A sequence from Drosophila melanogaster 18S rRNA bearing the conserved hypermodified nucleoside amψ: analysis by reverse transcription and high-performance liquid chromatography

Douglas C.Youvan* and John E.Hearst†+

*Department of Biophysics and Medical Physics, and †Department of Chemistry, University of California at Berkeley, Berkeley, CA 94720, USA

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ABSTRACT

The naturally occurring modified nucleoside 3-[3-amino-3-carboxypropyl]-1-methylpseudouridine (abbreviated amΨ) is found in eukaryotic 18S rRNA. We localized amΨ to sequence resolution in *D. melanogaster* 18S rRNA. This hypermodified base causes an absolute stop in cDNA elongation. The RNA sequence bearing amΨ was determined by dideoxy-sequencing with reverse transcriptase. The rDNA coding for this part of the 18S rRNA was sequenced by the Maxam-Gilbert method. Together these two sequencing methods can be used to position the cDNA stop (amΨ) in the rRNA sequence. Chemical evidence for the existence of amΨ in this RNA sequence was obtained by high-performance liquid chromatography (HPLC) of 18S rRNA nucleosides from radioactive-labeled cells. L-[2-^{14}C] methionine will selectively label amΨ in eukaryotic 18S rRNA. Using HPLC, we found a single ^{14}C-labeled nucleotide in digests of 18S rRNA. This nucleotide is in the RNA sequence bearing the cDNA stop since a restriction fragment which hybridizes to this sequence protects the modified base from RNase T1 digestion.

INTRODUCTION

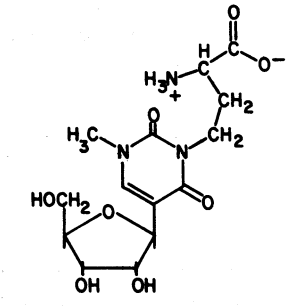
The hypermodified nucleoside amΨ has been found in all eukaryotic 18S rRNAs for which it has been examined, including Chinese-hamster (1), Hela, yeast (2,3), sycamore (4), rat, and chicken (5). Although amΨ has not been localized in any of these RNAs, it was identified in the RNase U2 fragment CamΨCA in yeast. The amΨ bearing sequence that we have determined in *D. melanogaster* 18S rRNA includes this oligomer and the cDNA stop is one base before the hypermodification.

[2-^{14}C] methionine uniquely labels amΨ in Chinese-hamster 18S rRNA (1). We have chemically localized amΨ to the vicinity of the cDNA stop sequence by hybridization of a small rDNA restriction fragment from pDmra56 (6) and purification of the RNase T1 resistant RNA-DNA hybrid. RNase T2 and bacterial alkaline phosphatase (BAP) were used to digest the hybrid RNA for analysis on reverse phase high-performance liquid chromatography (HPLC). A nucleotide uniquely labeled by [2-^{14}C]

methionine was detected by HPLC and tentatively identified as amΨp.

The structures of amΨ and the cellular methyl donor, S-adenosyl methionine (SAM), are shown in Figure 1. Saponara and Enger discovered amΨ in Chinese-hamster 18S rRNA as a hypermodified base which is uniquely labeled in cell culture by [2-^{14}C] methionine. They suggested that at least three enzymes are necessary to post-transcriptionally convert uridine to amΨ and that the methyl and 3-amino-3-carboxypropyl donor is SAM. The sulfonium ion of SAM activates both of these groups for donation; however, donation of the 3-amino-3-carboxypropyl residue to rRNA occurs only once in the case of 18S rRNA and not at all for 28S rRNA. SAM methyl transferase reactions occur much more frequently, a total of approximately 25 times in *D. melanogaster* 18S rRNA, most of which are 2'-O-ribose methylations (7).

Maden and coworkers (2,3) have found amΨ in all of the eukaryotic 18S rRNAs which they have investigated. They have evidence that the biosynthetic pathway proceeds through 1-methyl-pseudouridine and that



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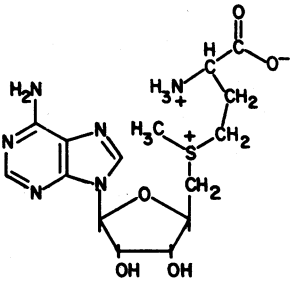


II

Figure 1. Structures of (I) 3-(3-amino-3-carboxypropyl)-1-methyl-pseudouridine, abbreviated amΨ, and (II) S-adenosylmethionine, SAM.

the addition of the 3-amino-3-carboxypropyl group is a late, cytoplasmic event. The conservation of amΨ through various eukaryotic species and the uniqueness of the hypermodification suggest an important function. The amount of DNA coding for the modification enzymes necessary for the synthesis of amΨ could exceed the length of DNA coding for the entire primary sequence of 18S rRNA.

The localization of base modifications in rRNAs has been accomplished in the case of *E. coli* 16S RNA by sequencing the rDNA and by correlation with modification data from RNA oligonucleotides (8) sequenced by classical methods (9). A more rapid method of localizing some base modifications utilizes reverse transcriptase, since the kinetics of cDNA elongation is sensitive to template base modifications which affect base-pairing (10). N^2-methylguanine (m^2G) was detected in *E. coli* 16S rRNA as a kinetic pause with a lifetime of three minutes. Previously, the conservation of the 3'-terminal $m_2{}^6Am_2{}^6A$ sequence was demonstrated by a cDNA stop (11). In the case of a base modification that affects base-pairing, the exact position can be found by observing the kinetics of cDNA elongation relative to a dideoxy-sequence (10,12). With the exception of the 3'-terminal $m_2{}^6Am_2{}^6A$ sequence and given the various types of base modifications found in eukaryotic 18S rRNA, we would expect, a priori, that reverse transcriptase would be inhibited only by amΨ. Experimentally, we find that there is one internal cDNA stop in *D. melanogaster* 18S rRNA. Using dideoxy-sequencing, we found that this cDNA stop occurs one base before the uridine in the sequence ACUCA. In yeast, amΨ is found in the RNase U2 fragment CamΨCA. No data is available for flies. Direct biochemical evidence for the existence of amΨ in *D. melanogaster* 18S rRNA was obtained by HPLC on methionine-labeled RNA digested with RNase T2 and BAP. A polar compound was resolved which occurs at about 1 part per 2000 and is labeled by [CH_3-^{3}H] methionine and [2-^{14}C] methionine. This compound is tentatively identified as amΨp. Base compositional analysis of the rDNA protected RNA localizes amΨ, chemically, to the RNA sequence where the cDNA stop occurs. The modified nucleotides localized to this hybrid are a subset of the modifications found in the total 18S rRNA.

MATERIALS AND METHODS

Plasmids and Primers. A bacterial clone of *D. melanogaster* rDNA (pDmra56, a gift from Igor Dawid) was grown in M9 medium plus 20 μg/μl

thiamine (6). The amplified plasmid was prepared by ethidium bromide-CsCl banding of a cleared bacterial lysate. Restriction enzymes were purchased from Bethesda Research Laboratory and plasmids were digested in the buffers specified by the company. Hind III, Hinf I, Eco RI and Sau 3A digested restriction fragments were 3'-end labeled by "filling in the sticky end" with [α-^{32}P] dATP (Amersham, 400 Ci/mmol) using 20-50 units of AMV reverse transcriptase (Joe Beard, Life Sciences, Inc.) for 1 hr at 37°C in the restriction enzyme buffer. Hind III, Eco RI double digests of pDmra56 end-labeled and electrophoresed on 6% polyacrylamide gels (30:1 Bis; 50 mM Tris, pH 8.3/1 mM EDTA) resolve three small fragments: 275 bp Hind III-Hind III, 305 bp Hind III-Eco RI and the pMB9 354 bp Hind III-Eco RI. The 275 bp and 305 bp fragments are complementary to the 3'-one-third of 18S rRNA. Hind III digestion of pDmra56 releases only the 275 bp fragment which migrates 50% as fast as xylene cyanol on 6% polyacrylamide gels. All fragments were extracted from polyacrylamide gels as previously described (13). An Eco RI-Sau 3A restriction fragment complementary to the 3'-terminal end on 18S rRNA was prepared by digesting pDmra56 with Eco RI, 3'-end-labeling with [α-^{32}P] dATP and reverse transcriptase, and digesting with Sau 3A. This fragment was extracted from an 8% polyacrylamide gel, as above. Primers I, II, III and IV were prepared by Hinf I digestion of the purified 275 bp Hind III fragment from pDmra56. Alternatively, the fragments were obtained directly from the subclones pDmdy01 or pDmdy19 by a double Hind III, Hinf I digest. Separation was on 8% polyacrylamide gels: fragment I migrates near xylene cyanol and fragment IV migrates 50% faster.

Strand-Separation and Primer Annealing. The 275 bp Hind III and 305 bp Eco RI-Hind III fragments were strand-separated on a 6% polyacrylamide gel by a method previously described (13). Fragments I, II, III and IV are not strand-separable by this method. For these smaller fragments we annealed them to excess rRNA: 1 µg of the restriction fragment and 20 µg of 18S rRNA were melted in 20 µl of H_2O at 100°C for 90 sec, 20 µl of 4 × RT buffer was added (1 × RT buffer is 50 mM Tris-HCl, pH 8.3/6 mM $MgCl_2$/40 mM KCl) and the mixture annealed at 65°C for 5 minutes and 37°C for 5 minutes. This annealed mixture was centrifuged 4 hours at 20°C on 15-30% sucrose gradients in LES buffer (0.1 M LiCl/10 mM EDTA, pH 7.6/0.1% SDS) and the 3'-^{32}P-end labeled strand sedimenting with 18S rRNA was pooled, ethanol precipitated with 3 volumes of ethanol, and the

RNA was hydrolyzed (0.3 N NaOH at room temperature, 12 hr). The hydrolysate was neutralized with a 5x molar excess of Tris-HCl and the DNA was ethanol precipitated.

Subclones. The 275 bp Hind III fragment of pDmra56 was subcloned into a derivative of pGL101 (a gift from Carl Thummel) in which the Pvu II site was converted to a Hind III site by the insertion of a linker. Random screening (14) of 24 ampicillin resistant colonies yielded 2 clones with single 275 bp inserts in opposite orientations relative to the adjacent lac promoter. Transcripts of pDmdy19 are the same sense and polarity as cellular rRNA, and pDmdy01 is opposite.

Ribosomal RNA and Methionine-Labeling. The ribosomal RNA from *D. melanogaster* Oregon R embryos was extracted as previously described (15) and fractionated by sedimentation for 4 hr at 45K rpm in an SW50.1 rotor or 16 hr at 25K rpm in an SW27.1 rotor on 15-30% linear sucrose gradients in LES buffer at 20°C. Methionine-labeled RNA was prepared from *D. melanogaster* KC cells cultured in serum-free Echalier medium (16). L-[CH_3-^3H] methionine (Amersham, 74 Ci/mmol) or DL [2-^{14}C] methionine (New England Nuclear, 47 mCi/mmole) was added to exponentially growing cells in T75 flasks (Falcon) 100 µCi/ml or 2.5 µCi/ml, respectively, for 2 to 3 days. Cells were collected by centrifugation, RNA extracted by phenol (buffer LES saturated) and 18S rRNA fractionated by sucrose gradient sedimentation as described above.

Reverse Transcription and Sequencing. The conditions for priming, reverse transcription, and dideoxy-sequencing rRNA have been previously described (10). Chemical sequencing of DNA has been described previously (13).

Preparation of RNA-DNA Hybrids. 30 µg of Hind III digested pDmdy01 or pDmdy19 in 40 µl of Hind III digestion buffer was melted with 100 µg of [CH_3-^3H] methionine-labeled 18S rRNA (2 KCPM/µg) dissolved in 40 µl of H_2O for 2 minutes at 100°C. 40 µl of 20 mM $MgCl_2$/100 mM Tris-HCl (pH 7.5) was added and the mixture annealed at 65°C for 5 minutes and 37°C for 5 minutes. RNase T1 (500 units, Boehringer Mannheim) was added for 1 hour at 37°C and then the 275 bp RNA-DNA hybrid was separated from small RNA fragments by gel filtration on a 15 cm G100 column (Pharmacia Sephadex) run in 0.2 N NaCl. The RNA-DNA hybrid and vector DNA peak eluting at the void volume was ethanol precipitated.

Digestion of RNA for HPLC. Up to 80 µg of RNA was resuspended in 20 µl of 2.5 mM sodium acetate (pH 4.5) and digested with 2 to 4 units

of RNase T2 (Sigma) for 12 to 24 hours at 37°C. Tris base was added to 25 mM and 0.2 units of bacterial alkaline phosphatase (P-L Biochemicals, 40 U/mg) digested 12 hours at 37°C. In the case of RNA-DNA hybrids, the RNase T2 digestion mixture was heated to 100°C for one minute, digested at 50°C for one hour, boiled again for one minute, two units of ribonuclease T2 added, and digested at 37°C for 12 hr. Vector DNA in this mixture was ethanol precipitated after the BAP digestion and the ethanol supernatant was evaporated and resuspended in 20 µl H_2O.

HPLC. RNA digests were injected without further treatment on a 25 cm analytical C_{18} reverse phase column (Altex, Ultrasphere), run at 1 ml/min with 10 mM ammonium phosphate (pH 5.1) and step increases in methanol (from 2.5% to 10% at 20 minutes; from 10% to 20% at 40 minutes). The retention times of yeast tRNA modified nucleosides have been determined under these conditions (17,18).

RESULTS

Scheme for Sequencing a Hypermodified Base. The experimental methodology and logic for sequencing and chemically identifying a hypermodified base are shown in Figure 2. Dideoxy-sequencing with reverse transcriptase primed by an annealed restriction fragment is used to determine the RNA sequence up to the hypermodified nucleoside. A Maxam-Gilbert sequence of the cloned DNA is read into an unmodified RNA sequence. Together these two sequences position the reverse transcriptase stop (hypermodified nucleoside) on the RNA sequence. Chemical identification of the hypermodification requires a base compositional analysis for this internal segment of RNA. A suitable restriction fragment is annealed to the RNA and the RNA is digested with RNase T1 under conditions for which the hybrid RNA is protected. After purification, the RNA-DNA hybrid is digested to ribonucleosides by RNase T2 and BAP and analyzed by reverse phase HPLC.

Sequencing the amΨ Stop. The rDNA clone pDmra56 (6) contains a 17kb rDNA transcriptional unit (Figure 3). There is one Eco R1 site in the transcriptional unit located approximately 230 bp from the 3'-end of the 18S rRNA. We have mapped a Sau 3A site closer to the 3' end that is consistent with the known 18S rRNA 3'-terminal sequences (23). This 110 bp Eco R1-Sau 3A fragment primes 18S rRNA and cDNA elongates approximately 500 bases and abruptly stops. The 305 bp Hind III-Eco R1 fragment from pDmra56 primes 18S rRNA and cDNA is transcribed up to an identical position. This places the cDNA stop (hypermodified base) "under" the 275 bp

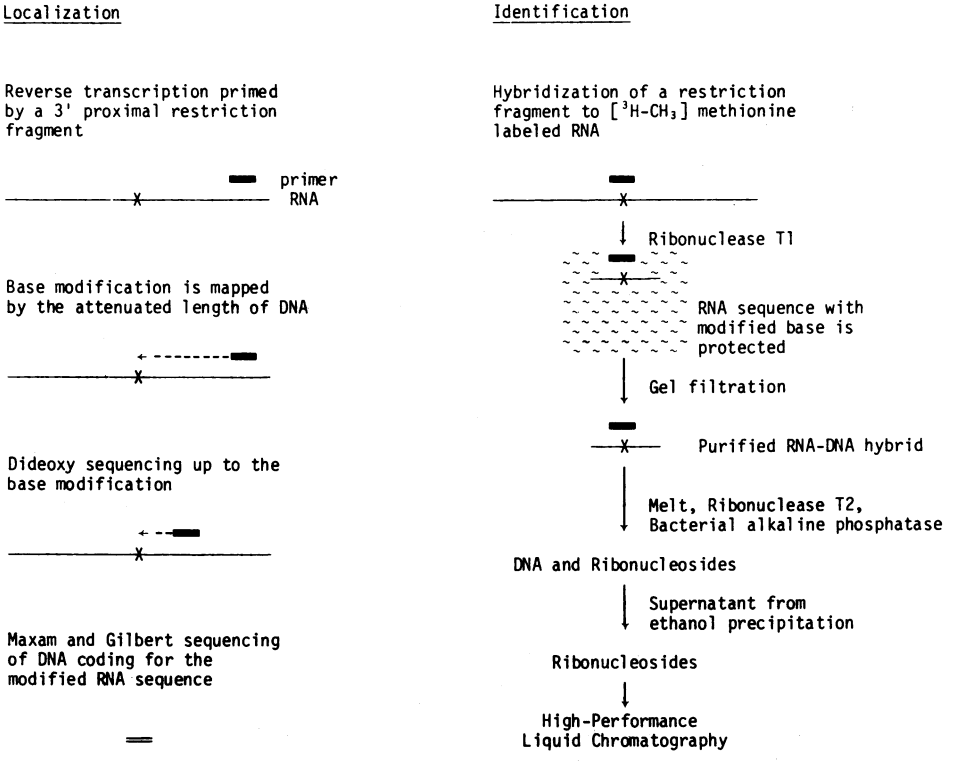


Figure 2. A scheme for the localization and identification of modified bases in high molecular weight RNAs. Modified bases affecting base-pairing stop reverse transcriptase or result in the accumulation of cDNA intermediates pausing at the modification. For example, the $m_2^6Am_2^6A$ sequence in the 3'-terminus of small subunit rRNAs is a cDNA stop (11). Psoralen photochemical adducts in RNA are also cDNA stops (R. Swanstrom, personal communication; Youvan and Hearst, unpublished data). The m^2G's in *E. coli* 16S rRNA are detected as 3 minute kinetic pauses in cDNA elongation (10). Pauses and stops are localized to sequence resolution in conjunction with dideoxy-sequencing. Chemical identification of modified nucleosides in the RNA sequence that attenuates cDNA synthesis is made by HPLC nucleoside analysis of restriction fragment protected ribonucleosides.

Hind III fragment. As a primer, this fragment does not encounter the stop and cDNA accumulates up to the end of the RNA. This data is summarized in Figure 3. The 275 bp Hind III fragment was subcloned into a derivative of pGL101 (19) and a finer restriction map was made in order to "bracket" the stop. The cDNA reverse transcripts primed with frag-

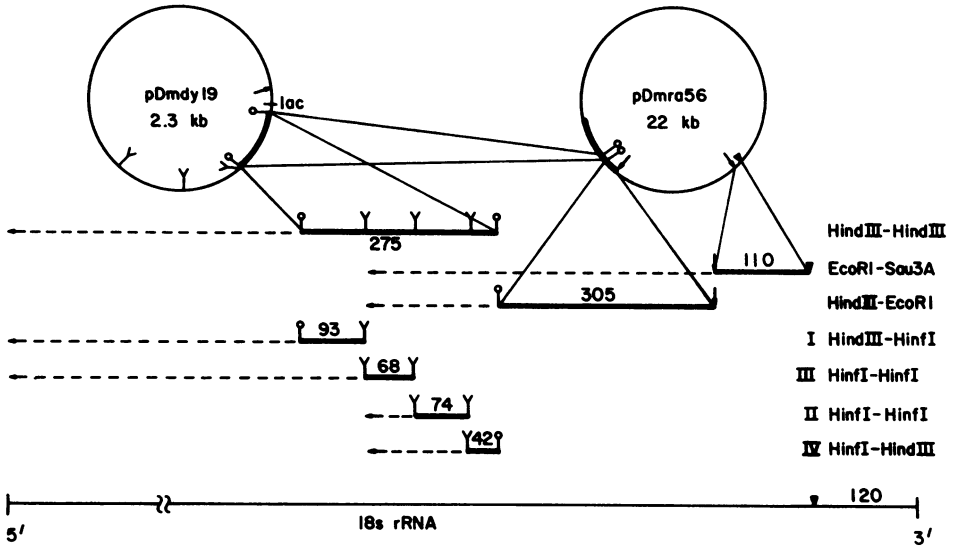


Figure 3. Summary of AMV reverse transcriptase synthesized cDNA on *D. melanogaster* 18S rRNA. 3'-proximal restriction fragment primers extend up to an absolute stop, but primers with 3'-OH ends past this point can be extended to the end of the template. Conditions for reverse transcription and kinetic analysis of cDNA elongation assayed by alkaline agarose gel electrophoresis have been previously described (10). The distance of the Sau 3A and Eco RI sites from the 3' terminus has been previously determined (23).

ments I, II, III and IV are summarized in Figure 3 and the experimental data is shown in Figure 4. We conclude that the 3'-end of primers II and IV are to the 3'-proximal side (in the RNA sense) of the hypermodification which is under fragment III. Primer II was used for dideoxy-sequencing up to the stop (Figure 5). Maxam-Gilbert sequencing starting approximately 50 bases from a 3'-end label on the sense strand of the 275 bp Hind III fragment is shown in Figure 6. Together these sequences position the cDNA stop at the 3'-C in the sequence GACUCA (see Figure 7), which includes the Hinf I site (G↓ANTC) for the 5'-end of fragment III. It also includes a sequence identical to the RNase U2 fragment from yeast bearing amΨ:CamΨCA. The *Drosophila* sequence ACamΨCA would generate such an RNase U2 fragment. This places the cDNA stop one base before amΨ and the 3'-OH [α-^{32}P] ATP end-label of primer III (an efficient primer) exactly opposite amΨ. Therefore, the mechanism of the reverse transcriptase stop involves the inability of monomer dATP to base-

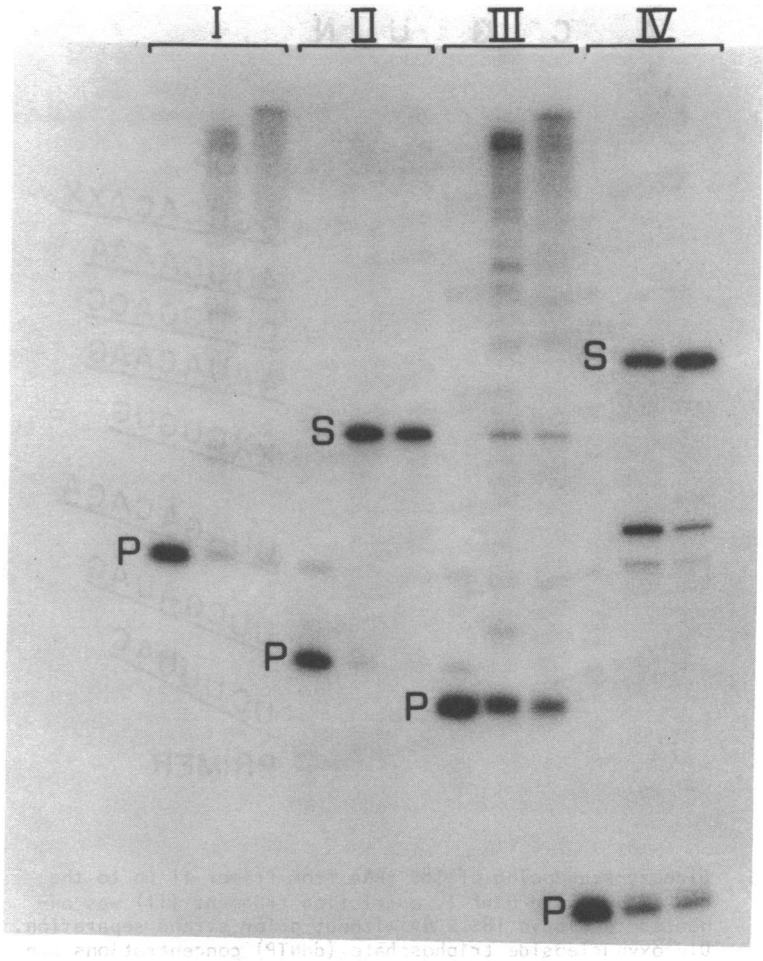


Figure 4. Reverse transcripts primed with restriction fragments (I, II, III, IV, see Figure 3) which bracket the stop. Primers were 3'-end labeled and strand separated. Reverse transcription was from 0, 5, 15 minutes (left to right) for each of the four primers. Electrophoresis of cDNA is on a denaturing 8% polyacrylamide gel (8 M urea, 1/20 Bis, 50 mM Tris-borate/pH 8.3, 1 mM EDTA). Abbreviations: P - primer, S - stop.

pair and incorporate into the cDNA. If it were incorporated, as it is in the fortuitous case of 3'-end labeled primer III, cDNA elongation continues (see the discussion and Figure 10).

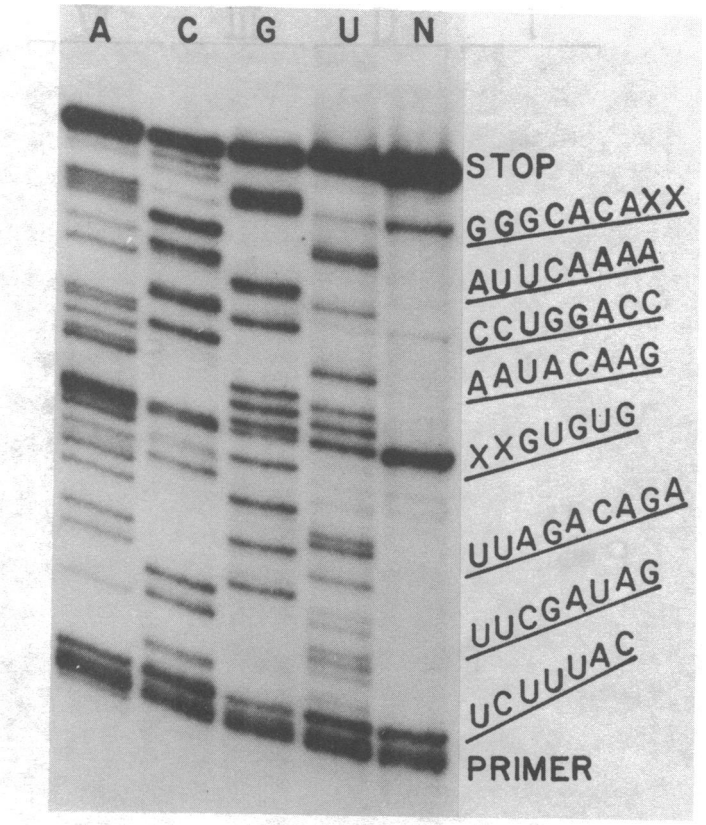


Figure 5. Dideoxy-sequencing of 18S rRNA from Primer II up to the cDNA stop. The Hinf I restriction fragment (II) was annealed to embryo 18S rRNA without prior strand separation. Dideoxynucleoside triphosphate (ddNTP) concentrations are higher than those previously reported (10); X mM ddNTP:Y mM of dNTP, A 25:25, C 25:12, G 25:25, and T 25:25. Electrophoresis of cDNA was on 40 cm long gels (0.3 mm thick; 8% polyacrylamide/ 1/20 Bis, 8 M urea, 50 mM Tris-borate/pH 8.3) for 3 hours at 1375 volts. Lane N has no ddNTP's.

HPLC Analysis of Methionine-Labeled 18S rRNA Digests. L-[CH_3-^{3}H] methionine added to cell culture medium is metabolically activated via SAM for methyl donation to various cellular substrates, including RNA. We prepared [CH_3-^{3}H] methionine 18S rRNA from *D. melanogaster* KC cells grown in Echalier medium without serum. The RNA was digested with RNase T2 and BAP for nucleoside compositional analysis using reverse

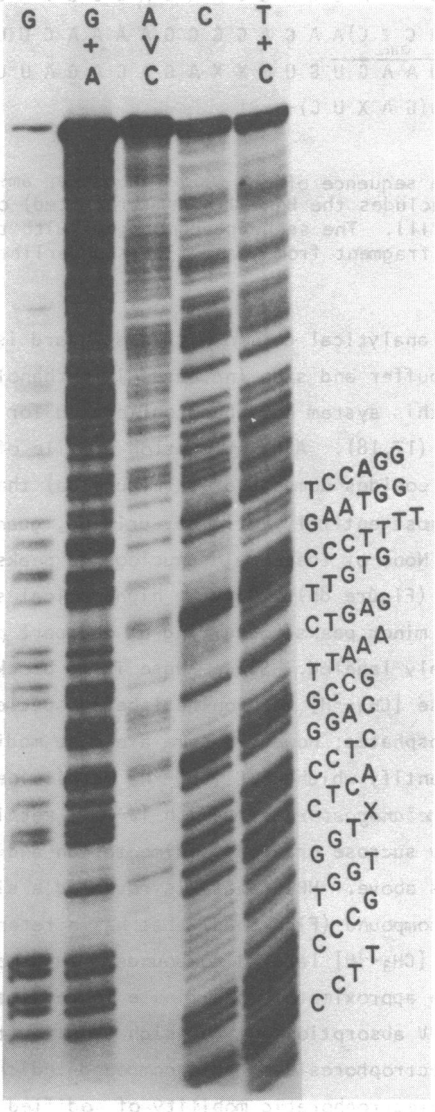


Figure 6. Chemical DNA sequencing of the 275 bp Hind III fragment from pDmdyl9. This fragment was 3'-end labeled with [α-^{32}P] dATP by reverse transcriptase. The sequence begins approximately 50 nucleotides from the 3'-end of the sense (primer) DNA strand (8% polyacrylamide gel, as in Figure 5).

5'---G G A A G G G C A C C A C C A X G A G U G G A G C C U G C G G C A
A U U U(G A C $\overset{\text{am}}{\Psi}$ C)A A C A C G G G A A A A C U U A C C A G G U C C
G A A C A U A A G U G U G X X A G A C A G A U U G A U A G C U U C
A U U U C U(G A X U C)---3'

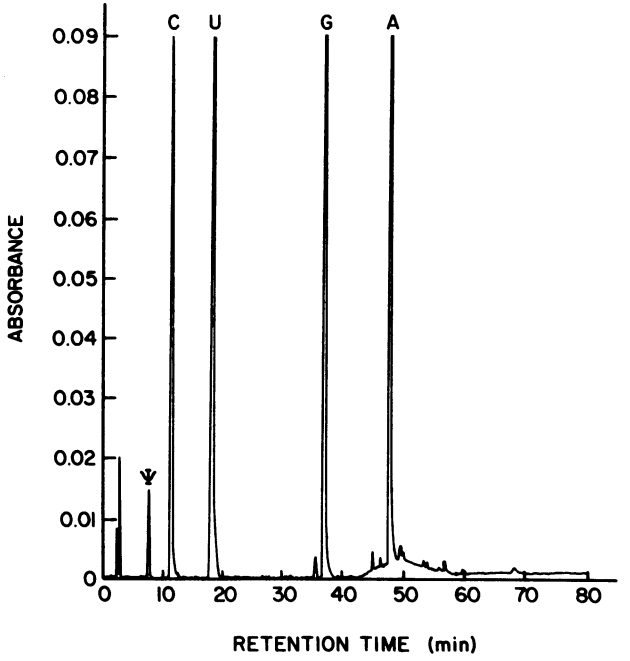
Figure 7. Tentative sequence of the *D. melanogaster* amΨ site. This sequence includes the Hinf I ends (bracketed) of restriction fragment III. The sequence homologous with the amΨ bearing RNase U2 fragment from yeast (3) is underlined.

phase HPLC on a C_{18} analytical column with a standard isocratic ammonium phosphate (pH 5.1) buffer and step increases in methanol concentration. Retention times in this system have been calibrated for the nucleosides found in yeast tRNA (17,18). A UV absorption profile of the *D. melanogaster* 18S rRNA nucleosides demonstrates (Figure 8a) that the composition of the digest is almost entirely cytidine, uridine, guanosine, adenosine, and pseudouridine. None of these major nucleoside peaks are labeled by [CH_3-^{3}H] methionine (Figure 8b). Only at high optical sensitivity do we observe (Figure 8c) minor peaks, occurring at one part per 1000 or less, that are radioactively labeled. Since RNase T2 is blocked by 2'-O-methyl ribose, most of these [CH_3-^{3}H] methionine labeled species are unidentified dinucleoside monophosphates; however, some are base modified nucleosides.

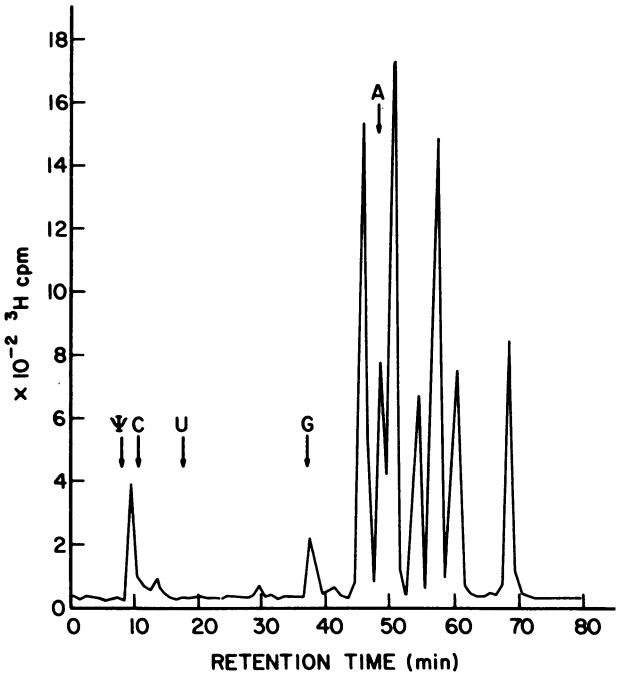
In order to identify which HPLC [CH_3-^{3}H] methionine-labeled peak is amΨ, we labeled *D. melanogaster* cells with [2-^{14}C] methionine. The 18S rRNA was isolated by sucrose gradient sedimentation and digested with RNase T2 and BAP, as above. HPLC analysis revealed a single [2-^{14}C] methionine-labeled compound (Figure 8d) that has a retention time identical to one of the [CH_3-^{3}H] labeled compounds. This modified nucleoside has a retention time approximately one minute longer than pseudouridine and is observed by UV absorption only at high sensitivity (Figure 8c). Analytical paper electrophoresis of this compound indicates that it is a nucleotide. The electrophoretic mobility of modified nucleotides on neutral paper is indicative of their charge to mass ratio as specified by an empirical equation (20). The electrophoretic mobility of this compound on paper electrophoresis at pH 3.5 is as expected for a charge to mass ratio of amΨ plus a phosphate.

The mobility of the *D. melanogaster* compound is identical to the mobility of amΨp from yeast that was previously reported (2). T2 ribonuclease digestion proceeds through a nucleoside 2'-3' cyclic phosphate

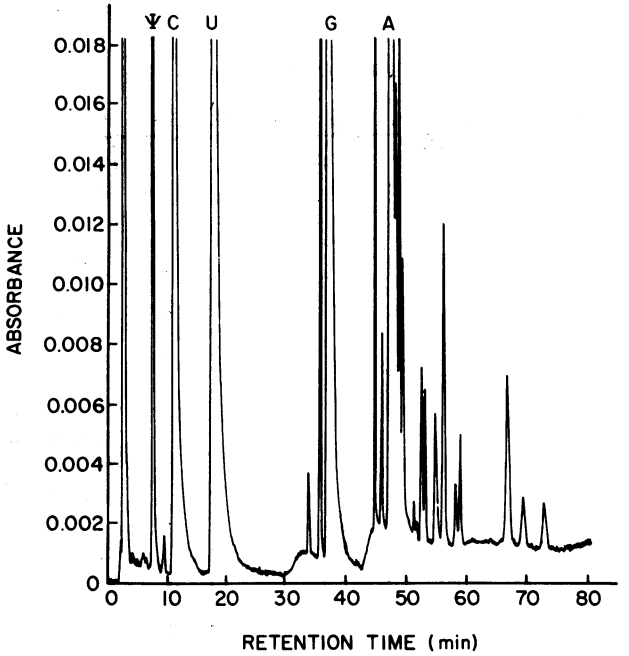
Figure 8. High-performance liquid chromatography of *D. melanogaster* 18S rRNA digested with RNase T2 and BAP. In all profiles 20 µl samples were injected on a 25 cm analytical Ultrasphere (Altex) C_{18} column (1 ml/min, 10 mM NH_4PO_4/pH 5.1, 2.5% v/v methanol to 20 min, 10% to 40 min, 20% to 80 min).



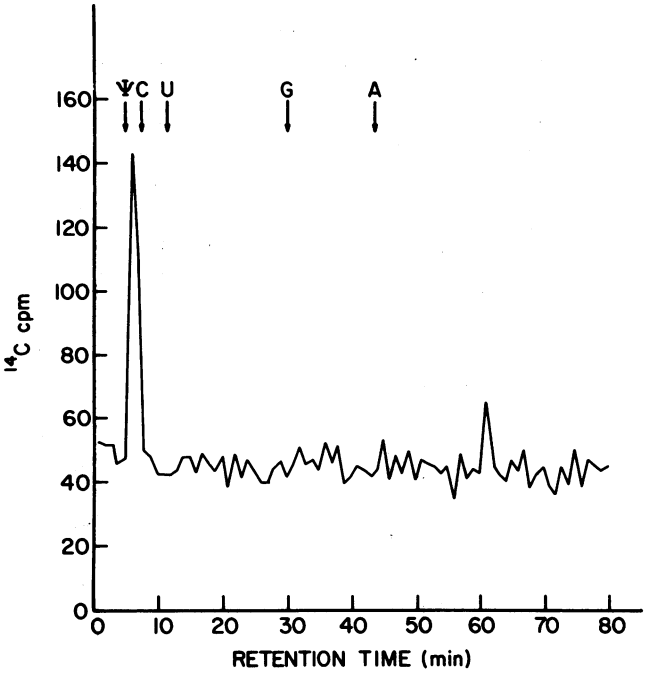
Profile 8a: A digest of 15 µg of [CH_3-^{3}H] methionine labeled KC cell 18S rRNA (2 KCPM/µg), 0.1 a.u.f.s., 254 nm (peak heights: Ψ - 0.015, C - 0.110, U - 0.150, G - 0.312, A - 0.460.



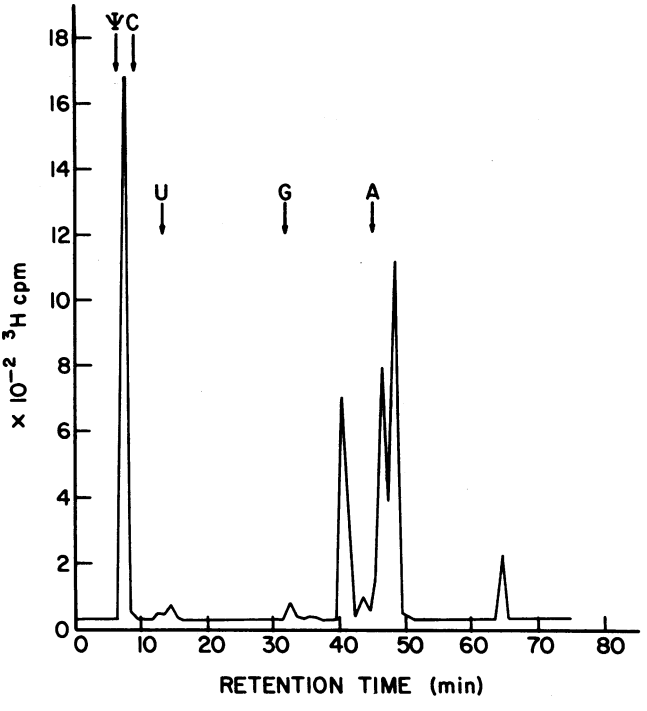
Profile 8b: ^{3}H profile for the above run; most of the tritium-labeled peaks are unidentified dinucleoside monophosphates with 2'-O-ribose methylation.



Profile 8c: 80 μg of embryo 18S rRNA, digested as above, 0.02 a.u.f.s.; major nucleosides are 30-120× full scale deflection; approximately 20 [CH_3-^3H] methionine labeled compounds are observed by UV absorbance.



Profile 8d: A digest of 100 μg of [2-^{14}C] methionine labeled rRNA from KC cells. Only amΨp is labeled; see text. Profiles are not corrected for background radiation.



Profile 8e: A digest of the 275 bp Hind III DNA-RNA hybrid. These [CH_3-^{3}H] labeled compounds are a subset of the modifications observed in total 18S rRNA and include amΨp (eluting between Ψ and C).

intermediate and some chemically modified uridines are not easily digested further (J.-P. Bachellerie, personal communication). We tentatively identify this compound as the 2'-3' cyclic phosphate form of amΨ, designated amΨp. BAP does not digest this cyclic phosphate nucleotide. The cyclic phosphate explains the alkali instability of amΨp that was previously observed (2).

HPLC Analysis of an Internal RNA Sequence. Verification that amΨ is located in the same RNA sequence which includes the reverse transcriptase stop necessitates a base compositional analysis of an internal segment of 18S rRNA. Restriction fragments annealed to RNA protect the complementary RNA from RNase T1 digestion (21). We annealed the 275 bp Hind III fragment from pDmdy19 to [CH_3-^{3}H] methionine-labeled 18S rRNA, and digested with RNase T1. Under appropriate digestion conditions, RNA-DNA hybrids of a length (electrophoretic mobility on nondenaturing polyacrylamide gels)

similar to the corresponding double-stranded restriction fragments are produced and the unprotected RNA is digested to small fragments. Although the RNA-DNA hybrid can be extracted from a polyacrylamide gel, we find that gel filtration (Figure 9) adequately separates the hybrid from the smaller, unprotected RNase T1 fragments. The hybrid elutes at the void volume of the column along with the vector DNA. RNA nucleosides in the hybrid are analyzed by reverse phase HPLC after heat denaturation and digestion with RNase T2 and BAP. The hybrid RNA nucleosides (Figure 8e) are a subset of the total 18S rRNA profile (Figure 8c) and include amΨp.

DISCUSSION

cDNA Pauses and Stops Mark Base Modifications. The major kinetic barriers in the reverse transcription of *E. coli* 16S rRNA are modified bases (10). The $m_2^6Am_2^6A$ sequence near the 3'-end of the RNA is a cDNA stop (11).

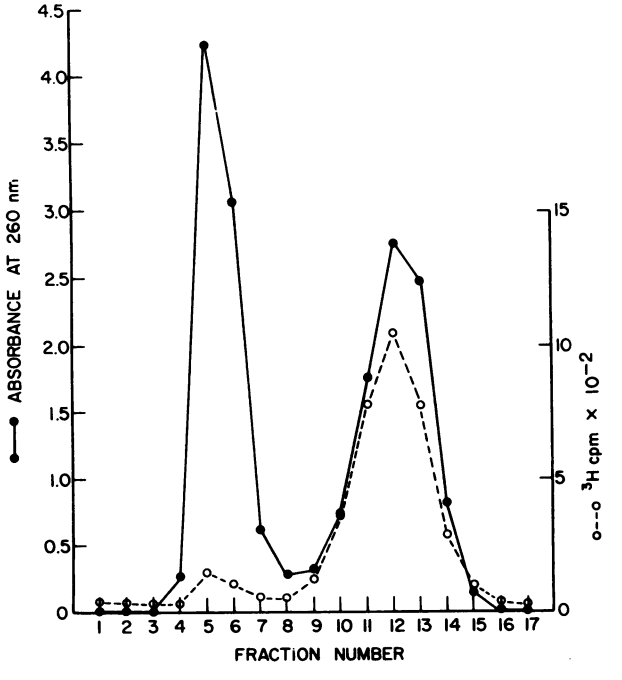


Figure 9. Separation of a restriction fragment DNA-RNA hybrid from RNase T1 digested RNA by gel filtration. Plasmid pDmdyl9 was digested with Hind III, annealed to [CH_3-^{3}H] methionine-labeled KC cell 18S rRNA, and digested with RNase T1. Protected RNA-DNA hybrids and vector DNA elute at the void volume.

We have shown that the internal m^2Gs are located at cDNA pauses with lifetimes of approximately 3 minutes. Unlike the cDNA pauses observed on the *E. coli* 16S rRNA template, an absolute stop in cDNA elongation occurs on the *D. melanogaster* 18S rRNA template approximately 765 nucleotides from the 3'-end. By restriction fragment protection of [CH_3-^{3}H] methionine labeled RNA and HPLC analysis of the protected nucleosides, we have localized a hypermodified base to this sequence. Since this nucleoside is labeled by [CH_3-^{3}H] methionine and uniquely labeled by [2-^{14}C] methionine, we have tentatively identified it as amΨ. The corresponding cDNA stop occurs in the sequence ACUCA which is identical to the yeast RNase U2 fragment sequence in which amΨ has been observed (2).

Mechanism of the amΨ cDNA Stop. Reverse transcripts from 3'-proximal primers stop one base before amΨ (Figure 10). This should be contrasted to the Hinf I restriction fragment III which is an efficient primer (Figures 4 and 10). After 3'-end labeling by "filling in the sticky end" of the Hinf I site (G↓ANTC) with one dATP, this primer hybridizes to the rRNA such that the 3'-terminal adenosine is exactly opposite amΨ. This suggests that the blocked step in cDNA elongation at amΨ involves base-pairing of monomer dATP with template amΨ. When this dATP is incorporated into the cDNA, as it is in the case of primer III, cDNA elongation can continue, apparently without perfect Watson-Crick base-pairing at the 3'-terminus. Another possibility is that the requirement of 3'-terminal base-pairing is less stringent for a primer than for cDNA elongation. Also, hybridization of primer III to the rRNA may cause a favorable conformational change in the quasi-base-pairing of amΨ with adenosine:

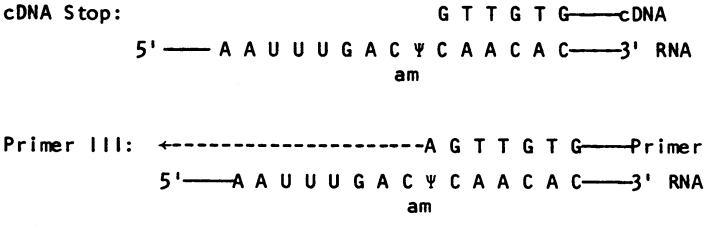


Figure 10. A possible mechanism for the reverse transcriptase stop at amΨ. Complementary DNA synthesis by AMV reverse transcriptase stops one nucleotide before amΨ; however, 3'-end labeled restriction fragment III is an efficient primer. This suggests that the blocked step in cDNA polymerization involves the base-pairing of monomer dATP with amΨ; see text.

rotation of the ribose-base bond of amΨ so that the bulky N-3-(3-amino-3-carboxypropyl) is away from the pseudo-base-pair.

Conservation of the amΨ-bearing Sequence. The CamΨCA sequence has been identified within a larger RNase T1 fragment in yeast by [1-^{14}C] methionine-labeling (2). This 10 nucleotide oligomer and the corresponding amΨ bearing sequence from *D. melanogaster* are identical in the positions of 8 out of 10 nucleotides; the only difference is the translocation of a single adenosine residue. An identical, long RNase T1 fragment has been observed in rat and chicken (5). These observations extend the conserved amΨ sequence from yeast to flies, birds, and mammals. Our searches of the *E. coli* 16S rRNA sequence for a similar oligomer have failed. The absence of amΨ in 16S rRNA is also suggested by our reverse transcription data (10), which indicates the lack of any internal absolute cDNA stop.

There is extensive homology between the eukaryotic amΨ sequence and the *E. coli* rRNA sequence bearing m^2Gm^5C at sequence position 965-966. By the reverse transcription assay, the former is an absolute stop and the latter is a kinetic pause site (10) for cDNA elongation. The chemical equivalence of amΨC and m^2Gm^5C within the ribosome is something to ponder.

While this manuscript was under review, the yeast 17S rDNA sequence was published (22). There are 18 bp of perfect homology between the *D. melanogaster* amΨ sequence and the yeast sequence at sequence position 1178. This homology is perfect from -8 bp to +9 bp in relationship to amΨ, and extensive homology exists over a larger region.

Purification of amΨ Modification Enzymes. pDmdyl9 bears a lactose operon promoter (19) adjacent to the 275 bp Hind III rDNA insert. *In vitro* transcription of this plasmid should make RNA of the same polarity as cellular rRNA with an unmodified amΨ site: CUCA. pDmdyOl is identical to pDmdyl9 except for a two-fold rotation of the inserted DNA, so that RNA complementary to cellular rRNA would be transcribed. These RNAs could be used as substrates for assaying modification enzymes involved in amΨ biosynthesis. [CH_3-^{3}H] SAM and [2-^{14}C] SAM labels would be transferred to the RNA transcribed from pDmdyl9 by N-1-methylpseudouridine transferase and N-3-(3-amino-3-carboxypropyl)-pseudouridine transferase, respectively.

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+To whom reprint requests should be addressed.

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