Polyadenylated, Noncapped RNA from the Archaeabacterium * Methanococcus vannielii *

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Polyadenylated [poly(A)⁺] RNA molecules have been isolated from * Methanococcus vannielii* by oligodeoxythymidylicate-cellulose affinity chromatography at 4°C. Approximately 16% of the label in RNA isolated from cultures allowed to incorporate [³H]uridine for 3 min at 37°C was poly(A)⁺ RNA. In contrast, less than 1% of the radioactivity in RNA labeled over a period of several generations was contained in poly(A)⁺ RNA molecules. Electrophoretic separation of poly(A)⁺ RNA molecules showed a heterogeneous population with mobilities indicative of sizes ranging from 900 to 3,000 bases in length. The population of poly(A)⁺ RNA molecules was found to have a half-life in vivo of approximately 12 min. Polyadenylate [poly(A)] tracts were isolated by digestion with RNase A and RNase T₁ after ³²P end labeling of the poly(A)⁺ RNA with RNA ligase. These radioactively labeled poly(A) oligonucleotides were shown by electrophoresis through DNA sequencing gels to average 10 bases in length, with major components of 5, 9, 10, 11, and 12 bases. The lengths of these poly(A) sequences are in agreement with estimates obtained from RNase A and RNase T₁ digests of [³H]adenine-labeled poly(A)⁺ RNA molecules. Poly(A)⁺ RNA molecules from * M. vannielii* were labeled at their ⁵’ termini with T₄ polynucleotide kinase after dephosphorylation with calf intestine alkaline phosphatase. Pretreatment of the RNA molecules with tobacco acid pyrophosphatase did not increase the amount of phosphate incorporated into poly(A)⁺ RNA molecules by polynucleotide kinase, indicating that the poly(A)⁺ RNA molecules did not have modified bases (caps) at their ⁵’ termini. The relatively short poly(A) tracts, the lack of ⁵’ cap structures, and the instability of the poly(A)⁺ RNA molecules isolated from * M. vannielii* indicate that these archaeabacterial poly(A)⁺ RNAs more closely resemble euabacterial mRNAs than eucaryotic mRNAs.

Polyadenylate [poly(A)] tracts are found at the ³’ termini of eucaryotic mRNAs. The function of poly(A) is unclear. It has been suggested that it is involved in the transport of mature mRNAs from the nucleolus to the cytosol (27), in controlling the in vivo half-life of mRNA molecules (18) and in regulating the translation of mRNAs (36, 38; for reviews, see references 5 and 28). Until recently, it was generally accepted that the post-transcriptional reactions leading to ³’ polyadenylation of eucaryotic mRNAs did not occur in euabacterial cells. However, techniques have now been developed that have demonstrated polyadenylated [poly(A)⁺] RNA molecules in RNA preparations from several euabacterial species (12). In contrast to eucaryotic poly(A)⁺ RNAs, euabacterial poly(A)⁺ RNAs have much shorter poly(A) tracts, are unstable, exhibit a wide range of sizes, and comprise a much smaller percentage of the total cellular RNA (6, 12, 19, 30, 37, 41). In several cases, euabacterial poly(A)⁺ RNAs have been shown by in vitro translation to be functional mRNAs (30, 31, 45).

The ³’ termini of eucaryotic mRNA molecules are usually modified by the addition of a cap structure consisting of a modified guanosine residue linked to the RNA molecule by a ⁵’-³’ triphosphate linkage (2, 4, 40). The mRNAs of euabacteria, on the other hand, have not been found to be capped. The presence or absence of cap structures in archaeabacterial mRNAs has not been reported.

Poly(A) sequences hybridize to oligodeoxythymidylicate [oligo(dT)] Properties have been used in affinity chromatography to separate poly(A)⁺ RNA molecules from total cellular RNA preparations and to provide a ³’-OH primer for synthesis of cDNA by reverse transcriptase. These techniques are of central importance in gene cloning procedures. We recently began cloning genes from methanogenic archaeabacteria (methanogens) and therefore have investigated the possibility of using oligo(dT) binding to isolate methanogen mRNAs. This technique was successfully used to isolate mRNAs from * Thermoplasma* and * Sulfolobus* species, indicating the presence of poly(A)⁺ RNA molecules in these thermoacidophilic archaeabacterial species (M. Ohba and T. Oshima, 1982, Abstract in Proceedings of the 1982 Symposium on Archaeabacteria, p. 353, O. Kandler [ed.]. Fisher Press, Stuttgart, Federal Republic of Germany). In contrast, RNA molecules encoding bacterio-opsin in * Halobacterium halobium* (an archaeabacterial species thought to be more closely related to methanogens than are the thermoacidophiles) do not appear to be polyadenylated and do not contain ⁵’ cap structures (7, 8). Our results demonstrate the presence of poly(A)⁺ RNA molecules in * Methanococcus vannielii* but indicate that these molecules are unstable, have relatively short ³’ poly(A) tracts, and do not have ⁵’ cap structures.

**MATERIALS AND METHODS**

Cell growth and labeling. * M. vannielii* cultures were grown at 37°C in 20 ml of ER minimal salts medium (15) in 100-ml vaccine bottles with an atmosphere of 80% H₂-20% CO₂ at 40 lb/in². The generation time was approximately 5 h. RNAs were labeled for 3 min at 37°C by injection of 200 μCi of [⁵,6-³H]uridine (28 Ci/mmol, 1 mCi/ml; Amersham Corp., Arlington Heights, Ill.) or [⁸-³H]adenine (22 Ci/mmol, 1 mCi/ml; Amersham) into exponentially growing cultures (absorbance at 580 nm [A₅₈₀] = 0.3). Cultures were cooled on ice and oxygenated (by bubbling with compressed air) to stop incorporation of the radioactive precursor. Cells were har-
vested by centrifugation at 6,000 × g for 5 min at 4°C and washed twice with 10 ml of oxygenated, ice-cold ER medium, and RNA was extracted from the cells as described below. RNAs were also labeled at 37°C for incorporation periods of approximately three generations by diluting an exponentially growing culture into fresh ER medium containing 2.5 μCi of [5,6-3H]uridine per ml and allowing continued growth.

*Escherichia coli* W3110 was grown in LB nutrient broth medium (35) at 37°C with continuous shaking. RNA was labeled for 30 s at 37°C by the addition of 20 μCi of [5,6-3H]uridine into 2 ml of exponentially growing cultures (A580 = 0.3). Incorporation was halted by the addition of NaN3 (final concentration, 25 mM) and rapid cooling on ice. Cells were harvested by centrifugation (2 min) in a microfuge (model 5412; Eppendorf) and washed twice with 1 ml of ice-cold LB medium containing 25 mM NaN3, and RNA was extracted as described below. RNA was also labeled for three generations by growing *E. coli* in LB medium containing 2.5 μCi of [5,6-3H]uridine per ml.

*Saccharomyces cerevisiae* a161 8" was grown in YEPD nutrient broth (3) at 30°C with continuous shaking. Exponentially growing cultures (A580 = 1.0) were cooled on ice, and the cells were removed from suspension by centrifugation at 4,000 × g for 5 min at 4°C and washed once with ice-cold water before RNA was extracted (see below).

**Nucleic acid extraction.** Labeled and unlabeled RNA was extracted from *M. vannielii* and *E. coli* by the method described by Gopalakrisna et al. (12). Washed cell pellets were suspended in 200 μl of lysis buffer (80 mM Tris-hydrochloride [pH 7.5], 10 mM MgCl2, 10 mM 2-mercaptoethanol [2ME]) per ml of original culture volume. Heparin (0.2 mg/ml), 1,10-phenanthroline (2 mM), EDTA (10 mM), sodium dodecyl sulfate (SDS; 0.5% [wt/vol]), and protease K (0.23 mg/ml) were added at the indicated final concentrations to give a final volume of 450 μl. The extraction mixture was incubated for 20 min at 37°C. Crude RNA extracts were stored at -20°C.

*S. cerevisiae* RNA was extracted from protoplasts prepared by zymolysate treatment (17). Cells (5 g [wet weight]) were suspended in 12 ml of 0.1 M EDTA (pH 8)–2.5% (vol/vol) 2ME, shaken for 15 min at 30°C, collected by centrifugation at 3,000 × g for 5 min at 4°C, and suspended in 12 ml of 0.1 M EDTA (pH 8)–2.5% (vol/vol) 2ME and 4.5 mg of zymolysate 20T (Sigma Chemical Co., St. Louis, Mo.) was added. After incubation for 1 h at 30°C with continuous shaking, the resulting protoplasts were pelleted at 3,000 × g for 5 min at 4°C and suspended in 30 ml of 1% (wt/vol) SDS–5 mM EDTA (pH 8). Diethyl pyrocarbonate (100 μl) was added, and the suspension was incubated for 5 min at 30°C to obtain lysis of the protoplasts. Potassium acetate was added (final concentration, 0.5 M), and the K+–SDS protein precipitate was removed from suspension by centrifugation at 12,000 × g for 5 min at 4°C. The supernatant was mixed vigorously with an equal volume of 50% phenol–48% chloroform–2% isoamyl alcohol and centrifuged at 12,000 × g for 10 min at 4°C. The upper, aqueous phase was collected and re-extracted with phenol–chloroform as described above. Nucleic acids were precipitated from the aqueous phase by the addition of 2.5 volumes of ethanol and incubation at -20°C overnight. The precipitate was collected by centrifugation at 27,000 × g for 30 min at 0°C, dissolved in AIN (0.1 M sodium acetate [pH 4.8], 15 mM sodium iodoacetate, 0.15 M NaCl) and stored at -20°C.

**Sucrose gradient analysis.** Unlabeled RNA (50 to 200 μg) was precipitated by the addition of sodium acetate (final concentration, 0.25 M) and 2.5 volumes of ethanol and incubation at -70°C for 1 h. The precipitate was collected by centrifugation at 4°C for 15 min in a microfuge and dissolved in 50 μl of 50 mM NaCl–50 mM sodium acetate (pH 5.1; hybridization buffer). [3H]Polyuridylicate ([3H]poly(U); 23 nCi (equivalent to 16 ng and 525 μCi/mmol; Miles Laboratories, Inc., Elkart, Ind.)] was added, and hybridization was allowed for 15 min at 45°C (26). Reaction mixtures were then cooled on ice, diluted by the addition of 300 μl of hybridization buffer, and layered onto 17-ml linear gradients of 5 to 20% (wt/vol) sucrose dissolved in 50 mM sodium acetate–150 mM NaCl (pH 5.1). After centrifugation at 27,000 rpm (SW28.1 rotor; Beckman Instruments, Inc.) for 19 h at 4°C (5.5 × 1011 rad/s), the gradients were fractionated by collecting 10-drop fractions from the bottom of each tube. The A260 of each fraction was measured, and the radioactivity in each fraction was counted after the addition of 5 ml of a scintillation fluid designed to permit efficient counting of aqueous samples (Formula 961; New England Nuclear Corp., Bedford, Mass.).

**Oligo(dT)-cellulose chromatography.** Oligo(dT)-cellulose chromatography was carried out by the column method with both [3H]-labeled and unlabeled RNA samples and by the centrifuge method with [3H]-labeled RNA (see below). Radioactivity in trichloroacetic acid (TCA)-precipitable material was determined in labeled RNAs before oligo(dT)-cellulose chromatography. Ice-cold TCA (final concentration, 5% [wt/vol]) was added to RNA solutions, the precipitates were collected on glass fiber filters, and the filters were dried and counted in a Beckman model 7500 liquid scintillation counter. Unlabeled RNA samples were precipitated with ethanol and collected by centrifugation in a microfuge (as described for the preparation of RNA for sucrose gradient analysis; see above), dissolved in water, ethanol precipitated, and pelleted a second time. The resulting pellet was dissolved in 10 mM Tris-hydrochloride–1 mM EDTA (pH 7.6) before column chromatography.

**Column chromatography.** All steps in this procedure were performed at room temperature. [3H]-labeled and unlabeled RNA samples were adjusted to 0.5 M NaCl and diluted to 10 ml with binding buffer (0.5 M NaCl, 10 mM Tris-hydrochloride [pH 7.5], 0.5% [wt/vol] SDS, 1 mM EDTA). Samples were applied to a 0.25-g oligo(dT)-cellulose (type II; Collaborative Research, Inc. Waltham, Mass.) column (0.38 cm2 by 2 cm) which had been preswathed with elution buffer (10 mM Tris-hydrochloride [pH 7.5], 0.05% [wt/vol] SDS, 1 mM EDTA) and 0.1 N NaOH and then equilibrated with binding buffer. Material bound to the column was washed with 20 ml of binding buffer and then eluted with 5 ml of elution buffer. Fractions (1 ml) were collected, and either the A260 or radioactivity in each fraction was determined.

**Centrifuge chromatography.** [3H]-labeled RNA samples were adjusted to 0.5 M NaCl and diluted to 1 ml with binding buffer (0.5 M NaCl, 10 mM Tris-hydrochloride [pH 7.5]). Oligo(dT)-cellulose (34 mg) was added, and the suspension was incubated for 1 h at 4°C with continuous shaking. The matrix was pelleted by centrifugation for 5 s in a microfuge and washed 10 times with 1-ml fractions of ice-cold binding buffer. Poly(A)" RNA was eluted from the matrix by four sequential 1-ml washes (15 min at 45°C) with 10 mM Tris-hydrochloride (pH 7.5) (12). Radioactivity released by each wash and elution was counted. The supernatants obtained after centrifugation were mixed with 9 ml of Formula 961 scintillation fluid before scintillation counting.

**Isolation of poly(A)" RNA molecules.** Preparative amounts of poly(A)" RNAs were obtained by a 10-fold scale-up of the
oligo(dT)-cellulose centrifuge chromatography method described above. Unlabeled yeast poly(A)+ RNA was also prepared by this method, except that hybridization was allowed for 30 min at room temperature. Eluted poly(A)+ RNA molecules were precipitated by the addition of sodium acetate (final concentration, 0.25 M) and 2.5 volumes of ethanol and incubation at −70°C overnight. Precipitates were collected by centrifugation at 95,000 × g for 1 h at 4°C and dissolved in sterile water.

**Half-life determination.** A 30-ml exponentially growing culture of *M. vannielii* was labeled for 3 min at 37°C by the addition of 100 μCi of [5,6-3H]uridine. A 1,000-fold excess of unlabeled uridine was then added (0.4 ml of an anaerobic solution containing 0.5 μM uridine). Incubation was continued at 37°C, and 5-ml fractions were removed at 2.5, 5, 10, 20, 40, and 80 min after the addition of unlabeled uridine. RNA was isolated immediately after removal of each fraction. The amount of radioactivity in poly(A)+ RNA molecules in each sample was assayed by the oligo(dT)-cellulose centrifuge chromatography method.

**RNAse A and RNAse T1 digestions.** Samples of *M. vannielii* poly(A)+ RNA labeled with [8-3H]adenosine were dissolved in 200 μl of 10 mM Tris-hydrochloride (pH 7.6)–0.33 M NaCl–2 mM MgCl2 containing 3 μg of RNAase A, 100 U of RNAse T1, and 15 μg of DNase I (13). After digestion for 30 min at 37°C, macromolecules were precipitated by the addition of ice-cold TCA (final concentration, 5% [wt/vol]), and the radioactivity in TCA-precipitable material was measured. These values were compared with the amounts of radioactivity in TCA-precipitable material determined before and after digestion of samples of the [8-3H]adenosine-labeled poly(A)+ RNA for 48 h in 0.3 N NaOH at 37°C.

**Poly(U) hybridization.** Amounts of poly(A) in RNA samples were determined by hybridization of [3H]poly(U) to unlabeled RNA samples and subsequent digestion of the nonhybridized material with RNAse A (12). Increasing amounts of unlabeled RNA were mixed with 2.3 nCi of [3H]poly(U) (1.6 ng) in 50 μl of hybridization buffer (10 mM Tris-hydrochloride [pH 7.6], 0.2 M NaCl, 5 mM MgCl2, 0.2% [wt/vol] SDS), and the mixtures were incubated at 25°C for 15 min. Reactions were stopped and SDS was precipitated by the addition of 50 μl of 200 mM KCl–5 mM MgCl2 and by incubation at 4°C for 20 min. The resulting K+–SDS precipitates were removed by centrifugation for 2 min in a microfuge at 4°C, and the supernatants were collected. RNAse A (final concentration, 2.6 μg/ml) and DNase I (final concentration, 33 μg/ml) were added to the supernatants, and the mixtures were incubated at 25°C for 30 min. Carrier DNA (20 μg of sonicated salmon sperm DNA) was added, and macromolecules were precipitated by the addition of ice-cold TCA (final concentration, 2.5% [wt/vol]). Precipitates were quickly collected by filtration under conditions minimizing TCA hydrolysis of poly(U) (44), and radioactivity in the precipitates was measured. A standard curve was constructed by hybridizing known amounts of synthetic poly(A) (Sigma) to the fixed amount of [3H]poly(U).

**Electrophoresis.** Samples of total *M. vannielii* RNA, labeled by incorporation of [3H]uridine at 37°C for 3 min or 16 h, and samples of [3H]uridine-labeled poly(A)+ RNA (isolated from total RNA samples labeled for 3 min) were glyoxylated by incubation in 16 μl of a buffer containing 50% [vol/vol] dimethyl sulfoxide, 1 M deionized glyoxal, and 10 mM NaH2PO4 (pH 7) for 1 h at 56°C (34). Tracking dye was added, and the samples were loaded into the wells of a 1.8% (wt/vol) agarose gel buffered with 10 mM NaH2PO4 (pH 7). Electrophoresis was carried out for 2 h at 100 V (75 mA).

**Poly(A) length determination.** The lengths of 3' poly(A) tracts in *M. vannielii* poly(A)+ RNA molecules were determined by the electrophoretic mobility of material resistant to digestion with RNAse A and RNAse T1. The 3' ends of poly(A)+ RNA molecules (0.6 μg) were labeled by incubation for 24 h at 4°C in 30-μl reaction mixtures containing 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 8.3), 10 mM MgCl2, 3.3 mM dithiothreitol, 0.1 mM ATP, 10% (vol/vol) dimethyl sulfoxide, 15% (vol/vol) glycerol, 0.5 mM (50 μCi) [5,6-3H]uridine (3,5′-diphosphate ([32P]CBP), 2,900 Ci/mmol; New England Nuclear), and 10 U of RNA ligase (New England Nuclear) (10). Reactions were stopped by incubation for 5 min at 65°C. Unincorporated label was removed by passage of the reaction mixture through a Sephadex G-50 column (equilibrated and eluted with 10 mM Tris-hydrochloride [pH 7.4]). Labeled RNA in the eluate was collected by ethanol precipitation.

Samples of the [32P]-labeled RNA were digested at 37°C for 30 min with RNAse A and RNAse T1 as described by Shultz et al. (41). Reaction mixtures (100 μl) contained 10 mM Tris-hydrochloride (pH 7.4), 0.33 M NaCl, 12.5 μg of yeast tRNA, 2.8 mM MgCl2, 0.1 μg of poly(A), 7.5 U of RNAse T1, and increasing amounts of RNAse A (0.012, 0.06, 0.3, and 1.5 μg). Control samples were incubated with only RNAse A, with only RNAse T1, and without either RNAse A or RNAse T1. Digestions were stopped by adding 100 μl of 50% phenol–48% chloroform–2% isoamyl alcohol, blending with a Vortex mixer, and centrifuging for 1 min in a microfuge. The aqueous phases were collected. The organic phases were mixed with 100 μl of 0.5 M sodium acetate and blended with a Vortex mixer, and the mixtures were centrifuged again for 1 min in a microfuge. The resulting aqueous phases were pooled with the original aqueous phases. Carrier RNA (1.2 μg of yeast tRNA) and 0.5 ml of ethanol were added, and the samples were placed at −70°C for 30 min. Precipitates were pelleted by centrifugation for 15 min in a microfuge at 4°C and dissolved in electrophoresis sample buffer (89 mM Tris-borate [pH 7.8], 89 mM boric acid, 2 mM EDTA, 5% [vol/vol] glycerol, 0.05% [vol/vol] xylene cyanol, 0.05% [wt/vol] bromophenol blue). Polynucleotides in the samples were separated by electrophoresis through a 10% polyacrylamide–7 M urea DNA sequencing gel (33), and their location, after electrophoresis, was detected by autoradiography.

### Table 1. Isolation of poly(A)+ RNA from *E. coli* and *M. vannielii*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Labeling period*</th>
<th>cpm retained (%)p</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> W3110</td>
<td>30 s</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>2 h</td>
<td>3.0</td>
</tr>
<tr>
<td><em>M. vannielii</em></td>
<td>3 min</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>16 h</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* RNAs were labeled with [5,6-3H]uridine as described in the text.

**Poly(A) length determination.** The lengths of 3' poly(A) tracts in *M. vannielii* poly(A)+ RNA molecules were determined by the electrophoretic mobility of material resistant to digestion with RNAse A and RNAse T1. The 3' ends of poly(A)+ RNA molecules (0.6 μg) were labeled by incubation for 24 h at 4°C in 30-μl reaction mixtures containing 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 8.3), 10 mM MgCl2, 3.3 mM dithiothreitol, 0.1 mM ATP, 10% (vol/vol) dimethyl sulfoxide, 15% (vol/vol) glycerol, 0.5 mM (50 μCi) [5,6-3H]uridine (3,5′-diphosphate ([32P]CBP), 2,900 Ci/mmol; New England Nuclear), and 10 U of RNA ligase (New England Nuclear) (10). Reactions were stopped by incubation for 5 min at 65°C. Unincorporated label was removed by passage of the reaction mixture through a Sephadex G-50 column (equilibrated and eluted with 10 mM Tris-hydrochloride [pH 7.4]). Labeled RNA in the eluate was collected by ethanol precipitation.
S' cap assay. The presence of S' cap structures in M. 
vannielii poly(A)+ RNA molecules was assayed by attempting 
to label the RNA molecules at their S' ends with T4 
polyadenylate kinase, after alkaline phosphatase treatment, 
with and without initial treatment of the RNA with tobacco 
acid pyrophosphatase (TAP), an enzyme which removes S' 
cap structures (9). Reaction mixtures of 10 µl containing 50 
mM sodium acetate (pH 6), 10 mM 2ME, 0.07 µg of M. 
vannielii poly(A)+ RNA, and 2 U of TAP (Bethesda Research 
Laboratories, Gaithersburg, Md.) were incubated at 
37°C for 30 min; 2 µl of 0.5 M Tris-hydrochloride (pH 8), 7 µl 
of water, and 1 µl (4 U) of calf intestine alkaline phosphatase 
(Boehringer Mannheim Biochemicals, Indianapolis, Ind.) 
were then added. After incubation at 56°C for 30 min, 
enzyme activity was stopped by the addition of 1 µl of 0.25 
M K2HPO4 (pH 9.5). The reaction mixture was transferred 
to a tube containing 2 µCi of [γ-32P]ATP (4,500 Ci/mmol; ICN 
Radiochemicals, Irvine, Calif.), 200 pmol of unlabeled 
ATP, 1 µl of 0.25 M MgCl2, 2 µl of 50 mM dithiothreitol, and 
1 µl (10 U) of T4 polynucleotide kinase (Pharmacia Bio- 
chemicals, Piscataway, N.J.), and the mixture was incu- 
bated at 37°C for 30 min. The reaction was stopped, and 
protein was removed by adding 200 µl of 2 M ammonium 
acetate and 200 µl of phenol-chloroform, blending with a 
Vortex mixer, and centrifuging for 1 min in a microfuge. The 
aqueous phase was collected, and RNA was precipitated by 
the addition of 28 µg of carrier tRNA and 600 µl of ethanol 
and incubation at −70°C for 30 min. The precipitate 
was collected by centrifugation for 15 min at 4°C in a microfuge, 
washed with 70% ethanol, and denatured by glyoxylation as 
described above. A fraction was removed, and the amount 
of radioactivity incorporated into material precipitated by 
ice-cold 5% TCA was determined. RNAs in the remainder 
of the sample were then separated by electrophoresis through a 
vertical 2% (wt/vol) agarose gel. The gel was dried and used 
to expose Kodak X-A-R film. Reaction mixtures containing 
S. cerevisiae poly(A)+ RNA, S. cerevisiae tRNA (Sigma), 
and M. vannielii poly(A)+ RNA, in place of M. vannielii 
poly(A)+ RNA, were also prepared and handled by identical 
procedures.

RESULTS
Detection of poly(A)+ RNA molecules in M. vannielii. M. 
vannielii RNA was rapidly labeled in vivo with either 
[5,6-3H]uridine or [8-3H]adenine. The percentages of the 
total labeled RNA that bound to oligo(dT)-cellulose at 4°C 
after labeling with [3H]uridine for periods equivalent to 0.01 
and 3 generations are given in Table 1. Approximately 16

FIG. 1. Poly(A) assay curves of total RNAs from S. cerevisiae, E. coli, and M. vannielii. Unlabeled RNA preparations were allowed to hybridize with [3H]poly(U), and the mixtures were then subjected to digestion with RNase A. Undigested material was precipitated by TCA, and the amount of radioactivity in these molecules was measured. A standard curve was constructed with synthetic poly(A) in place of total RNA (A). The slopes of the lines obtained from S. cerevisiae (B), E. coli (C), and M. vannielii (D) total RNA preparations were compared with the standard curve to determine what percentages of the total RNAs were poly(A) sequences (see Table 2).
and 21% of the RNA labeled for 0.01 generations in M. vannielii and E. coli, respectively, bound to oligo(dT)-cellulose, i.e., RNA molecules which contained poly(A) sequences. This value obtained for E. coli W3110 is very close to the value (18%) reported by Gopalakrisna et al. (12) for E. coli B. In comparison, only 0.68 and 0.55% of the RNA labeled for three generations in E. coli and M. vannielii, respectively, bound to oligo(dT)-cellulose. Of the radioactive material retained by oligo(dT)-cellulose in samples of RNA labeled for 0.01 generations, 98% was converted to TCA-soluble material by digestion with 0.3 M NaOH at 37°C for 16 h.

Results obtained by assaying RNA samples from S. cerevisiae, E. coli, and M. vannielii for the amounts of poly(A) are given in Fig. 1 and Table 2. The amount of poly(A) as a fraction of total S. cerevisiae RNA is approximately 75-fold higher than the equivalent amount in preparations of either E. coli RNA or M. vannielii RNA. Assuming an average length of 1,200 bases for poly(A)+ RNA molecules in M. vannielii (a value obtained by electrophoresis; see below and Fig. 2), and given that 0.55% of M. vannielii total RNA is poly(A)+ RNA molecules (Table 1) and that poly(A) tracts comprise 0.0065% of total M. vannielii RNA (Table 2), the mean length of poly(A) tracts can be calculated. Total RNA contains 0.55% poly(A)+ RNA molecules, or 5.5 ng of poly(A)+ RNA molecules per μg of total RNA. In addition, 1 μg of total RNA contains 0.065 ng of poly(A) tracts. The fraction of the poly(A)+ RNA molecules which is composed of poly(A) tracts is therefore 0.065/5.5 or 1.2%. This would be 14 bases of poly(A) for an average poly(A)+ RNA molecule with a length of 1,200 bases.

Electrophoretic analysis of M. vannielii poly(A)+ RNA molecules. Electrophoretic separations of denatured M. vannielii RNA samples are shown in Fig. 2. Samples of total RNA, radioactively labeled for several generations, had radioactivity located in sharp peaks corresponding to 23S, 16S, and 4–5S RNA species (Fig. 2A). M. vannielii apparently also converts uridine to deoxynthymidy late-3′-tri phosphate, as radioactivity was detected in the chromosomal DNA band. Radioactivity in total RNA samples, labeled for only 3 min, was also found in 23S and 16S molecules, but, in addition, radioactivity was found in molecules with mobilities indicating sizes intermediate between 23S (3,000 bases) and 9S (900 bases) (Fig. 2B). Radioactivity was found predominantly in molecules in this size range, with an average length of approximately 1,200 bases, after electrophoresis of labeled molecules preselected by their ability to bind oligo(dT) (Fig. 2C).

Length of poly(A). The quantity of poly(A) sequences in [3H]adenine-labeled M. vannielii poly(A)+ RNA molecules was estimated by resistance of labeled material to digestion with RNase A (which cleaves 3′ to pyrimidine ribonucleotide residues) and RNase T1 (which cleaves 3′ to guanosine residues). Of the radioactivity in [8–3H]adenine-labeled M. vannielii poly(A)+ RNA molecules, 4.3% was in oligonucleotides resistant to digestion. By assuming a mean length of 1,200 bases for poly(A)+ RNA molecules (Fig. 2C) and an average of 35% adenosine residues in the non-poly(A) regions of the molecules (M. vannielii DNA is 71% A+T [11]), the length of poly(A) tracts in these molecules again can be calculated. The average poly(A)+ RNA molecule contains 1,200 × 0.35, or 420, adenosine residues, 4.3% (18 bases) of which were resistant to digestion with RNase A and RNase T1 and therefore represent the poly(A) tracts.

![FIG. 2. Electrophoretic separation of denatured 3H-labeled RNAs prepared from M. vannielii. RNA molecules from cells labeled with [5,6-3H]uridine for 3 generations (A) and 0.01 generations (B) and poly(A)+ RNA molecules isolated from cells labeled for 0.01 generations (C) were denatured by glyoxylation and separated by electrophoresis through a 1.8% agarose gel. The locations of DNA and stable RNAs were determined by examination of the gel after staining with acridine orange. The gel in each lane was then sliced into fractions, and the radioactivity in each fraction was determined. The total radioactivity in A, B, and C was 22,857, 12,180 and 6,216 cpm, respectively.](http://jb.asm.org/)
The location of M. oligomers were digested G) without standards (H poly(A)+ RNA of RNase 6, 10, 11, 12, adds therefore the indicating are bases [6-, 7-, 8-, 16-, 22-, and 27-base poly(A) tracts]. These minor components are more readily detected after less thorough digestion with RNase A. Similar overall results were obtained by digestion with RNase A alone, except that there was much less radioactivity detected in the 6-base oligonucleotide and more radioactivity detected in the 11- and 12-base oligonucleotides.

**Half-life measurement of poly(A)+ RNA molecules in vivo.** The half-life of poly(A)+ RNA molecules in M. vannielii was determined by pulse-chase experiments. Values of 11, 12, and 12.5 min were obtained in three separate experiments (Fig. 4).

**Comparison of M. vannielii, E. coli, and S. cerevisiae poly(A)+ RNA molecules.** The method routinely used to isolate eucaryotic poly(A)+ RNA molecules is oligo(dT)-cellulose affinity chromatography at room temperature. The ability of oligo(dT)-cellulose columns to retain 3H-labeled and unlabeled RNAs at room temperature was therefore investigated. Although 3.4% of the total RNA preparations from S. cerevisiae was retained by the column, only traces (<0.1%) of material in preparations of total RNA from E. coli and M. vannielii, from cultures labeled over several generations, were retained. After pulse-labeling for 0.01 generations, 1.6 and 0.4% of radioactivity in RNA preparations from E. coli and M. vannielii cells, respectively, were retained by oligo(dT)-cellulose columns at room temperature. In contrast, 21 and 16% of incorporated radioactivity in these same pulse-labeled preparations were retained by oligo(dT)-cellulose at 4°C when binding was assayed by the centrifuge method (Table 1).

The size of eucaryotic poly(A)+ RNA molecules can be estimated, after hybridization of total unlabeled RNA to [3H]poly(U), by sedimentation of the hybridized products through aqueous neutral sucrose gradients. For example, S.

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**FIG. 3.** Electrophoretic separation of 3'-end-labeled poly(A) tracts. M. vannielii poly(A)+ RNA molecules were 3' end labeled and then digested with RNase A and RNase T1. Digestion-resistant oligomers were separated by electrophoresis in a DNA sequencing gel. The location of [32P]CPB and the locations of molecular weight standards from a known DNA sequence separated in tracks of the same gel are shown on the right. Labeled RNA was treated as follows: (A) no treatment; (B) incubated in the reaction mixture without RNase A or RNase T1; (C) digested with KOH; (D through G) digested with 1.5, 0.3, 0.06, and 0.012 μg of RNase A, respectively; (H through K) digested with RNase A as in lanes D through G but with the addition of 7.5 U of RNase T1 to each reaction mixture.

The lengths of the 3' poly(A) tracts were determined directly by electrophoresis of the oligonucleotide products of RNase A and RNase T1 digestions of 3'-end-labeled poly(A) RNA molecules (Fig. 3). The majority of radioactive molecules detected had mobilities indicating lengths of 6, 10, 11, 12, and 13 bases. End labeling with RNA ligase added 1 base (in this case cytosine, from the [32P]CPB), and therefore the results indicate that the majority of poly(A) tracts are 5, 9, 10, 11, and 12 bases. Fainter bands are evident, indicating chain lengths of 7, 8, 9, 17, 23, and 28 bases [6-, 7-, 8-, 16-, 22-, and 27-base poly(A) tracts]. These

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**FIG. 4.** Determination of the half-life of poly(A)+ RNA molecules in M. vannielii cells. M. vannielii cells were allowed to incorporate [5,6-3H]uridine for 3 min at 37°C. Excess unlabeled uridine was then added, and fractions were removed at various times during subsequent incubation at 37°C. Radioactivity in poly(A)+ RNA molecules was measured in each fraction and is expressed as a percentage of the amount of radioactivity in poly(A)+ RNA molecules in the RNA preparation isolated immediately after the labeling period.
POLYADENYLATED, NONCAPPED RNA FROM M. VANNIELII

FIG. 5. Sucrose gradient separation of [3H]poly(U) after hybridization to total RNAs. Unlabeled total RNA preparations from E. coli (A), S. cerevisiae (B), and M. vannielii (C) were allowed to hybridize with [3H]poly(U) at 45°C. Molecules in the reaction mixture were then separated by sedimentation through 5 to 20% sucrose gradients. Absorbance at 260 nm (●) and radioactivity (○) in each fraction was measured.

cerevisiae RNA preparations were found to contain poly(A)^+ RNA molecules exhibiting a broad range of sizes with a most frequent size of approximately 10S (Fig. 5B). In contrast, E. coli and M. vannielii RNA preparations, when tested in the same manner, lacked molecules capable of hybridizing to [3H]poly(U), as demonstrated by the inability of these RNA preparations to significantly alter the sedimentation profile of the added [3H]poly(U) (Fig. 5A and C, respectively).

5' cap assay. The absence of 5' cap structures in M. vannielii poly(A)^+ RNA molecules was demonstrated by the finding that these molecules could be 5' end labeled with and without prior treatment of the RNA molecules with TAP (Table 3). S. cerevisiae poly(A)^+ RNA, which contains 5' caps (42), was poorly labeled without prior removal of caps by treatment with TAP. M. vannielii poly(A)^+ RNA molecules, however, were labeled equally well both with and without prior treatment with TAP, as were M. vannielii nonadenylated RNA molecules and S. cerevisiae tRNA molecules. Autoradiographic analysis of the labeled RNAs separated by electrophoresis through 2% agarose gels demonstrated that the poly(A)^+ RNA preparations contained a mixture of RNA molecules exhibiting a broad range of molecular weights, which is consistent with the results shown in Fig. 2 and 5. Radioactivity incorporated into M. vannielii nonpolyadenylated RNA was mostly located in 16S and 23S rRNAs (data not shown).
TABLE 3. Effect of TAP pretreatment on T4 polynucleotide kinase directed incorporation of [γ-32P]ATP

<table>
<thead>
<tr>
<th>RNA used as substrate†</th>
<th>γ-32P incorporated (cpm)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+TAP</td>
</tr>
<tr>
<td>M. vannielii poly(A)+</td>
<td>6005</td>
</tr>
<tr>
<td>M. vannielii poly(A)-c</td>
<td>7978</td>
</tr>
<tr>
<td>S. cerevisiae poly(A)+</td>
<td>9560</td>
</tr>
<tr>
<td>S. cerevisiae RNA</td>
<td>4146</td>
</tr>
</tbody>
</table>

† Reaction mixtures contained 70 ng of RNA.
‡ Incorporation of [γ-32P]ATP by T4 polynucleotide kinase with (+) and without (−) pretreatment of the RNA sample with TAP (see text).

Poly(A)+ RNA is RNA that does not bind oligo(dT)-cellulose and remains in the supernatant after the initial pelleting of the oligo(dT)-cellulose during the isolation of poly(A)+ RNA.

**DISCUSSION**

In this study, we report the detection, isolation, and characterization of poly(A)-containing RNA molecules from the methanogenic archaebacterium *M. vannielii*. Almost 16% of the radioactivity incorporated into RNA preparations from *M. vannielii* cells labeled for very short periods of time (0.01 generations) with [5,6-3H]uridine is located in molecules which bind to oligo(dT)-cellulose at 4°C. In contrast, only 0.55% of the radioactivity in RNA preparations from *M. vannielii* cells labeled for several generations is located in molecules which bind to oligo(dT)-cellulose. These values are similar to the percentages of labeled RNA which bind to oligo(dT)-cellulose in preparations of *E. coli* W3110 (20.9 and 0.68%) when *E. coli* cells were labeled for the same fractions of their generation time. These values are also similar to those reported for various eubacteria (12, 13, 19, 23, 39) and cyanobacteria (6). *M. vannielii* poly(A)+ RNA molecules were found to be unstable, with an in vivo half-life of 12 min or 1/24th of a generation time, at 37°C. *E. coli* mRNA has been shown to have a half-life of approximately 2 min, or 1/20th of a generation time, at 37°C (16). 3'–terminal poly(A) tracts isolated from *M. vannielii* were found to vary from 5 to 27 bases in length, averaging approximately 10 bases in length. This value is in the same range as those reported for a variety of eubacteria (6, 37, 41). The occurrence in *M. vannielii* of poly(A) tracts with lengths that primarily increase by multiples of 5 bases suggests the possibility that poly(A) is added in units of 5 bases. The decrease in radioactivity in the higher/molecular weight poly(A) tracts (Fig. 3) on exhaustive digestion with RNase A may indicate the presence of pyrimidine bases located at approximately 5-base intervals in some of the 3' poly(A) tracts of the poly(A)+ RNA molecules.

The size distribution of *M. vannielii* poly(A)+ RNA molecules was found to be heterogeneous, ranging from about 900 to 3,000 bases in length. Assuming that these are mRNA molecules, they could encode single polypeptides ranging from 30,000 to 100,000 daltons. Similar size ranges for poly(A)+ RNA molecules have been found in a number of eubacterial species (19, 37, 41), all of which contain polycistrionic mRNAs. Therefore, it is probable that many of the *M. vannielii* poly(A)+ RNA molecules are also polycistrionic mRNA molecules, although corroborating evidence for such molecules is as yet only preliminary (32, 43; U. Konheiser, G. Pasti, C. Bollschweiler, and A. Klein, Mol. Gen. Genet., in press).

The presence of 5' cap structures could not be detected in poly(A)+ RNA molecules prepared from *M. vannielii*. These results suggest that *M. vannielii* probably employs the eubacterial system of 16S rRNA:mRNA hybridization rather than the eucaryotic system of 5' cap recognition to bind mRNAs to ribosomes for initiation of translation. DNA sequencing studies of *M. vannielii* genes support this conclusion. We have shown the presence of putative ribosomal binding sequences preceding ATG translation initiation codons (G. S. Beckler and J. N. Reeve, unpublished data).

Knowledge of the physical structure and metabolism of archaebacterial mRNA molecules currently is very limited. Cloning and sequencing studies have provided basic documentation of the structure of several rRNA (11, 20, 21, 29) and tRNA molecules (14, 24, 25), including the demonstration of introns in some archaebacterial TRNAs (22), but it remains to be determined whether archaebacterial mRNAs have such eucaryotic characteristics as introns or 3' cleavage before polyadenylation. Results of a preliminary report indicate that the thermoadophilic archaebacteria *Thermoplasma* and *Sulfolobus* species have poly(A)+ RNAs that are very similar to those of eucaryotes (M. Ohta and T. Oshima, 1982, Abstract in Proceedings of the 1982 Symposium on Archaeabacteria, p. 352), whereas results of detailed studies of the bacterio-opsin mRNA from *H. halobium*, which is an archaebacterium related to the methanogens, indicate that this molecule is not polyadenylated. The discrepancy between the results reported here and the results of analysis of the bacterio-opsin encoding mRNA may well reside in the method used to isolate RNA. The method which was used to isolate mRNA from *H. halobium* is not one which has yielded poly(A)+ RNA from procaryotes (7, 8). The extent of polyadenylation in the total population of RNA molecules in *H. halobium* has not been reported. The results presented here are the first documentation of poly(A)+ RNA molecules in the halophilic and methanogenic grouping of archaebacteria and indicate that, at least in *M. vannielii*, such molecules exhibit eubacterial rather than eucaryotic features.

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