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trans-Spliced *Caenorhabditis elegans* mRNAs Retain Trimethylguanosine Caps

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The nematode *Caenorhabditis elegans* has an unusual small nuclear RNA, containing a 100-nucleotide RNA molecule, spliced leader RNA, which donates its 5′ 22 nucleotides to a variety of recipient RNAs by a *trans*-splicing reaction. The spliced leader RNA has a 5′ trimethylguanosine (TMG) cap, which becomes the 5′ end of *trans*-spliced mRNAs. We found that mature *trans*-spliced mRNAs were immunoprecipitable with anti-TMG cap antibodies and that TMG-containing dinucleotides specifically competed with the *trans*-spliced mRNAs for antibody binding. We also found that these mRNAs retained their TMG caps throughout development and that the TMG-capped mRNAs were polysome associated. Since the large majority of *C. elegans* mRNAs are not *trans*-spliced, the addition of the spliced leader and its TMG cap to a limited group of recipient RNAs may create a functionally distinct subset of mRNAs.

In the nematode *Caenorhabditis elegans*, approximately 10% of mRNAs receive a 5′ noncoding exon (the spliced leader [SL]) by *trans*-splicing (1, 2, 9). The 22-nucleotide SL corresponds to the 5′-most portion of its precursor, the SL RNA (9). Previous work has shown that the SL RNA has a 2,2,7-trimethylguanosine (TMG) cap and is associated with the Sm antigen (3, 17, 18). These two features are hallmarks of small nuclear RNAs, the RNA components of the small nuclear ribonucleoprotein particles that are required for splicing standard (*cis*-spliced) introns. Sm is bound to the intron region of the SL RNA and is therefore not associated with mature mRNAs (17). The 5′ TMG cap, on the other hand, would be present on mature mRNAs unless it were modified or removed during or after splicing. Our laboratory previously reported (17) that mature, *trans*-spliced mRNAs were not precipitated with anti-TMG antibodies, whereas small nuclear RNAs were efficiently precipitated in the same experiments. However, we have now found that mature, *trans*-spliced mRNAs are efficiently immunoprecipitated if we shorten them by using a RNase H cleavage protocol before exposure to the antiserum. We demonstrate that *trans*-spliced mRNAs retain their TMG caps throughout development and that these TMG-capped mRNAs are associated with polysomes.

MATERIALS AND METHODS

Materials. RNase H and T4 polynucleotide kinase were purchased from Bethesda Research Laboratories, Inc. m^7GpppG (MMG) was obtained from Pharmacia, Inc. $m_2^{2,7}$GpppG (DMG) and $m_3^{2,2,7}$GpppG (TMG) were gifts from Edward Darzynkiewicz and Stan Tahara. The K121 anti-TMG antibody was a gift from Adrian Krainer. The oligonucleotides used were as follows: act 73, which is complementary to all four actin mRNAs from nucleotides 73 through 94 downstream of the translation initiation sites (10); act1/3, which is complementary to *act-1* and *act-3* mRNAs from nucleotides 4 to 23 upstream of the translation initiation site; act4, which is complementary only to *act-4* mRNA from nucleotides 12 to 31 upstream of the translation initiation site; and SL-3′, which is complementary to the 3′ portion of the SL RNA from nucleotides 71 to 89 (9).

Preparation of RNA. *C. elegans* (strain Bristol-N2) was grown in liquid culture and harvested by the method of Sulston and Brenner (15). Total RNA was prepared from a developmentally mixed population of worms or, in some cases, from each developmental stage of worms: embryos, larvae (L1, L2, L3, L4, and dauer larvae), and adults. All RNA preparations were made by using the guanidinium hydrochloride extraction protocol of Macleod et al. (12).

Oligonucleotide-directed RNase H cleavage and anti-TMG immunoprecipitation. A 10-μg sample of each RNA preparation was mixed with 1 μg of the oligonucleotide act73. This mixture was allowed to anneal and then treated with RNase H (5). After deproteinization and ethanol precipitation, this mixture was heated to 75°C and quick-cooled in an ice water bath. Anti-TMG immunoprecipitation was performed, and the resultant pellets and supernatants were extracted for RNA, which was electrophoresed, blotted, and probed as previously described (17).

Immunocompetition experiment. For each competitor, 60 μg of RNA prepared from a developmentally mixed population of *C. elegans* was subjected to oligonucleotide-directed RNase H cleavage. After deproteinization, the RNA was apportioned to six tubes, mixed with the indicated amounts of cap dinucleotides (MMG, DMG, and TMG), heated, and quick-cooled in an ice water bath. Subsequent immunoprecipitation and Northern (RNA) analysis were performed as described above. The blot was then hybridized with ^{32}P-labeled act1/3 probe and SL-3′ probe. The intensity of each band in the autoradiographs was quantitated by densitometry.

Polysome preparation. Polysomes were prepared from young adult *C. elegans*, using conditions recommended for purification of plant polysomes by Larkins (11). A total of 10^5 worms were routinely used for each sucrose gradient. After harvest, worms were washed repeatedly with M9 buffer, frozen in liquid nitrogen, and ground into fine powder with a prechilled mortar and pestle in the presence of liquid nitrogen. The worm powder was then suspended in 3 volumes of polysome buffer [0.2 M Tris hydrochloride (pH 8.5), 35 mM $MgCl_2$, 200 mM KCl, 5 mM dithiothreitol, 15 mM

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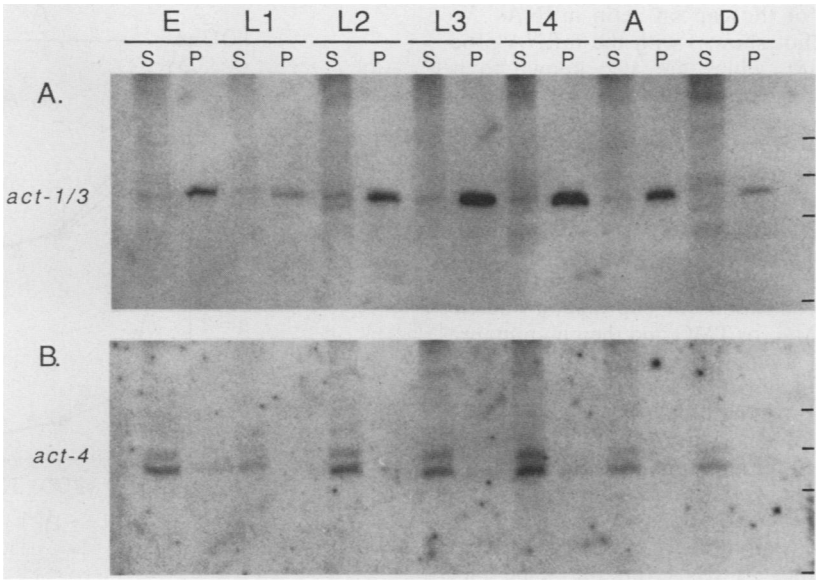

FIG. 1. Anti-TMG immunoprecipitation of actin mRNAs isolated from staged nematodes. Total RNA prepared from each developmental stage of *C. elegans* (E, embryos; L1, L2, L3, and L4, larval stages; A, adult hermaphrodite; D, dauer larvae) were subjected to oligonucleotide-directed RNase H cleavage, anti-TMG immunoprecipitation, and Northern blotting. The blot was hybridized with ^{32}P-labeled act1/3 probe (A); after removal of the act1/3 probe, the same blot was probed with ^{32}P-labeled act4 (B). Lanes: S, RNA in the supernatant; P, RNA in the precipitate after anti-TMG immunoprecipitation. Bars indicate positions of end-labeled *Msp*I fragments of pBR322, which were (from top to bottom) 180, 147, 123, and 90 nucleotides long.

ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*^{1},*N*^{1}-tetraacetic acid (EGTA), 0.5% Nonidet P-40], and debris and organelles were sedimented at 4°C for 2 min at 5,000 rpm in a Beckman JS13 rotor (4,000 × *g*). This supernatant was layered directly on a 20 to 50% (wt/wt) linear sucrose gradient in polysome buffer. Gradients were centrifuged in a Beckman SW27 rotor at 25,000 rpm for 2 h at 4°C. Fractions of 1.4 ml were collected, and optical density at 254 nm was scanned during fractionation. In a separate tube, the postnuclear supernatant was adjusted with EDTA to a final concentration of 100 mM, incubated on ice for 15 min with occasional mixing, and layered on a sucrose gradient as described above. For preparation of polysomal RNA, gradient fractions in the region delineated by the two arrows located on the bottom of the polysome profile (see Fig. 3A) were pooled, and 1/25 volume of 0.5 M EDTA, 1/10 volume of 10% sodium dodecyl sulfate, and 1/400 volume of proteinase K (20 mg/ml) were added. This solution was incubated at 37°C for 30 min, extracted with phenol-chloroform, and precipitated with ethanol. The RNA pellet was redissolved and reprecipitated from an LiCl solution (0.1 M Tris [pH 7.4], 0.2 M LiCl, 2 mM EDTA) to eliminate trace amounts of sodium dodecyl sulfate. These RNA pellets were suspended in TE buffer (pH 8.0).

RESULTS

***C. elegans trans*-spliced mRNAs retain TMG caps throughout development.** In our previous experiments, SL RNA was efficiently precipitated by anti-TMG cap antiserum, whereas *trans*-spliced mRNAs were not (17). Since SL RNA is a much smaller and presumably more compact molecule than are mature mRNAs, we became concerned that the difference between the behavior of SL RNA and the mRNAs in this assay might be due to their size difference rather than their having a different cap structure. To circumvent this possible problem, *trans*-spliced actin mRNAs were shortened to a homogeneous population of 5′ ends similar in size to small nuclear RNAs.

The mRNAs encoded by *act-1*, *act-2*, and *act-3* are *trans*-spliced, whereas the mRNA encoded by *act-4* is not (9). A DNA oligonucleotide, act73, complementary to a region in the 5′ portion of all four species of actin mRNA, was incubated with *C. elegans* RNA. This mixture was then treated with RNase H, which specifically cleaves DNA-RNA hybrids and should generate 5′ actin mRNA fragments of about 135 nucleotides. After phenol extraction and ethanol precipitation, the resultant RNA was immunoprecipitated by anti-TMG antibodies coupled to protein A-Sepharose and analyzed on RNA blots. The initial experiments demonstrated that at least a portion of these shortened mRNAs were in the precipitated fraction, suggesting that they were TMG capped. Furthermore, if these shortened *act-1* and *act-3* mRNAs (which are identical in sequence) were first heated and quickly cooled before immunoprecipitation, most of the *act-1* and *act-3* mRNAs was present in the precipitate, whereas the *act-4* mRNA remained in the supernatant (data not shown).

These initial experiments were performed with *C. elegans* RNA prepared from a population of mixed developmental stages. Since a stage might have been under- or overrepresented in that population, RNA was prepared from synchronized populations of nematodes in embryonic, larval, and adult stages of development. These RNA preparations were analyzed as described above. The RNA blot analyses of these immunoprecipitations are shown in Fig. 1. In all RNA preparations, most of the *act-1* and *act-3* mRNAs was present in the pellet, whereas the *act-4* mRNA was present in the supernatant. Densitometric analysis confirmed that despite differences in the levels of actin mRNA during development, the ratio of *act-1* and *act-3* mRNAs in the pellet to that in the supernatant was roughly equivalent in all RNA preparations (not shown). Thus, there was no apparent

developmental variation of the cap on actin mRNAs. We obtained similar results (not shown) with the mRNA products of the *gpd* genes (8), which are also known to be *trans*-spliced (1). Hence, it seems generally true that the TMG caps placed on *C. elegans* mRNAs by *trans*-splicing are not removed.

Immunocompetition experiments fail to distinguish between the caps on SL RNA and the *act-1* and *act-3* mRNAs. To determine whether *trans*-spliced RNAs were indeed capped with TMG, we performed immunocompetition experiments. Shortened mRNAs, prepared as described above, were mixed with various concentrations of cap analog dinucleotides of either MMG, DMG, or TMG and then immunoprecipitated with a constant amount of anti-TMG antibodies. After incubation, the resultant pellets and supernatants were analyzed on RNA blots probed with radiolabeled oligonucleotides that hybridized to the shortened RNAs. Densitometric analysis of resultant autoradiographs revealed that the precipitabilities of *act-1* and *act-3* mRNAs were inhibited only moderately by high concentrations of MMG or DMG cap, whereas TMG cap was a relatively efficient competitor (Fig. 2A). Analysis of the SL RNA (on the same blot) showed that the efficiency of precipitation of SL RNA was the same as for the shortened *act-1* and *act-3* mRNAs regardless of which competitor was used (Fig. 2B). Shortened *act-4* mRNA, which lacks SL, was not precipitated even in the absence of competitor (Fig. 1). Since the SL RNA is TMG capped and since shortened *act-1* and *act-3* mRNAs behaved identically to it in this assay, we conclude that mature *trans*-spliced mRNAs remain TMG capped.

The TMG-capped mRNAs are associated with polysomes. To determine whether the TMG-capped mRNAs are translated, we immunoprecipitated RNA isolated from polysomes. Polysomes were prepared by a procedure designed to minimize RNase-catalyzed degradation (11) due to the high level of degradative enzymes present in whole-worm extracts. Because the experiment was performed at the preparative level, the polysome gradient contained a broad peak rather than incremental polysome peaks (Fig. 3A). Northern blot analysis of individual fractions of this gradient showed that the amounts of actin mRNA and vitellogenin mRNA (a non-*trans*-spliced control) correlated with the optical density in the polysome peak. When a similar gradient was performed in the presence of high concentrations of EDTA, the polysome peak was missing (Fig. 3A), and no mRNAs were present in this region of the gradient (data not shown). Since these fractions of the non-EDTA gradient were enriched for mRNA but were lacking in spliceosomal components, and since EDTA eliminated virtually all detectable RNA from these fractions, we believe that they contained mostly or entirely polysomes. RNA extracted from the polysome fraction and total RNA isolated from the initial worm lysate were subjected to oligonucleotide-directed RNase H cleavage, anti-TMG immunoprecipitation, and Northern analysis as described above. The same percentage of *act-1* and *act-3* mRNAs was immunoprecipitable from polysomal preparations as from total RNA (Fig. 3B). The fact that these *trans*-spliced mRNAs were no less efficiently immunoprecipitated from polysomal RNA preparations than they were from total RNA demonstrates that TMG-capped mRNAs are associated with polysomes. Thus, it is very likely they are translated in this form.

DISCUSSION

The data in this and the accompanying paper (19) demonstrate that *trans*-spliced actin mRNAs in *C. elegans* retain the TMG caps that they receive along with the 5′ 22 bases of the SL RNA. Although a few viral RNAs are known to have TMG caps (7, 20), our results provide the only known example of TMG-capped cellular mRNAs. The possibility that we have failed to detect the presence of a small amount of MMG-capped *act-1* and *act-3* mRNAs cannot be eliminated. However, in all of our experiments we find the shortened actin transcripts to be precipitated to the same extent as SL RNA (Fig. 2 and 3) and U2 RNA (unpublished observations), which are both known to be TMG capped. Furthermore, the TMG-capped mRNAs are associated with polysomes.

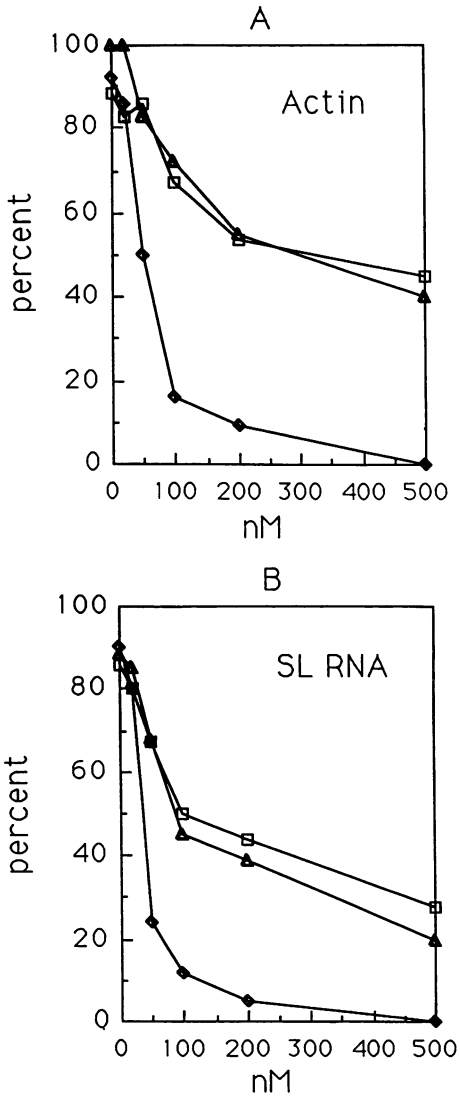

FIG. 2. Anti-TMG immunoprecipitation of actin mRNAs: competition by capped dinucleotides. Total RNA prepared from a developmentally mixed population of *C. elegans* was subjected to oligonucleotide-directed RNase H cleavage, anti-TMG immunoprecipitation, and Northern blotting in the presence of the indicated concentrations of capped dinucleotides (see Materials and Methods). The percentage of total radioactive counts, which were quantitated by densitometry from the autoradiograph, was plotted versus the final concentration of cap dinucleotide present in the immunoprecipitation. After removal of the act1/3 probe (A), the blot was hybridized with the SL-3′ probe (B). Competitors used were synthetic dinucleotides of the form X(5′)ppp(5′)G: △, X = MMG; □, X = DMG; ◇, X = TMG.

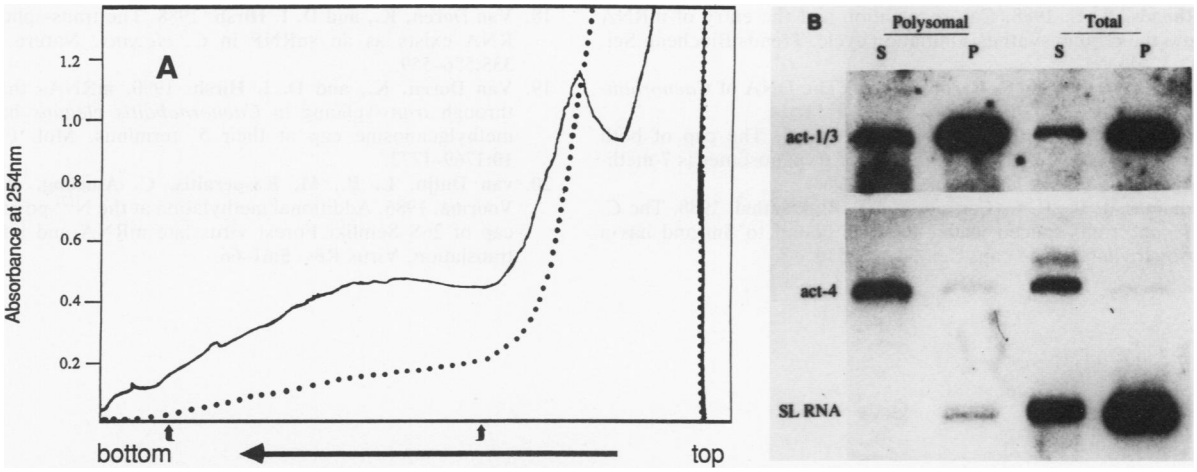

FIG. 3. Anti-TMG immunoprecipitation of polysomal actin mRNAs. Polysomes were prepared as described in Materials and Methods. (A) UV absorbance profile. Symbols: ——, no EDTA; ·····, 100 mM EDTA; ◆ delineates gradient fractions used for the polysomal RNA preparation. (B) Anti-TMG immunoprecipitation of polysomal RNA. Polysomal RNA and total RNA from the same worm population were subjected to oligonucleotide-directed RNase H cleavage, anti-TMG immunoprecipitation, and Northern analysis. The upper panel shows the blot probed with act1/3; the lower panel shows the same blot, after removal of the act1/3 probe, probed with a mixture of act4 and SL-3′.

Normally, recognition of the MMG cap by eIF-4E is the first step in mRNA-ribosome association (14). Thus, the presence of a TMG cap might be expected to have repercussions for the initiation of translation. Whether a special mechanism for translating *trans*-spliced mRNAs exists in *C. elegans* is not yet clear. It has recently been demonstrated that a TMG-capped β-globin mRNA, synthesized in vitro, is translatable in a reticulocyte lysate albeit at reduced efficiency (4). We can envision at least two specialized mechanisms for the translation of TMG-capped mRNAs: first, *C. elegans* may have a unique initiation factor that binds preferentially to TMG-capped mRNAs; second, the sequence of the SL may allow cap-independent initiation of protein synthesis, perhaps by interaction with ribosomal sequences.

A rationale for the existence of *trans*-splicing in nematodes has not yet been offered. The creation of a subset of mRNAs containing both a TMG cap and a unique 22-nucleotide sequence could create a functionally distinct subset of mRNAs. For instance, these mRNAs might have a specialized subcellular localization, an unusual stability, or a unique mode of translation. Any of these processes could be regulated by a molecule that interacts with the TMG cap, the SL sequence, or both.

In contrast to *trans*-spliced *C. elegans* mRNAs, typanosome mRNAs (all of which are presumed to be *trans*-spliced) are capped with MMG (6, 13, 16). Since there are several differences between nematode and trypanosome *trans*-splicing (2), the significance of the different cap structures is not clear. Despite this difference, it seem worthwhile to search for nuclear mRNA *trans*-splicing in other eucaryotes by using anti-TMG antibodies.

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