Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 709–713, January 1990
Biochemistry

Transcription and cap trimethylation of a nematode spliced leader RNA in a cell-free system

(RNA polymerase II/small nuclear RNAs/capping)

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Communicated by Joan A. Steitz, October 30, 1989 (received for review April 19, 1989)

ABSTRACT **Maturation of a fraction of mRNAs in nematodes involves the acquisition of a common 5′ terminal spliced leader sequence derived from a nonpolyadenylylated spliced leader RNA by trans splicing. We have developed a cell-free system prepared from *Ascaris lumbricoides* embryos that accurately and efficiently synthesized the spliced leader RNA of *A. lumbricoides*. Transcription of the spliced leader RNA was catalyzed by RNA polymerase II, and the majority of the spliced leader RNAs synthesized *in vitro* possessed a trimethylguanosine cap structure identical to that found on *in vivo*-synthesized spliced leader RNA.**

In nematodes, a fraction of mRNAs contain an identical 22-nucleotide (nt) spliced leader (SL) sequence at their 5′ ends. Several lines of evidence indicate that the SL is donated from a small nonpolyadenylylated transcript (SL RNA) through a transsplicing reaction (for review, see refs. 1 and 2). SL RNAs have been characterized in some detail in the free-living nematode *Caenorhabditis elegans* and in the parasitic nematodes *Brugia malayi* and *Ascaris lumbricoides* (3–5). These RNAs are of similar size (approximately 100 nt) and all contain the 22-nt SL sequence at their 5′ ends.

Studies in several laboratories have shown that these SL RNAs bear striking similarities to the vertebrate U small nuclear RNAs (snRNAs) known to be essential for cis splicing. They contain trimethylguanosine ($m_3^{2,2,7}G$) cap structures and functional binding sites for Sm, a group of related proteins associated with small nuclear ribonucleoproteins (5–8). Furthermore, like U snRNAs, nematode SL RNAs are transcribed by RNA polymerase II (5). Extensive *in vivo* analysis of U snRNA transcriptional control elements has revealed that snRNA genes have unique features that distinguish them from other genes transcribed by RNA polymerase II (for review, see ref. 9). However, transcription of these genes has generally been inaccessible to the biochemical manipulations made possible with *in vitro* systems.

Here, we describe a cell-free extract prepared from *A. lumbricoides* embryos that efficiently synthesized the SL RNA of *A. lumbricoides*. Direct analysis of SL RNA transcripts synthesized *in vitro* indicated that they contained authentic 5′ and 3′ termini. Furthermore, a substantive proportion of the SL RNAs synthesized *in vitro* contained a trimethylguanosine cap structure. The availability of this cell-free system will permit a detailed examination of the sequence elements that direct both initiation and 3′ end formation of nematode SL RNAs. In addition, this system may provide an opportunity to biochemically characterize the components that catalyze SL RNA synthesis and cap trimethylation.

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MATERIALS AND METHODS

Preparation of Extracts. Fertilized *Ascaris* eggs capable of synchronous division were prepared from uteri of mature females as described (10). To prepare extracts, fertilized eggs were allowed to develop to the 32-cell stage by incubation at 30°C. After removal of egg shells by treatment with hypochlorite, the eggs were concentrated by centrifugation and washed several times in ice-cold isotonic phosphate-buffered saline. After two additional washes in 10 mM KCl/1.5 mM $MgCl_2$/0.5 mM dithiothreitol/10 mM Tris·HCl, pH 7.9 (buffer A), the eggs were resuspended in a minimal amount of buffer A and homogenized by 20 strokes in a Dounce homogenizer. The homogenate was made 200 mM KCl by the addition of 0.25 vol of 1 M KCl, mixed gently for 1 min, and centrifuged at 15,000 rpm for 10 min in an SS 34 rotor (Sorvall). The resultant supernatant was dialyzed versus 100 vol of 100 mM KCl/1 mM dithiothreitol/0.2 mM EDTA/20% (vol/vol) glycerol/20 mM Tris·HCl, pH 7.9 for 4 hr. After dialysis, the extract was centrifuged at 10,000 rpm for 15 min. The supernatant was divided into 100-μl aliquots and stored at −90°C.

***In Vitro* Transcription.** *In vitro* transcription reaction mixtures (25 μl) contained 15 μl of extract, 3.0 mM $MgCl_2$, 60 mM KCl, 2 mM dithiothreitol, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 50 μM UTP, 10 μCi [α-^{32}P]UTP, 20 mM creatine phosphate, 12 mM Tris·HCl (pH 7.9), and 500 ng of supercoiled template. After a 2-hr incubation at 30°C, reaction mixtures were diluted to 250 μl in 250 mM NaOAc/20 mM Tris·HCl, pH 7.5/1 mM EDTA/0.25% SDS, digested with proteinase K (100 μg/ml) for 30 min at 37°C, and extracted with phenol/chloroform, 1:1 (vol/vol). Reaction products were analyzed on 8 M urea/6% polyacrylamide gels. After electrophoresis, labeled transcripts were visualized by autoradiography. Transcription templates were produced by subcloning segments of the *Ascaris* 5S rRNA/SL RNA gene repeat (5) into pBS-m13$^+$ (Stratagene).

To construct a phage T7 transcription template encoding the *A. lumbricoides* SL RNA, the T7 promoter sequence (11) was fused to the coding sequence of the SL RNA such that transcription by T7 RNA polymerase would initiate synthesis with the first guanosine residue of the 22-nt sequence. This was accomplished by using the polymerase chain reaction technique with two oligodeoxynucleotides, one containing the T7 promoter and the first 10 nt of the SL sequence, the other complementary to the 3′ end of the *A. lumbricoides* SL RNA coding sequence. This construct was inserted into pBS-m13+ such that transcription by T7 RNA polymerase yielded a 116-base synthetic transcript containing 109 bases

Abbreviations: nt, nucleotide(s); SL, spliced leader; snRNA, small nuclear RNA.

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of SL RNA sequence and 7 bases of polylinker sequence (GGGGAUC).

Preparation of *A. lumbricoides* SL RNA Labeled *in Vivo*. *A. lumbricoides* embryos, obtained as described above, were allowed to develop for 10 days at 30°C (L1 stage) at which point the larval worms were freed from their egg shells by gentle Dounce homogenization after hypochlorite treatment. Freed worms were concentrated by centrifugation (1000 × *g* for 1 min) and resuspended in phosphate-free Dulbecco's modified Eagle's medium (GIBCO) containing carrier-free [^{32}P]orthophosphate (5 mCi/ml; ICN). After a 3-hr incubation at 30°C, the worms were again collected by centrifugation and the resulting pellet was lysed by addition of 1% SDS/250 mM NaOAc/1 mM EDTA/20 mM Tris·HCl, pH 7.5. After a 30-min incubation at 65°C, the suspension was diluted 1:2 and proteinase K (200 μg/ml) was added. After a 2-hr incubation at 30°C, the lysate was extracted several times with phenol/chloroform and twice with chloroform alone. The resulting aqueous phase was made 66% (vol/vol) ethanol and DNA was removed by "spooling." Precipitated RNA was recovered by centrifugation and resuspended, and labeled RNAs were hybrid-selected by using a single-stranded M13 clone containing sequence complementary to the *A. lumbricoides* SL RNA. Selected RNA was eluted from the DNA by heat denaturation and fractionated on a denaturing polyacrylamide gel. A single labeled band, corresponding to SL RNA, was recovered from this gel.

RNA Secondary Analysis. For nearest-neighbor analysis, individual oligonucleotides were recovered from fingerprints and digested with RNase T2 or nuclease P1, as described (12). Digestion products were analyzed by one-dimensional thin layer chromatography on PEI cellulose plates (Brinkman) (13).

For analysis of capped oligonucleotides, transcripts as detailed in the text were digested to completion with RNase T2 and digestion products were resolved in two dimensions on cellulose plates (Kodak), as described (14). Capped oligonucleotides were recovered and further digested with nuclease P1 and nucleotide pyrophosphatase (Sigma, P7383). Digestion products were resolved in two dimensions on cellulose (see above) in the presence of unlabeled marker nucleotides (see text).

To compare capped oligonucleotides present on SL RNAs synthesized *in vitro* or *in vivo*, transcripts were digested to completion with either RNase T2 or nuclease P1. Digestion products were resolved in two dimensions on cellulose plates, as detailed (15).

RESULTS

Transcription of *A. lumbricoides* SL RNA in Embryo Extract. Using nuclear run-on experiments, it has been shown (5) that the *A. lumbricoides* SL RNA is actively synthesized in early embryos. The SL RNA gene was presumably transcribed by RNA polymerase II since SL RNA synthesis was inhibited by low levels of α-amanitin. To assay SL RNA synthesis *in vitro*, we prepared extracts from synchronous 32-cell *A. lumbricoides* embryos by using a variety of conditions. In transcription reaction mixtures containing whole-cell extract, we observed the appearance of an ≈110-base transcript in response to addition of cloned templates containing the *A. lumbricoides* SL RNA gene (Fig. 1, lanes 3 and 5). Synthesis of this transcript was dependent upon the inclusion of the entire SL RNA coding sequence and was completely inhibited by α-amanitin at 1 μg/ml (Fig. 1, lanes 4 and 6). As expected, the synthesis of 5S rRNA was insensitive to this level of inhibitor (Fig. 1, lanes 2 and 4). The size of the *in vitro* transcript, specificity of template utilization, and sensitivity to α-amanitin suggested that the SL RNA gene of *A. lumbricoides* was faithfully transcribed by RNA polymerase II in the embryo extract.

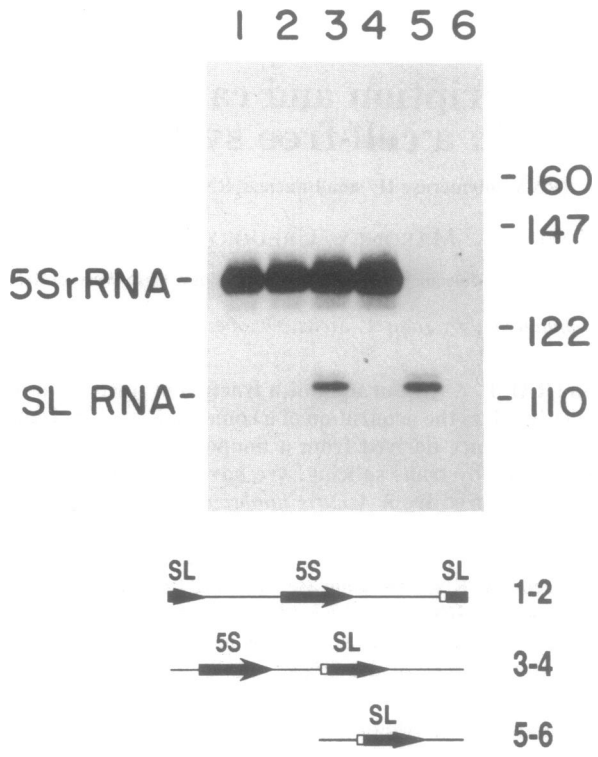

FIG. 1. Transcription of the *A. lumbricoides* SL RNA in a cell-free system. Supercoiled plasmid templates, as schematically depicted, were included in reaction mixtures containing [^{32}P]UTP in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of α-amanitin (1 μg/ml). The indicated sizes correspond to the mobility of labeled restriction fragments electrophoresed in parallel lanes.

RNA Fingerprint Analysis of *A. lumbricoides* SL RNA Synthesized *in Vitro*. To assess the accuracy of transcription, we used two-dimensional RNase T1 fingerprint analysis. For purposes of comparison, the *A. lumbricoides* SL RNA coding sequence was fused to a phage T7 promoter and transcribed by T7 RNA polymerase in the presence of each of the four α-^{32}P-labeled ribonucleoside triphosphates. Individual RNase T1-resistant oligonucleotides were recovered from two-dimensional fingerprints of these RNAs. Using RNA secondary analysis coupled with knowledge of the DNA sequence of the template, each oligonucleotide was identified by nearest-neighbor analysis. A fingerprint of the synthetic transcript labeled with [α-^{32}P]GTP is shown in Fig. 2*C*. Oligonucleotide a is pppGp and oligonucleotide b is CACUUGp, which contains a terminal guanosine derived from the vector.

A fingerprint of *A. lumbricoides* SL RNA synthesized in embryo extract is shown in Fig. 2*F*. A comparison of this fingerprint with that of the T7-synthesized RNA indicated that the SL RNA synthesized in extract contained a complement of RNase T1-resistant oligonucleotides that exactly corresponded to those of the T7 RNA with the following three exceptions: (*i*) the SL RNA synthesized in extract contained a capped oligonucleotide (spot 15), whose sequence was determined as described below; (*ii*) it contained neither pppGp nor other vector-derived sequences; and (*iii*) extract-synthesized SL RNA contained a 3′ terminal oligonucleotide $CACU_{OH}$ (spot 20) (see below). A comparison of this fingerprint with the fingerprint of SL RNA synthesized *in vivo* (Fig. 2*G*) indicated that these RNAs contained a strikingly similar pattern of fingerprint spots. In particular, the two RNAs appeared to share the same 5′ capped oligonucleotide and to terminate at the same 3′ position. The minor differences between these RNAs are discussed below.

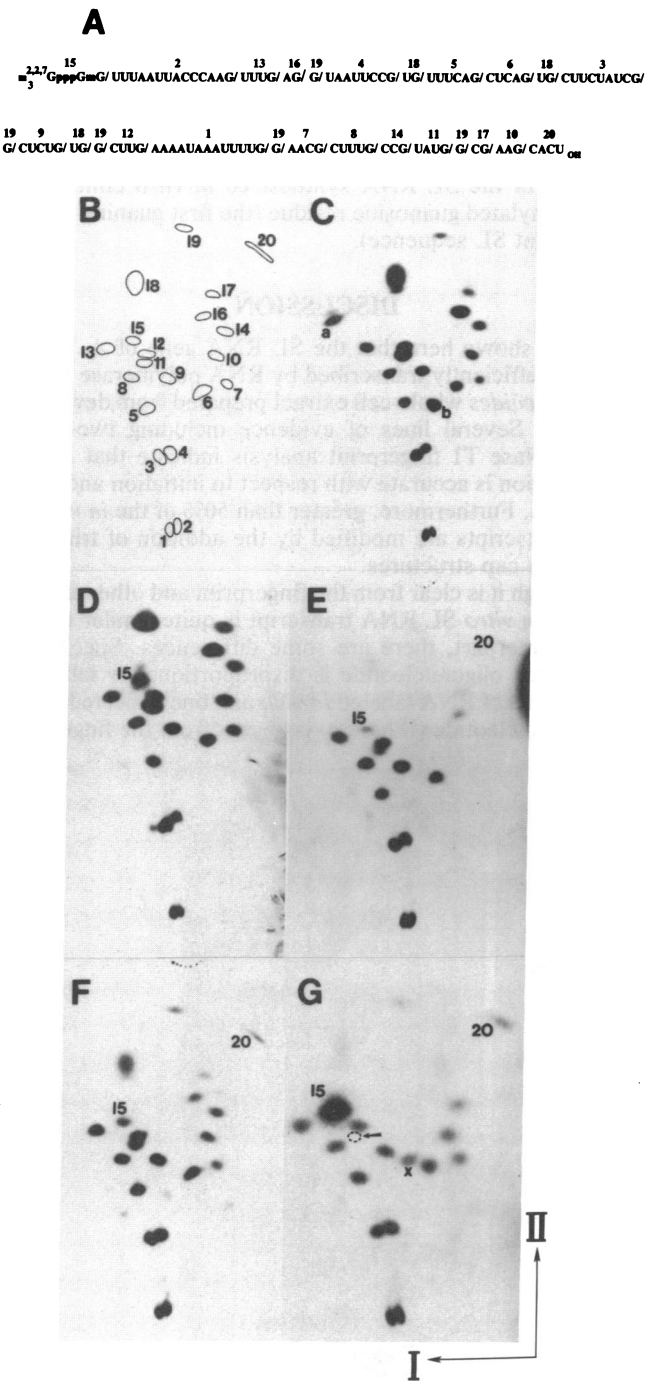

FIG. 2. RNase T1 fingerprint analysis of *A. lumbricoides* SL RNA synthesized *in vitro* and *in vivo*. (*A*) Sequence of the *A. lumbricoides* SL RNA. Slashes separate oligonucleotides resulting from digestion of *in vitro*-synthesized SL RNA with RNase T1. Identification of the cap structure is described in the text and in Fig. 3. (*B*) Schematic representation of an RNase T1 fingerprint of *A. lumbricoides* SL RNA in which oligonucleotides are numbered with reference to *A*. Identification of oligonucleotides is described in the text. (*C*) RNase T1 fingerprint of a synthetic SL RNA synthesized by T7 RNA polymerase. Oligonucleotide a is pppGp and oligonucleotide b is CACUUGp, which contains vector sequence. (*D*) Fingerprint of SL RNA synthesized in extract in the presence of [α-^{32}P]GTP. (*E*) Fingerprint of SL RNA synthesized in extract in the presence of [α-^{32}P]UTP. (*F*) Fingerprint of SL RNA synthesized in extract in the presence of all four [α-^{32}P]rNTPs. (*G*) Fingerprint of SL RNA synthesized *in vivo* in the presence of [^{32}P]orthophosphate. SL RNA synthesized *in vivo* was prepared. Oligonucleotide X is not present on fingerprints of SL RNAs synthesized in extract and has not been identified. The dotted circle indicates the position of an expected oligonucleotide that was absent from this fingerprint. Arrows I and II indicate the first dimension (gel electrophoresis) and second dimension (homochromatography), respectively (12). Extract-synthesized SL RNAs used for fingerprint analysis were prepared from transcription reactions similar to those shown in Fig. 1, lane 5. After deproteinization, reaction products were precipitated with a monoclonal antibody directed against trimethylguanosine (16). Precipitated RNAs were then fractionated on denaturing polyacrylamide gels. After autoradiography, labeled RNAs were recovered from gels prior to two-dimensional fingerprint analysis.

Identification of 3′ and 5′ Terminal Oligonucleotides of *A. lumbricoides* SL RNA Synthesized *in Vitro*. It seemed likely that fingerprint spot 20 in Fig. 2 *B*, *E*, *F*, and *G* was the 3′ terminus of the *in vitro*-synthesized SL RNA, since this oligonucleotide was absent from fingerprints of both the T7-synthesized RNA and [^{32}P]GTP-labeled SL RNA synthesized in extract (Fig. 2 *C* and *D*) but was present in extract-synthesized RNAs labeled with [^{32}P]UTP (Fig. 2*E*), [^{32}P]-ATP, or [^{32}P]CTP (data not shown). To confirm the identity of this spot, it was excised from fingerprints of uniformly labeled extract-synthesized RNA and subjected to further analysis. The mobility of this oligonucleotide on a 20% polyacrylamide gel was unaltered by treatment with phosphatase, indicating that it had no terminal phosphate groups. Digestion with RNase T2 yielded 2 mol of Cp and 1 mol of Ap, whereas digestion with nuclease P1 yielded 1 mol of pU, 1 mol of pC, and 1 mol of pA. Again, taking into account the sequence of the template and the fact that all other oligonucleotides were identified, we concluded from these analyses that the SL RNA synthesized in extract terminated with the 3′ oligonucleotide $CACU_{OH}$ 108 nt downstream of the first base of the SL sequence. Two lines of evidence indicated that this represented authentic 3′-end formation *in vitro*. (*i*) A fingerprint spot with identical mobility was observed with *in vivo*-labeled SL RNA (Fig. 2*G*). (*ii*) Direct sequence analysis of [^{32}P]pCp-end-labeled SL RNA indicated that authentic SL RNA terminated in the sequence CACU (data not shown).

Similarly, the 5′ terminal oligonucleotide of the *in vitro*-synthesized SL RNA (spot 15) was identified by several criteria. This spot was not present in fingerprints of the T7 transcript (Fig. 2*C*) or on fingerprints of extract synthesized SL RNA labeled with [^{32}P]CTP or [^{32}P]ATP (data not shown) but was present on fingerprints of [^{32}P]GTP-labeled or [^{32}P]UTP-labeled SL RNA synthesized in extract (Fig. 2 *D* and *E*). Furthermore, when SL RNA labeled with [^{32}P]GTP was digested with RNase T1 and the digestion products were precipitated with a monoclonal antibody directed against trimethylguanosine cap structures (16), only this oligonucleotide was bound by the antibody (data not shown, and see below). We concluded from these analyses that spot 15 represented a capped oligonucleotide derived from the 5′ terminus of the SL RNA. This appeared to correspond to the authentic 5′ terminus of *A. lumbricoides* SL RNA since an oligonucleotide of identical mobility was present in the fingerprint of SL RNA labeled *in vivo* (compare Fig. 2 *F* and *G*); although in the fingerprint of *in vivo*-labeled SL RNA, the presumptive capped oligonucleotide had a much greater relative intensity than the corresponding spot in fingerprints of SL RNA transcribed in extract. We cannot presently explain this difference in intensity and have not been able to recover sufficient amounts of the *in vivo*-labeled spot for detailed secondary analysis. However, two additional lines of evidence suggested that the 5′ terminus of SL RNA was faithfully generated *in vitro*. Both *in vivo*- and *in vitro*-labeled SL RNAs were prepared and digested to completion with RNase T2 or nuclease P1 and the digestion products were resolved by two-dimensional thin layer chromatography (15). In both cases the capped oligonucleotides of the two RNAs migrated identically (data not shown).

Structure of the Cap on *in Vitro*-Synthesized SL RNA. To examine in detail the cap structure present on the SL RNA synthesized in the whole-cell extract, [α-^{32}P]GTP-labeled transcripts were precipitated with the monoclonal antibody specific for trimethylguanosine caps (16). This treatment resulted in the precipitation of approximately 60% of the SL RNA transcripts, suggesting that this fraction of the SL RNAs synthesized *in vitro* contained a trimethylguanosine cap structure (Fig. 3*A*, lanes 1 and 2). To confirm this interpretation and to determine the cap structure present on the RNA that was not immunoprecipitated, bound and unbound RNAs were digested to completion with RNase T2 (Fig. 3*B*, chromatographs 1 and 2) and digestion products were resolved in two dimensions (14). Capped oligonucleotides were recovered for further digestion with nuclease P1 and nucleotide pyrophosphatase, and the redigestion products were again resolved by two-dimensional thin layer chromatography (Fig. 3*B*, chromatographs 3 and 4). Upon digestion the capped oligonucleotides of antibody-bound and unbound RNAs yielded three products each. The three products generated from unbound RNA exactly comigrated with pG, pGm, and pm^{7}G. Digestion of capped oligonucleotide derived from antibody-bound material yielded pG, pGm, and pm^{2,2,7}G. We concluded from these analyses that the SL RNAs synthesized *in vitro* contained a mixture of cap structures; the unbound RNA had the cap m^{7}GpppGmGp and the bound RNA had the cap m^{2,2,7}GpppGmGp. When these results were combined with the fingerprint analysis described above we concluded that transcription of the SL RNA gene in whole-cell extract was initiated at the first guanosine residue of the 22-nt SL sequence and that a large proportion of these transcripts were further modified to contain an authentic trimethylguanosine cap structure. In addition, most if not all of the SL RNA synthesized *in vitro* contained a 2′-O-methylated guanosine residue (the first guanine residue of the 22-nt SL sequence).

DISCUSSION

We have shown here that the SL RNA gene of *A. lumbricoides* is efficiently transcribed by RNA polymerase II in an *A. lumbricoides* whole-cell extract prepared from developing embryos. Several lines of evidence including two-dimensional RNase T1 fingerprint analysis indicate that *in vitro* transcription is accurate with respect to initiation and 3′ end formation. Furthermore, greater than 50% of the *in vitro* SL RNA transcripts are modified by the addition of trimethylguanosine cap structures.

Although it is clear from the fingerprint and other analyses that the *in vitro* SL RNA transcript is quite similar to its *in vivo* counterpart, there are some differences. Specifically, the capped oligonucleotide is disproportionately labeled in fingerprints of RNA labeled *in vivo* and one expected RNase T1 oligonucleotide (UAUGp) is absent from the fingerprints

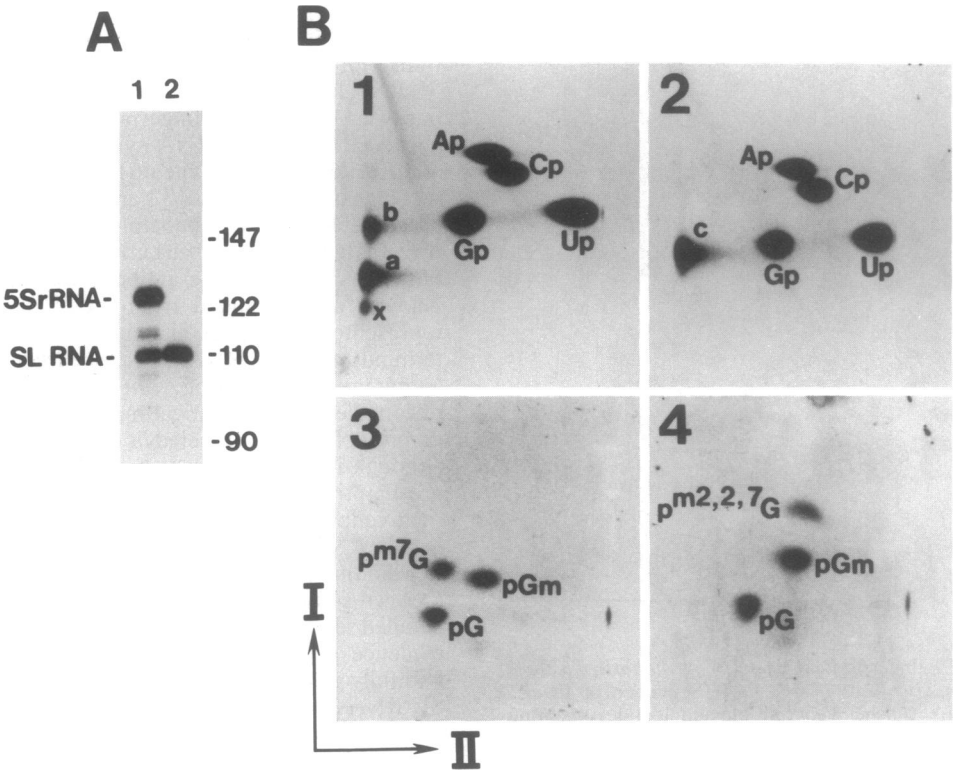

FIG. 3. SL RNA synthesized in extract contains a trimethylguanosine cap structure. SL RNA and 5S rRNA were synthesized in extract in the presence of [α-^{32}P]GTP in reactions similar to those shown in Fig. 1, lane 3. After deproteinization, the mixture of RNAs was precipitated with a monoclonal antibody specific for trimethylguanosine, and aliquots of unbound and bound fractions were analyzed by denaturing gel electrophoresis. (*A*) Unbound (lane 1) and bound (lane 2) RNAs. Sizes correspond to the mobility of labeled restriction fragments electrophoresed in parallel lanes. To analyze cap structures, bound and unbound SL RNAs were recovered from gels similar to those in *A* and digested to completion with RNase T2. Digestion products of unbound (*B*, chromatograph 1) or bound (*B*, chromatograph 2) RNAs were resolved by two-dimensional thin layer chromatography, as described (14). Capped oligonucleotides from each RNA (spot a, unbound, and spot c, bound) were recovered and further digested with nuclease P1 and nucleotide pyrophosphatase. Digestion products were again resolved by two-dimensional TLC (unbound in *B*, chromatograph 3; bound in *B*, chromatograph 4). The positions of unlabeled standards that were mixed with the labeled nucleotides prior to chromatography are indicated. Trimethylguanosine containing cap standards were synthesized (15) and provided by S. Tahara and E. Darzynkiewicz (Department of Microbiology, University of Southern California, Los Angeles); remaining standards were purchased from Pharmacia. Spots b and x in *B*, chromatograph 1, represent residual trimethylguanosine cap remaining after immunoprecipitation and an unidentified minor cap present in the unbound RNA, respectively.

of this RNA (see Fig. 2). Furthermore, one unidentified oligonucleotide is present on this fingerprint (see Fig. 2). It might be possible to explain the disproportionate cap spot intensity if the guanosine pool used for cap structures equilibrates more rapidly with the orthophosphate used in labeling. The other differences could be accounted for if the SL RNA synthesized *in vivo* is subject to additional modifications, which are not catalyzed in the extract; however, support for these conjectures awaits a more detailed analysis of the SL RNA synthesized *in vivo*.

In addition to the studies presented regarding transcription of the *A. lumbricoides* SL RNA gene, we have used indirect analysis (such as primer extension and oligodeoxynucleotide-directed RNase H digestion) to show that the SL RNA genes of both *B. malayi* and *C. elegans* are faithfully transcribed in the *A. lumbricoides* extract (data not shown). The embryo extract will thus prove useful to define the transcription control elements of a variety of nematode SL RNA genes. In this regard, it has been noted (5, 7) that some potential control elements for initiation of transcription and 3′ end formation of nematode SL RNAs resemble those known to be important for the synthesis of vertebrate U snRNAs.

The cell free system should also be useful in determining the elements that direct cap trimethylation. All three nematode SL RNAs examined have a consensus binding site (RA U_n GR, where $n > 3$ and R is a purine) for proteins with Sm antigenic determinants (5–8) and it has been demonstrated that these sites are functional (6–8). Using enucleated *Xenopus* oocytes, Mattaj (17) has shown that trimethylation of U snRNA caps is catalyzed by a cytoplasmic activity and that the Sm binding site was necessary and sufficient to direct cap trimethylation. In experiments similar to those described by others (6–8), we have used human Sm antisera to precipitate a fraction of the *A. lumbricoides* SL RNA synthesized *in vitro* (unpublished observations). It will be of interest to determine whether SL RNA cap trimethylation requires the binding of the *A. lumbricoides* proteins containing Sm antigenic determinants.

Finally, although it is well established that snRNAs are synthesized by a "modified" form of RNA polymerase II, the biochemical analysis of this polymerase and associated factors has been frustrated by the lack of cell-free transcription system (for review, see ref. 9). Only three systems, two derived from sea urchin embryos and one from *Xenopus*, have been described which synthesize U snRNAs (18–20). Thus, in addition to providing information relevant to SL RNA synthesis, the *A. lumbricoides* extract may be of more general significance in providing access to the components of snRNA synthesis.

We thank Drs. Adrian Krainer and Joan Steitz for generously providing antibodies used in these studies, Stan Tahara for cap standards and nucleotides, Joseph Shambaugh for making and sequencing constructs, and Andrea Branch for helpful discussions. This work was supported by a grant from the John D. and Catherine T. MacArthur Foundation and a Presidential Young Investigator award of the National Science Foundation (T.W.N.).

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