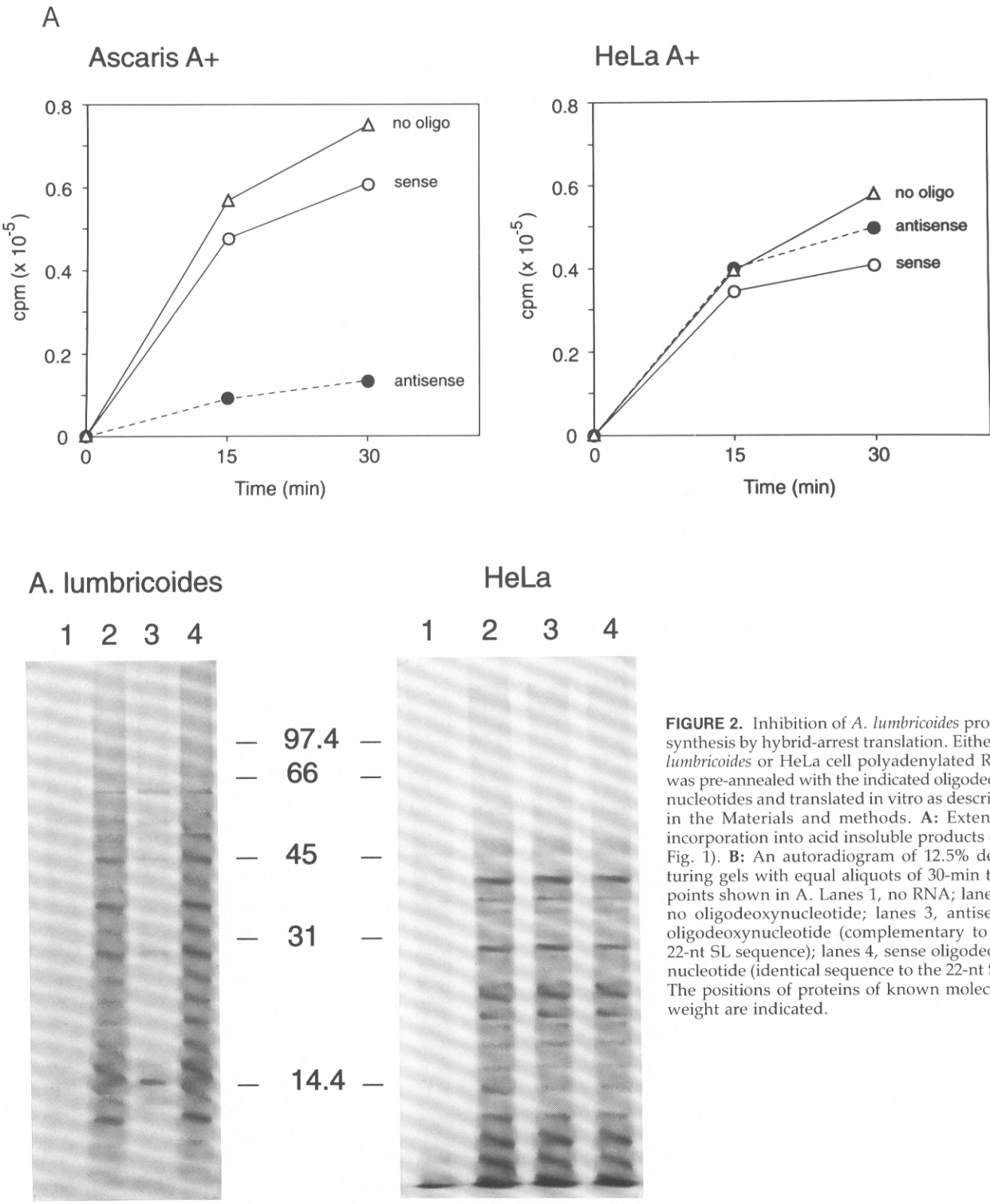


FIGURE 2. Inhibition of *A. lumbricoides* protein synthesis by hybrid-arrest translation. Either *A. lumbricoides* or HeLa cell polyadenylated RNA was pre-annealed with the indicated oligodeoxynucleotides and translated in vitro as described in the Materials and methods. **A:** Extent of incorporation into acid insoluble products (see Fig. 1). **B:** An autoradiogram of 12.5% denaturing gels with equal aliquots of 30-min time points shown in A. Lanes 1, no RNA; lanes 2, no oligodeoxynucleotide; lanes 3, antisense oligodeoxynucleotide (complementary to the 22-nt SL sequence); lanes 4, sense oligodeoxynucleotide (identical sequence to the 22-nt SL). The positions of proteins of known molecular weight are indicated.

polyadenylated (should such mRNAs exist). Finally, extremely low abundance or inefficiently translated mRNAs would not be analyzed.

Figure 4 compares autoradiograms of two-dimensional gels of translation products synthesized from either total polyadenylated RNA or those synthesized from hybrid-selected mRNAs. Approximately 350 protein species are evident when total mRNA is translated (Fig. 4A). Of these, approximately 300 (85%) can be visualized in the translation products of the hybrid-selected RNAs (compare Fig. 4B and C). Acknowledging the experimental caveats expressed above, this

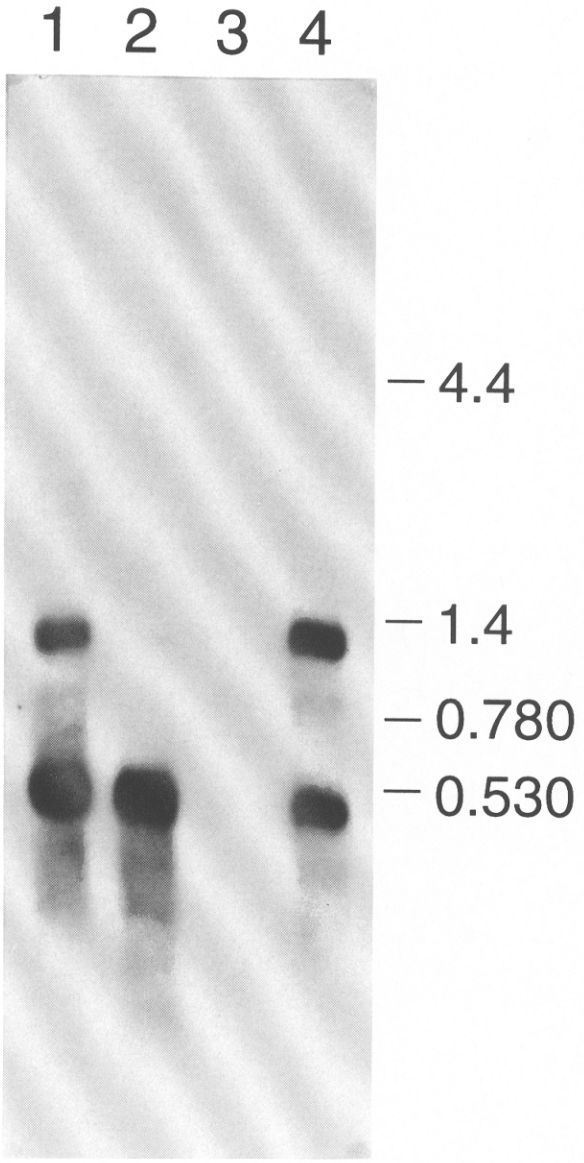


FIGURE 3. Hybrid selection of a *trans*-spliced mRNA. A northern blot of *A. lumbricoides* mRNAs is shown. The blot was probed simultaneously with labeled cDNA clones derived from gene 4 mRNA (*trans*-spliced) and gene 9 mRNA (non-*trans*-spliced) as described in the Materials and methods. The corresponding mRNAs are ~0.5 kb (gene 4) and 1.3 kb (gene 9). In lanes 1 and 4, 1 μg of *A. lumbricoides* polyadenylated was fractionated. Lane 2 contains the RNA selected with the antisense SL oligodeoxynucleotide from 5 μg of the same polyadenylated RNA and lane 3 contains RNA selected with the sense SL oligodeoxynucleotide from 5 μg of *A. lumbricoides* polyadenylated RNA. Hybrid selections are described in detail in the Materials and methods. Positions of RNAs of known size electrophoresed in parallel are indicated in kilobases.

percentage must be considered approximate. Nevertheless, it seems clear that the vast majority of *A. lumbricoides* mRNAs are processed by *trans*-splicing.

Influence of SL sequence and cap structure on translational efficiency

The availability of a homologous in vitro translation system afforded the opportunity to address two other questions relevant to *trans*-splicing. As noted in the Introduction, *trans*-spliced mRNAs acquire both the SL sequence and a 2,2,7-trimethylguanosine cap structure. It has long been a matter of speculation whether either or both of these features contribute to translational efficiency. To address this question, we synthesized four variants of an mRNA that is normally *trans*-spliced and encodes a protein of ~16.3 kDa (gene 12) (Hannon et al., 1990b). One pair of variants contained the SL sequence and either a 2,2,7-trimethylguanosine or 7-methylguanosine cap structure. A second pair of mRNAs had either of the two cap structures, but lacked the SL sequence. All of the synthetic mRNAs were polyadenylated (see the Materials and methods) and each directed the synthesis of the expected protein (see Fig. 5B,C).

Accumulation of the gene 12 protein over time in extracts programmed with equal concentrations of SL-containing synthetic mRNAs with different cap structures is shown in Figure 5A. Interestingly, the mRNA containing trimethylguanosine cap was translated significantly more efficiently than the mRNA containing a monomethylguanosine cap (~5-fold); an uncapped mRNA was not translated at a detectable level (Fig. 5). The same cap dependence was not observed when translation of synthetic mRNAs lacking the SL sequence was compared. Here the mRNA with the monomethyl cap was actually translated somewhat better (~2 fold) than the same RNA with a hypermethylated cap. Both of the SL-minus mRNAs were translated less well than the trimethylguanosine capped SL-containing mRNA and both were translated somewhat more efficiently than the monomethyl capped SL-containing mRNA (Fig. 5C; Table 1).

These results suggested the possibility of some form of functional collaboration between the hypermethylated cap and the SL sequence, presumably in initiation of protein synthesis. To explore this further, two separate 10-base substitution mutations were introduced into positions 7–16 of the SL sequence in the SL-containing mRNA (see the Materials and methods). Translation of each of these mRNAs (containing either of the two cap structures) was then compared to the same four mRNA variants described above (Table 1). Strikingly, both mutations abolished the cap structure dependence observed with the wild-type (wt) SL sequence (Table 1). These results indicated that the identity of the SL sequence was an important determinant in cap recognition and excluded the possibility that the cap specificity observed with SL-containing mRNAs was due to proximity to the initiator AUG (in gene 12 the *A* of the initiator AUG is 12 nt downstream of the SL sequence). It is important to note that the experiments described here have been carried out with a single mRNA. Thus, the generality of these observations remains to be established. Nevertheless, it is clear that the SL sequence and its accompanying cap *can* influ-

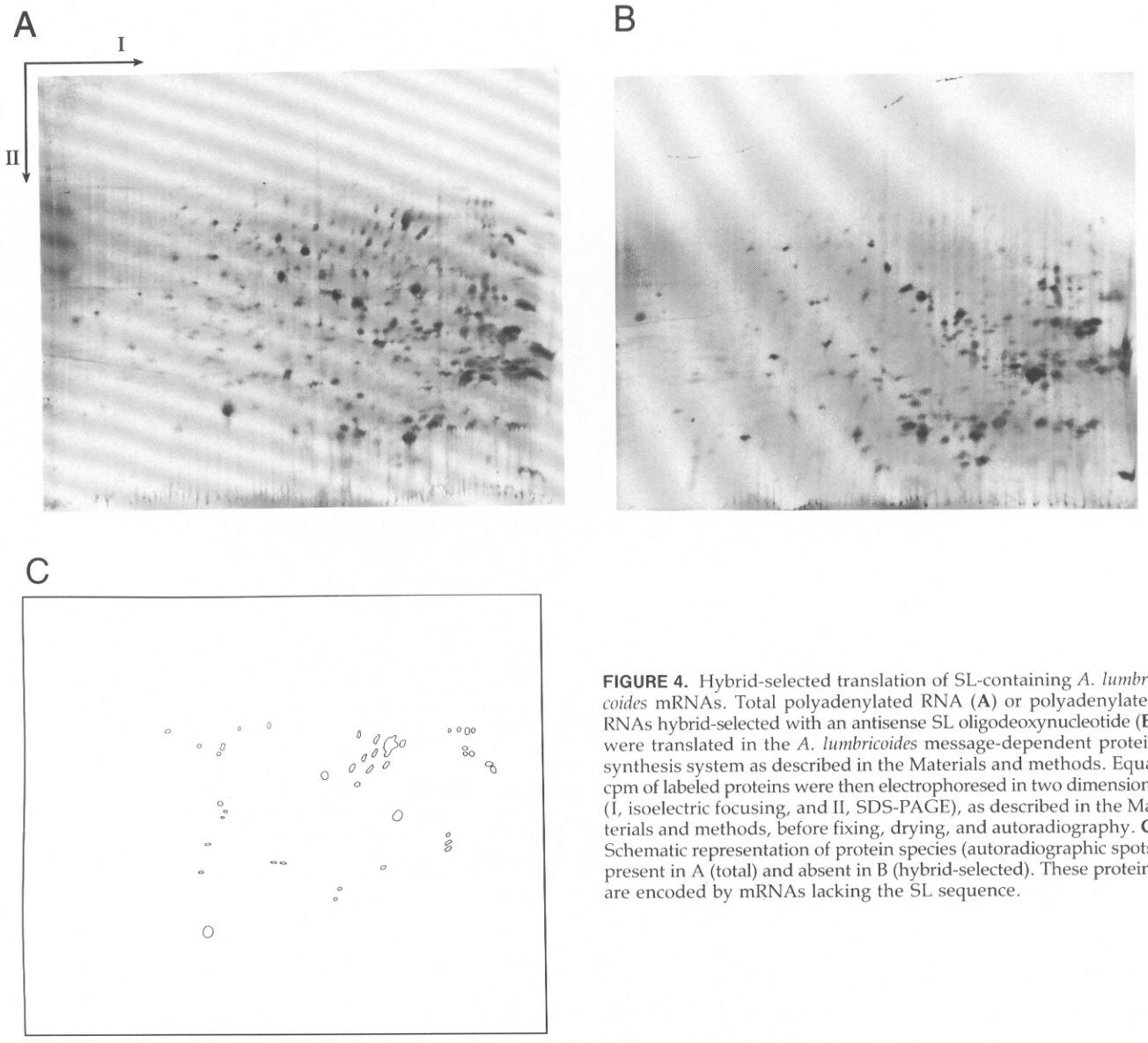


FIGURE 4. Hybrid-selected translation of SL-containing *A. lumbricoides* mRNAs. Total polyadenylated RNA (**A**) or polyadenylated RNAs hybrid-selected with an antisense SL oligodeoxynucleotide (**B**) were translated in the *A. lumbricoides* message-dependent protein synthesis system as described in the Materials and methods. Equal cpm of labeled proteins were then electrophoresed in two dimensions (I, isoelectric focusing, and II, SDS-PAGE), as described in the Materials and methods, before fixing, drying, and autoradiography. **C:** Schematic representation of protein species (autoradiographic spots) present in A (total) and absent in B (hybrid-selected). These proteins are encoded by mRNAs lacking the SL sequence.

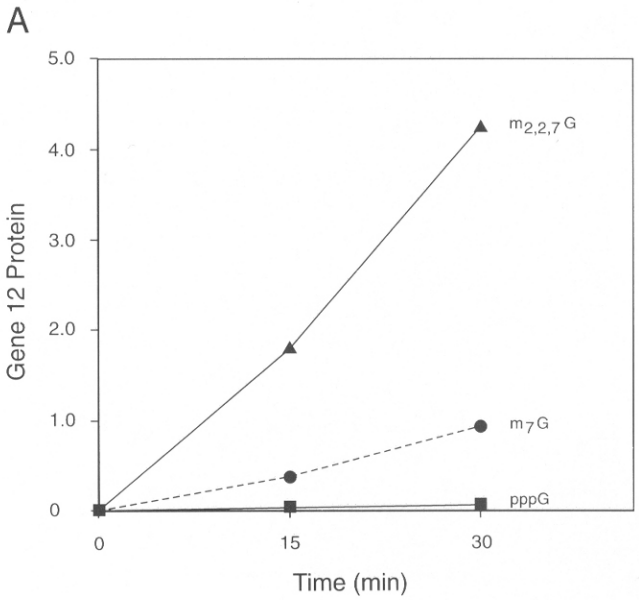
ence translational efficiency and it is intriguing that the 5′ end found on most mRNAs appears to be optimal for initiation of protein synthesis.

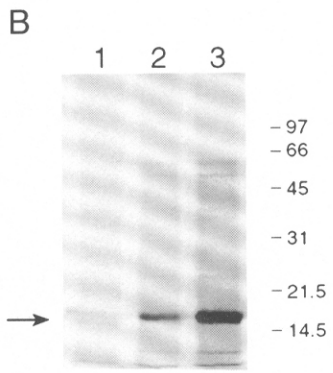
DISCUSSION

We have prepared a message-dependent in vitro translation system from developing embryos of the parasitic nematode *A. lumbricoides*. Previously, cell-free systems derived from this organism have proven to be useful in studying various aspects of nematode RNA processing and transcription. Availability of extracts capable of protein synthesis provides biochemical access to a third component of nematode gene expression.

Here, a variety of approaches were used to estimate the fraction of mRNAs generated via processing by *trans*-splicing. Both hybrid-arrest and hybrid-selection techniques indicate that a vast majority (80–90%) of *A. lumbricoides* mRNAs contain the 22-nt SL sequence. These results reinforce the view (Blumenthal, 1995) that *trans*-splicing plays a much larger role in nematode gene expression than originally suspected. In this regard it seems highly unlikely that our results apply only to *A. lumbricoides* because the available evidence suggests that the prevalence of *trans*-spliced mRNAs in other nematodes is also quite high. As noted in the Introduction, a recent estimate of the fraction of *C. elegans* mRNAs processed by *trans*-splicing is ~70% (reviewed in Blumenthal, 1995). Furthermore, examination of published cDNA clones from other nematode species indicates that most mRNAs contain an SL sequence (our unpubl. obs.).

The original perception that relatively few nematode mRNAs contained an SL sequence led to persistent





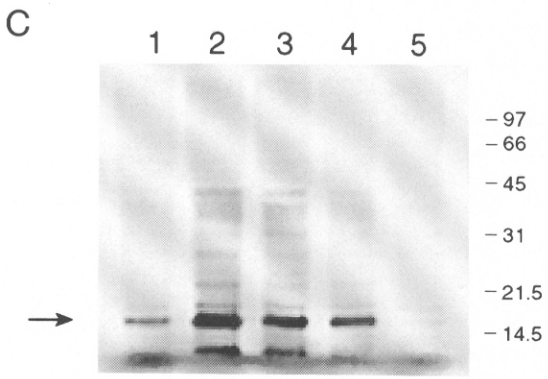


FIGURE 5. Translation of variant gene 12 mRNAs in vitro. Equal amounts (400 ng) of synthetic mRNAs encoding the gene 12 protein were synthesized and translated in the mRNA-dependent *A. lumbricoides* cell-free system as described in the Materials and methods. Equal aliquots of 30-min time points were electrophoresed on a 17.5% denaturing polyacrylamide gel, which was then fixed, dried, and visualized with a phosphorimager (**B**). The position of the gene 12 protein (~16.3 kDa) is indicated by the arrow, as are the positions of proteins of known molecular weight electrophoresed in parallel. Lane 1, uncapped RNA; lane 2, SL-plus monomethyl capped RNA; lane 3, SL-plus trimethylguanosine capped mRNA. The labeled bands migrating above the gene 12 protein may reflect nondenatured or trapped species. They do not reflect stimulation of nonspecific protein synthesis, because an identical pattern of bands is evident when the same mRNAs are translated in a wheat germ cell-free system (data not shown). **A:** Phosphorimage quantitation of gels of a 15-min time point (not shown) and of gels of the 30-min time point (see B). Amount of gene 12 protein is expressed in arbitrary units of pixel density. **C:** Two-hundred nanograms of synthetic mRNAs were translated and equal aliquots of a 30-min time point were analyzed as in B. Lane 1, SL-plus monomethylguanosine capped mRNA; lane 2, SL-plus trimethylguanosine capped mRNA; lane 3, SL-minus monomethylguanosine capped mRNA; lane 4, SL-minus trimethylguanosine capped mRNA; lane 5, no added RNA.

TABLE 1. Relative translational efficiency of synthetic gene 12 mRNAs.[a]

	wt SL	SL-minus	SL mut 1	SL mut 2
Trimethylguanosine cap	1.00	0.20	0.36	0.27
Monomethylguanosine cap	0.13	0.39	0.40	0.37

[a] Variant gene 12 mRNAs were synthesized and equal amounts (200 ng) of each were translated in the *A. lumbricoides* message-dependent cell-free extract as described in the Materials and methods. Translation reactions were stopped after 30 min and proteins were fractionated on a 17.5% denaturing gel such as that shown in Figure 5B and C. Synthesis of the 16.3-kDa protein was determined by quantitative phosphorimaging. Net synthesis of this protein from the trimethylguanosine capped wt SL-containing mRNA was arbitrarily set at 1.0 and the net synthesis of the protein from each of the other mRNAs (as indicated) are expressed as fractions of this amount. The same measurements were made multiple times at two different concentrations of mRNA (10 μg or 20 μg/mL) and at two time points (15 and 30 min). The relative order of translational efficiency was invariant, but occasionally the range of values between the most efficiently translated mRNA (SL-containing trimethylguanosine capped) and the least efficiently translated (SL-containing monomethylguanosine capped) was compressed, i.e., the ratio (least efficient/most efficient) varied from 0.13 to 0.22. Quantitation of [^{32}P]-labeled RNAs remaining after 30 min indicated that all of the RNAs had equivalent stabilities in vitro (data not shown).

speculation that *trans*-splicing could play some regulatory role in the control of gene expression or that specific classes of proteins might be synthesized from SL-containing mRNAs. In this regard, there is no evidence to date in support of these notions, either in *C. elegans* or in *A. lumbricoides* (for recent discussion, see Nilsen, 1993).

What then is the biological function of *trans*-splicing in nematodes? Blumenthal and colleagues have previously shown that SL addition (or absence) is not obligatory for the expression of certain specific genes in *C. elegans* because, using transformation, they were able to convert a normally *trans*-spliced mRNA into a functional non-*trans*-spliced mRNA (and vice versa) (Conrad et al., 1991, 1993). New insight into the role of *trans*-splicing in *C. elegans* has come from the recent discovery of multicistronic transcription units (operons) (Spieth et al., 1993). Indeed, it is now evident that such transcription units are remarkably prevalent in the *C. elegans* genome and that *trans*-splicing is used to process the primary transcripts (Zorio et al., 1994). As noted in the Introduction, *C. elegans* is unusual in that

it possesses two SL RNAs, SL1, common to all nematodes studied to date, and SL2, unique to *C. elegans*. The evidence indicates that SL2 *trans*-splicing is confined to downstream mRNAs in multigenic transcription units (Zorio et al., 1994). SL1 can also be added to such downstream mRNAs (Hengartner & Horvitz, 1994), but the rules that govern the choice between SL1 and SL2 addition remain to be deciphered. It has been postulated that the primary role of SL1 *trans*-splicing is to truncate unwieldy 5′ untranslated sequences or to remove in-frame upstream initiation codons from 5′ terminal mRNAs in operons or from monocistronic mRNAs (reviewed in Blumenthal, 1995).

It is tempting to speculate that *trans*-splicing serves the same functions in nematodes other than *Caenorhabditis* spp. Because no equivalent of SL2 has been found in these organisms, it seems possible that SL1 serves both functions. Unfortunately, this notion is difficult to verify experimentally, because nothing is known about mRNA transcription units in any other nematode. Furthermore, the experimental tool (transformation) used in the analysis of transcription units in *C. elegans* is not available in these organisms.

The cell-free translation system we have described should prove useful in dissecting components involved in nematode protein synthesis. In this regard, it will be of interest to investigate the factor(s) involved in cap recognition. In *Xenopus* oocytes, 2,2,7-trimethylguanosine caps serve as part of a bipartite nuclear localization signal for U snRNPs and the presence of a hypermethylated cap causes nuclear retention of synthetic mRNAs (see Izaurralde & Mattaj, 1995, for recent review and references). Clearly, such caps cannot serve this particular function in nematodes because mRNAs containing trimethylguanosine caps must be exported to the cytoplasm for translation. Furthermore, extracts from higher eukaryotic cells translate mRNAs with hypermethylated caps very poorly (Darzynkiewicz et al., 1988). Conversely, and not surprisingly, the *A. lumbricoides* extract translates such mRNAs quite efficiently. This observation, coupled with the fact that monomethyl capped mRNAs are also translated, raises the possibility that cap recognition in nematodes may be more complex than in other systems. In this regard, the apparent functional interplay between the SL sequence and the trimethylguanosine cap in translational efficiency is intriguing and suggests the existence of a factor(s) that recognizes these two elements in concert. In terms of SL recognition, we have previously described a transcription factor that binds to the 22-nt SL sequence as DNA; however, this factor does not bind to RNA (Hannon et al., 1990a; Denker & Nilsen, 1994). A requirement for a factor that binds the 22-nt sequence as RNA would help to explain the extraordinary sequence conservation of the SL among diverse nematodes (Nilsen, 1993). We are currently employing a variety of approaches in an attempt to identify such a factor in *A. lumbricoides* extracts.

The fact that SL addition can affect translational efficiency raises an interesting (if teleological) problem, i.e., is *trans*-splicing used to enhance protein synthesis or has the nematode translation apparatus evolved to accommodate *trans*-spliced mRNAs? Because *trans*-splicing appears to be required in the resolution of multigenic transcription units both in nematodes and in trypanosomes (reviewed in Nilsen, 1995), we believe the latter scenario to be much more likely.

MATERIALS AND METHODS

Preparation of extracts and micrococcal nuclease treatment

Whole-cell extracts were prepared from synchronous 32-cell *A. lumbricoides* as described (Maroney et al., 1990b), with the following modifications. KCl was not added to the initial homogenate and the KCl concentration in dialysis buffer was 50 mM. To make extracts message-dependent, $CaCl_2$ was added to 1 mM, and 200-μL aliquots were treated with 38 units of micrococcal nuclease (Worthington Biochemical Corp.) for 2.5 min at 20 °C. Nuclease digestion was stopped by addition of EGTA to a final concentration of 2 mM.

Protein synthesis assays and gel electrophoresis

Translation reactions contained in 20 μL, 10 μL extract, 125 mM KOAc, 2.75 mM MgOAc, 0.5 mM GTP, 0.5 mM ATP, 10 mM creatine phosphate, 50 μg/mL creatine phosphokinase, 2 mM DTT, 50 μM amino acids minus methionine (Promega), 20 μCi [^{35}S]-methionine (NEN), and 10 mM Tris, pH 7.6. Incorporation of labeled methionine was monitored as described (Weber et al., 1976). Analysis of labeled proteins on 12.5% denaturing one-dimensional gels was as described (Weber et al., 1976). For two-dimensional gels, the Multiphor II system (Pharmacia) was used. The first dimension (isoelectric focusing) was run on an Immobiline Dryplate (pH 4–7) and the second dimension on a 15% homogeneous ExcelGel per the manufacturer's instructions.

Hybrid-arrest and hybrid selection

For hybrid-arrest, ~3 μg of polyadenylated RNA (*A. lumbricoides* or HeLa) was annealed to 100 ng of either of two oligodeoxynucleotides (identical or complementary to the 22-nt SL sequence) in 100 mM KCl, 10 mM Tris, pH 7.6. Preannealed RNA was then added to message-dependent extracts to reach a final concentration of 25 μg/mL.

For hybrid selection, two oligodeoxynucleotides (identical in sequence to those above) were synthesized with a modifiable C12 linker and were biotinylated as described (Yu et al., 1993). One-hundred seventy-five nanograms of the biotinylated oligodeoxynucleotides (sense or antisense) were then separately annealed as described above to ~30 μg of *A. lumbricoides* polyadenylated RNA. Selected RNAs were recovered by binding to preblocked (Yu et al., 1993) streptavidin-

agarose (Sigma) beads. Binding was for 90 min at 4 °C in 300 mM NaCl, 0.1% NP-40, 20 mM Tris, pH 7.5. The beads were then recovered by centrifugation and washed three times with binding buffer. Washed beads were then incubated in 2 mg/mL proteinase K, 0.5% SDS, 100 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 7.5, for 45 min at 65 °C prior to a 20-min incubation at 80 °C to release bound RNAs. Released RNAs were recovered by phenol extraction and ethanol precipitation. Recovered RNAs were analyzed on denaturing northern blots using random primed cDNA probes (gene 4-*trans*-spliced and gene 9-non-*trans*-spliced). These cDNAs were previously characterized in an extensive analysis of *trans*-spliced and non-*trans*-spliced *A. lumbricoides* mRNAs (Hannon et al., 1990b).

Preparation and translation of synthetic mRNAs

For analysis of translation of specific mRNAs, a full-length cDNA clone (730 bp, gene 12) (Hannon et al., 1990b) was used. This cDNA was derived from an mRNA that is normally *trans*-spliced and encodes a protein of ~16.3 kDa. PCR was used to fuse the T3 promoter to the cDNA such that in vitro transcription would begin either with the first base of the SL sequence or the base immediately 3′ of the sequence, and both PCR products were inserted into the pSP64 Poly(A) vector (Promega). In vitro transcription with T3 RNA polymerase yielded polyadenylated RNAs plus or minus the SL sequence. m7GpppG or m2,2,7GpppG caps (Darzynkiewicz et al., 1990) (cap10: GTP1) were used to prime transcription of both constructs. Synthesis was quantitated by incorporation of [α^{32}P] GTP (NEN) and efficiency of capping was determined to be ~90% by two-dimensional TLC (Maroney et al., 1990b).

Alteration of the SL sequence in the gene 12-SL plus polyA+ construct was achieved by moving the entire construct into pBSM13+ (Stratagene) prior to site-directed mutagenesis as described (Kunkel et al., 1987; Viera & Messing, 1987). Resultant mutant sequences (in bases 7–16 of the SL) were: mutant 1, GCACTGTCCT; mutant 2, CACCATTCCC; wt, ATTACCCAAG.

Synthetic RNAs were added at a concentration of 10 or 20 μg/mL to assess translational efficiency.

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