MOLECULAR AND CELLULAR BIOLOGY, Jan. 1984, p. 101–109
0270-7306/84/010101-09$02.00/0
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Vol. 4, No. 1

Saccharomyces cerevisiae Killer Virus Transcripts Contain Template-Coded Polyadenylate Tracts

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The M double-stranded RNA component of type 1 killer strains of the yeast *Saccharomyces cerevisiae* contains an internal 200-base pair adenine- and uracil-rich region. The plus strands of this viral genomic RNA contain an internal adenine-rich region which allows these strands to bind to polyuridylate-Sepharose as tightly as do polyadenylated RNAs with 3′-terminal polyadenylated tracts of 70 to 100 residues. Internal template coding of an adenine-rich tract in positive polarity in vivo and in vitro transcripts of M double-stranded RNA may serve as an alternate method of transcript polyadenylation. The 3′-terminal residue of the in vitro m transcript is a non-template-encoded purine residue. The 5′ terminus of this transcript is involved in a stem-and-loop structure which includes an AUG initiation codon, along with potential 18S and 5.8S rRNA binding sites. Except for the 3′-terminal residue, transcription in vitro shows complete fidelity.

Killer strains of the yeast *Saccharomyces cerevisiae* secrete a protein toxin which is lethal to sensitive strains, but to which killers themselves are resistant. The genetic information required for toxin production and resistance is encoded on a linear, double-stranded (ds) RNA molecule denoted M (1,830 base pairs). A number of killer types of different toxin (and resistance) specificity exist; killer strains of the type 1 specificity were used in this study. All killer strains, as well as most nonkiller strains, also harbor a larger species of dsRNA denoted L (4,980 base pairs). Both M and L dsRNAs are cytoplasmically inherited and are encapsidated within virus-like particles; no infectious cycle has yet been demonstrated (reviewed in references 15, 17, 54).

Virus particles from both killer and nonkiller strains have been shown to copurify with a DNA-independent RNA polymerase activity which catalyzes the synthesis of full-length (as judged by denaturing gel electrophoresis), asymmetric positive polarity transcripts of L and M dsRNAs (13, 51, 53), designated l and m, respectively. Denatured L dsRNA and l transcript can be translated in vitro to produce the major capsid protein of L- and M-containing virions (10, 13, 29). Denatured M dsRNA and m transcript encode M-p32, a 32,000-dalton putative toxin precursor (9, 52). L dsRNA has recently been found to consist of at least three distinct forms, L_A, L_B, and L_C, which are present in various combinations in different yeast strains (21, 23, 45).

Electron microscopy of M dsRNA has shown an internal, readily denaturable region of approximately 200 base pairs, which appears to be almost 100% adenine plus uracil (A+U) base pairs (26). M dsRNA can be selectively cleaved at this internal region by S1 nuclease treatment or high temperature (48, 52) to yield two double-stranded fragments, designated M-1 (1,000 base pairs) and M-2 (630 base pairs). The A,U-rich region lies between M-1 and M-2. Denatured M-1 encodes M-p32, and denatured M-2 encodes variable amounts of a 19,000-dalton protein (M-p19) in a rabbit reticulocyte lysate protein synthesis system (52). S3 dsRNA, an internal deletion mutant of M dsRNA, lacks the 200-base pair, A,U-rich region along with flanking sequences (26; D. J. Thiele, E. M. Hannig, and M. J. Leibowitz, manuscript in preparation).

The strands of M dsRNA can be electrophoretically separated. The plus polarity strand has been identified as that having the same polarity as the in vitro-synthesized m transcript (47). Sequence analysis of the 3′ termini of M dsRNA has predicted an extensive open reading frame at the 5′ terminus of the plus strand. An AUG triplet at positions 14 to 16 of the plus strand is preceded by potential 18S and 5.8S rRNA binding sites in a region of the RNA predicted to be involved in a stem-and-loop structure (32, 47, 48). Such sequences may regulate the translation of M-p32. In vivo, M dsRNA is transcribed to produce both full-length (1,830 bases) and subgenomic (approximately 1,200 bases) transcripts (8; this work). Yeast mRNA synthesized in vivo which is homologous to M dsRNA has been shown to program the in vitro synthesis of M-p32, the putative killer toxin precursor (8).

We have analyzed yeast viral dsRNAs, separated strands, and in vitro transcripts for polyadenylate poly(A) based upon their affinity for oligodeoxythymidylate [oligo(dT)]-cellulose. Despite the affinity of plus genomic strands and the m in vitro transcript, sequence analysis reveals these molecules to lack 3′-terminal poly(A). We propose that the 200-base pair, A,U-rich internal region of M dsRNA consists primarily of AMP residues on the plus strand and UMP residues on the minus strand. This internal A-rich region may be responsible for the binding of plus genomic strands and of in vitro-synthesized m transcript to oligo(dT)-cellulose and polyuridylate [poly(U)]-Sepharose. This region may also be responsible for the similar behavior of full-length and subgenomic in vivo transcripts of M dsRNA, whose chromatographic behavior does not necessarily reflect post-transcriptional polyadenylation.

MATERIALS AND METHODS

***S. cerevisiae* strains and growth conditions.** Cells of the diploid prototrophic type 1 killer A364A × S7 (**a**/α *ade1*/+ *ade2*/+ *ura1*/+ *tyr1*/+ *his7*/+ *lys2*/+ *gal1*/*gal1* [KIL-k_1]) were used as a source of L and M dsRNAs (53). Cells were grown to stationary phase (5 days), unless otherwise specified, at 28°C with constant shaking in a medium containing

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1% yeast extract, 2% peptone, and 5% ethanol. The suppressive nonkiller T132B NK-3 (a *ade2-1 his4-864* [KIL-s]) (46) grown in YPAE medium (yeast extract-peptone medium with 0.04% [wt/vol] adenine sulfate plus 3% ethanol) was the source of S3 dsRNA.

Purification of *S. cerevisiae* dsRNA. Double-stranded RNA was extracted from cells by a modification (51) of the method described previously (26). Individual species of dsRNA were purified by preparative 1.5% agarose slab gel electrophoresis, ethanol precipitated twice, and stored at −20°C in sterile glass-distilled water.

Terminal modification of ds-RNAs. The 3′ termini of dsRNA species were reacted with [5′-^{32}P]pCp (cytidine 3′,5′-[^{32}P]bisphosphate; Amersham Corp.) in the presence of bacteriophage T4 RNA ligase (P-L Biochemicals, Inc.) as described previously (48). Incorporation of ^{32}P from [γ-^{32}P]ATP (Amersham; specific activity, 3,000 Ci/mmol) onto the 5′ termini of dsRNA in a reaction catalyzed by T4 polynucleotide kinase (P-L Biochemicals), after treatment with alkaline phosphatase, was performed as described previously (20). All labeling reactions were extracted with phenol (90%; aqueous)-chloroform-isoamyl alcohol (50:49:1), followed by chloroform-isoamyl alcohol (24:1) extraction of the aqueous phase. RNA was precipitated from the aqueous phase overnight at −20°C after the addition of 0.1 volume of 3 M sodium acetate (pH 6.0) and ethanol (2.5 to 3 volumes). These methods resulted in plus and minus polarity strands of M with equivalent 3′-terminal radioactivity. Incorporation of ^{32}P onto 5′ termini resulted in plus strands with at least 10 times the specific activity of minus strands, which may reflect conformational differences due to base composition.

Strand separation of dsRNAs. Strands of M and L_A dsRNA were separated as described previously (47). S3 dsRNA was strand separated in a similar manner, except that a 10% polyacrylamide gel was used. RNA species were excised and extracted from the gels as described previously (33), except that magnesium acetate was omitted. Eluted RNA was precipitated twice overnight at −20°C from 0.3 M sodium acetate by the addition of 2.5 to 3 volumes of ethanol. The pellets were washed twice with 70% ethanol, vacuum dried, and stored at −20°C in sterile glass-distilled water.

Preparation of in vitro transcripts. Virus particles were purified as described previously (53). Full-length, single-stranded l_A, m, and s transcripts were synthesized in the presence of nonradioactive or [α-^{32}P]UTP (Amersham) in an in vitro reaction catalyzed by the virus-associated DNA-independent RNA polymerase (53). Products were extracted as described previously (51) and were fractionated by electrophoresis on 1% agarose slab gels. Individual RNA species were visualized by ethidium bromide (1 μg/ml) staining or autoradiography, excised from the gel, and extracted as described above. Radioactive [^{32}P]UMP-m transcript was further purified through a strand separation gel as described above.

Terminal modification of in vitro-synthesized m transcript. The m transcript was purified from phenol extracts (51) of transcription reactions by oligo(dT)-cellulose chromatography (see below) and agarose gel electrophoresis. [5′-^{32}P]pCp was linked to its 3′ terminus as described above, except that dimethyl sulfoxide (10%) was present during the reaction. Incorporation of ^{32}P onto its 5′ terminus was performed as described above. Radioactive m transcript was then purified by polyacrylamide gel electrophoresis (47), visualized by autoradiography, and eluted from the gel as described above.

End analysis of RNA. RNA with one radioactive terminus (2,000 to 4,000 cpm) was lyophilized in 1.5-ml tubes in the presence of 5 μg of *Escherichia coli* B tRNA (GIBCO Laboratories). Terminal analysis of 3′-[5′-^{32}P]pCp RNA by digestions with RNase T2 (Calbiochem) was as described previously (50). For end analysis of [5′-^{32}P]RNA, the RNA pellet was redissolved in 10 μl of P1 enzyme mix (0.5 mg of P1 nuclease [Sigma Chemical Co.] per ml in 8.5 mM sodium acetate [pH 6.0]) and incubated for 45 min at 37°C. The released 5′ nucleotides were separated by polyethyleneimine cellulose chromatography (50), individual 5′ nucleotides were visualized by fluorescence under UV illumination (254 nm), and the radioactivity in each was determined by scintillation counting in a toluene-based scintillant.

RNA sequence analysis. Chemical sequence analysis of RNA was performed according to the method of Peattie (39). Enzymatic hydrolysis for sequence determination was carried out as described previously (48), except that pretreatment of RNA at 100°C was omitted and RNase U2 reactions were performed at pH 3.5. The specificities for the enzymatic reactions are as follows: RNase T_1, G, and RNase U2, A (44); RNase Phy M, A+U (18); and RNase CL-3, C (7). All sequencing enzymes, except for CL-3 (Bethesda Research Laboratories), were from P-L Biochemicals. Formamide ladders were generated as described previously (44); gels were run as described previously (19).

Purification of yeast mRNA. Cells of strain A364A × S7 growing logarithmically (optical density at 650 nm = 1.0 to 4.0) in yeast extract-peptone-ethanol medium at 28°C were collected at 0°C in a GSA rotor, washed once with ice-cold extraction buffer (50 mM Tris-hydrochloride [pH 7.5]–0.1 M NaCl–10 mM disodium-EDTA), and suspended in extraction buffer plus 1% Sarkosyl (sodium-*N*-lauroylsarcosinate) at approximately 10 ml per liter of original culture. Bentonite was added to 0.5 mg/ml, and an equal volume of ice-cold, acid-washed glass beads (0.45 to 0.50 mm) was added. Immediately after the addition of an equal volume of phenol (90% aqueous)-chloroform-isoamyl alcohol (50:49:1), cells were broken by high-speed vortexing (five 20-s bursts) at 4°C. After centrifugation, the phenol phase was re-extracted with extraction buffer (plus Sarkosyl). Pooled aqueous phases were then extracted with phenol-chloroform-isoamyl alcohol, then chloroform-isoamyl alcohol (24:1) repeatedly until the interface was clear. RNA was precipitated twice overnight at −20°C from 0.3 M sodium acetate with 2.5 volumes of ethanol.

Oligo(dT)-cellulose chromatography. Oligo(dT)-cellulose columns (type 3, Collaborative Research, Inc.) were used to select poly(A)-enriched RNAs (3). RNA samples were suspended in binding buffer, consisting of 10 mM Tris-hydrochloride (pH 7.4), 0.5 M NaCl, 1 mM disodium-EDTA, 0.5% sodium dodecyl sulfate (SDS), heated at 65°C for 3 min, chilled in an ice bath, and applied to the column after the addition of glycerol to 10% (vol/vol). After 5 min, columns were washed with at least 7 column volumes of binding buffer. Bound RNA was eluted in 10 mM Tris-hydrochloride (pH 7.4)–1 mM disodium-EDTA–0.05% SDS. For quantitative analysis of binding of [^{32}P]RNA species, 1-ml fractions were collected, and the radioactivity in each was determined by Cerenkov counting. RNA was precipitated (with or without salt addition) by the addition of 2.5 to 3 volumes of ethanol.

Thermal elution from poly(U)-Sepharose. Thermal elution of RNA from columns of poly(U)-Sepharose (Pharmacia Fine Chemicals, Inc.) was performed as described previously (37).

Electrophoresis of denatured RNA, transfer to nitrocellulose paper, and hybridization. Electrophoresis of RNA on denaturing agarose (1%)-formaldehyde gels was as described previously (31). Transfer of RNA to nitrocellulose paper was as described previously (4). Bound RNA was hybridized with 3′-[5′-^{32}P]pCp M dsRNA minus strand or with m transcript synthesized in vitro in the presence of [α-^{32}P]UTP. Radioactive RNA probes were preheated at 65°C for 5 min in hybridization solution without SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate). Hybridization proceeded at 65°C for 24 h in a solution containing 50% formamide, 5× SSC, 20 mM sodium phosphate (pH 7.0), 0.1% SDS. After incubation, the nitrocellulose sheets were washed as follows: twice in 2× SSC–0.1% SDS at room temperature; once in 1× SSC–0.1% SDS at 65°C; once in 0.5× SSC–0.1% SDS at 65°C; twice in 0.25× SSC–0.1% SDS at 65°C. All washes were for 15 min. The nitrocellulose "blots" were then dried and exposed to X-ray film (XAR-5; Kodak) at −70°C with an intensifying screen (Du Pont).

S1 nuclease analysis. Secondary structural analysis of M plus strand bearing 5′-terminal ^{32}P was performed as described previously (38, 47).

RESULTS

Polarity of in vivo M dsRNA-specific transcripts. Killer cells grown in glucose synthesize both full-length (1,830 bases) and subgenomic (1,200 bases) M-specific transcripts which bind to poly(U)-Sepharose (8). Figure 1 shows that poly(A)-enriched RNA from ethanol-grown cells of strain A364A × S7 contains these full-length and subgenomic transcripts, as detected by hybridization with radioactive M dsRNA minus strands. However, when in vitro-synthesized m transcript is used as the probe, neither of these in vivo transcripts hybridizes with the radioactive probe. Therefore, the poly(A)-enriched RNA preparations are free from M dsRNA, and the in vivo transcripts are of plus polarity. Traces of M dsRNA hybridizing with both plus and minus polarity probes can be detected in the material not binding to the second oligo(dT)-cellulose column.

Analysis of in vitro products for polyadenylation. ^{32}P-labeled dsRNA, separated strands, and in vitro-synthesized transcripts of M, S3, and L_A dsRNAs were tested for their ability to bind to oligo(dT)-cellulose (Table 1). Only the plus genomic strand of M dsRNA and the m transcript were retained on the columns to any significant degree. Since the M dsRNA plus strand is known not to have a polyadenylated 3′ terminus (16, 47), an A-rich region must exist internally to explain this binding. Both the binding and the nonbinding fractions of in vitro-synthesized m transcript and genomic plus strand contain full-length molecules, as judged by denaturing gel electrophoresis (data not shown). When in vitro m transcript is passed for a second time over oligo (dT)-cellulose, more than 95% of the material in the binding and nonbinding fractions shows the same chromatographic behavior as on the first column. This demonstrates the existence of a small number of nonbinding transcripts. The relationship of this transcript heterogeneity to the four subspecies of M found in agarose gel-purified M dsRNA remains unknown. The M dsRNA subspecies have identical sequences at their 3′ termini, and have been suggested to represent different "conformers" of M dsRNA (48). Genomic RNA strands and transcripts derived from L_A dsRNA or from the S3 deletion mutant of M dsRNA were not retained on the column, and therefore presumably have no A-rich region longer than 30 AMP residues (36).

To further measure the binding strength of the A-rich region of purified m transcript, [^{32}P]UMP m transcript synthesized in vitro was bound to poly(U)-Sepharose and eluted stepwise with increasing temperature in 25% formamide. Most of the transcript (80%) bound to the column, and most eluted at 45°C (63% of input counts per minute), with a minor elution peak at 50°C. We obtained the same elution profile with in vivo M-specific transcripts run under identical conditions (Fig. 2), confirming the results of Bostian et al. (8). Therefore, both the in vitro and the two major in vivo transcripts of M dsRNA elute with polyadenylated RNAs having a poly(A) length of 70 to 100 residues (37).

m transcript is not polyadenylated in vitro. The ability of in

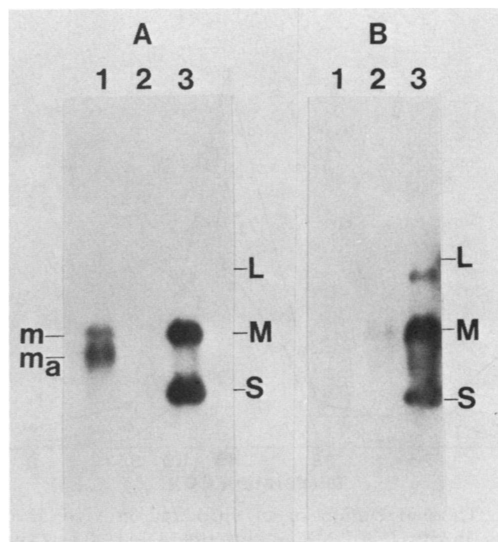

FIG. 1. Polarity of in vivo M dsRNA transcripts. Poly(A)$^+$ RNA (lane 1) purified twice by oligo(dT)-cellulose chromatography of A364A × S7 total RNA and RNA not binding to the second column (lane 2) were subjected to electrophoresis on agarose (1%)-formaldehyde gels, transferred to nitrocellulose paper, and hybridized with: (A) 3′-([5′-^{32}P]pCp)-M dsRNA minus strand, or (B) [^{32}P]UMP in vitro m transcript of plus polarity. Lane 3 contains denatured L, M, and S3 dsRNAs. The mobilities of denatured L, M, and S3 dsRNAs are indicated. These autoradiograms were purposely overexposed to demonstrate a lack of ssRNA of minus polarity and the relative absence of contaminating dsRNA. The nature of the species migrating slightly ahead of authentic L dsRNA and hybridizing with plus strands in lane B3 is unknown.

TABLE 1. Oligo(dT)-cellulose analysis of *S. cerevisiae* dsRNAs[a]

RNA species	% Unbound	% Bound
M dsRNA (native)	98.7	1.3
M plus strand	13.1	86.9
M minus strand	98.9	1.1
S dsRNA (native)	95.0	5.0
S plus strand	98.6	1.4
S minus strand	98.7	1.3
L_A dsRNA (native)	96.7	3.3
L_A plus strand	98.1	1.9
L_A minus strand	99.2	0.8
m transcript	14.8	85.2
s transcript	98.8	1.2
l_A transcript	95.8	4.2

[a] ^{32}P-labeled *S. cerevisiae* viral RNA species (20,000 to 120,000 cpm) were tested for their ability to bind on columns of oligo(dT)-cellulose (0.3 mg of dry powder) as described in the text. Recovery in all cases was quantitative. Strands of L_A dsRNA were purified as described elsewhere (46a).

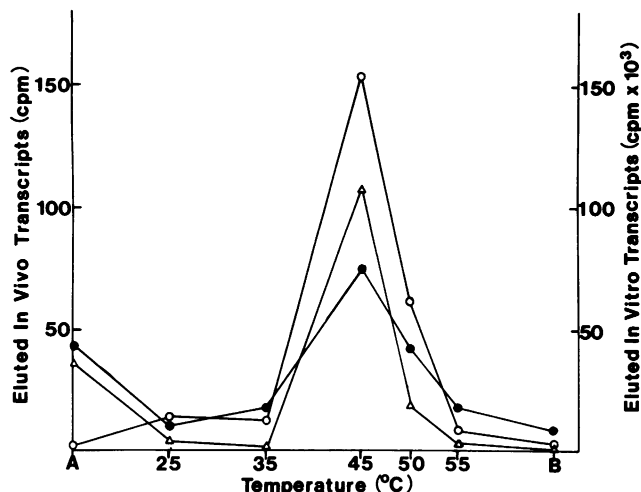

FIG. 2. Thermal elution of in vitro and in vivo M dsRNA transcripts. In vitro [^{32}P]UMP m transcript (4 μg [△]) was applied to a 2.5-ml column of poly(U)-Sepharose which was previously equilibrated in 50 mM Tris-hydrochloride (pH 7.5)–0.7 M NaCl–10 mM disodium-EDTA–25% formamide. RNA was eluted stepwise with increasing temperature in the same buffer with 0.1 M NaCl. Flow rate was 10 to 12 ml/h. Fractions (2 ml) were collected, and the radioactivity in a portion of each fraction (200 μl) was determined by Cerenkov counting. For analysis of in vivo transcripts of M dsRNA, total RNA from strain A364A × S7 was similarly fractionated. RNA eluting at each temperature was ethanol precipitated, subjected to electrophoresis on agarose (1%)-formaldehyde gels, transferred to nitrocellulose paper, and hybridized with 3′-([5′-^{32}P]pCp) M dsRNA minus strand. Hybridized RNA species corresponding to full-length m (●) and subgenomic m_a (○) in vivo transcripts were excised from the paper, and the radioactivity in each was determined by scintillation counting. Fraction A represents unbound material; fraction B was eluted at 55°C in 90% formamide. Recovery of in vitro-synthesized m transcript was quantitative.

vitro-synthesized m transcript to bind to oligo(dT)-cellulose was used as a step in a fractionation method to obtain transcript pure enough for nucleotide sequence determination. Oligo(dT)-cellulose-purified transcript was further purified by electrophoresis through a 1% agarose gel (Fig. 3). The transcript was eluted from the gel, and [5′-^{32}P]pCP was linked to its 3′ terminus, as described above, followed by further purification by polyacrylamide gel electrophoresis (47) before 3′-terminal sequence analysis.

Analysis of the 3′-terminal nucleotide of m transcript gave the following results (in percent total radioactivity in each base): 52.8% A, 38.5% G, 6.8% C, 1.9% U (average from three separate experiments). This contrasts sharply with the 3′ termini of the plus and minus strands of genomic M dsRNA, which are almost entirely adenosine residues (16, 48). Chemical sequence analysis of the 3′ terminus of the m transcript shows a sequence identical, for at least 100 bases, to that of the plus genomic strand (47), with the exception of the terminal base (Fig. 4). The doublets resolved on the lower portion of the gel indicate the presence of two populations of molecules, identical except for the 3′-terminal base, i.e., one population ending in . . .pA^{32}pCp(3′), the other in . . .pG^{32}pCp(3′). In vitro-synthesized m transcript is not terminally polyadenylated and therefore, as is the case with the plus genomic strand, contains an internal A-rich region responsible for binding to oligo(dT)-cellulose and poly(U)-Sepharose.

Primary and secondary structure at the 5′ terminus of m transcript. The sequence at the 5′ terminus of in vitro-synthesized m transcript was determined directly by partial enzymatic digestions, with base-specific endonucleases, of 5′-^{32}P-labeled m transcript. The sequence obtained (Fig. 5) is unique and agrees entirely with the sequence determined for the 5′ terminus of the genomic plus strand of M dsRNA (data not shown) and with the sequence predicted from the 3′-terminal sequence of the genomic minus strand (47). The 5′ terminus of the m transcript contains few G residues, a property similar to other yeast messages (28). In vitro transcription by the virion-associated RNA polymerase appears to initiate at a unique site with a G residue, resulting in the production of a full-length copy of the plus strand of M dsRNA (51). The degree of phosphorylation of the terminal G residue of the transcript before 5′-terminal labeling is unknown.

The single strand-specific S1 nuclease was used to test for secondary structure at the 5′ terminus of genomic plus strands, as previously used to analyze the 3′ termini of separated strands of M dsRNA (47). There were three main regions of S1 nuclease sensitivity of the plus strand 5′ terminus: bases 1 through 12, 28 through 36, and 65 through 67 (Fig. 6 and 7). Sensitivity at positions 1 to 9, which can be involved in a stem structure, presumably occurs because the presence of eight A-U base pairs of a possible nine in the stem allows the region to "breathe" in solution and exist transiently in single-stranded form. Positions 10 to 12 and 28

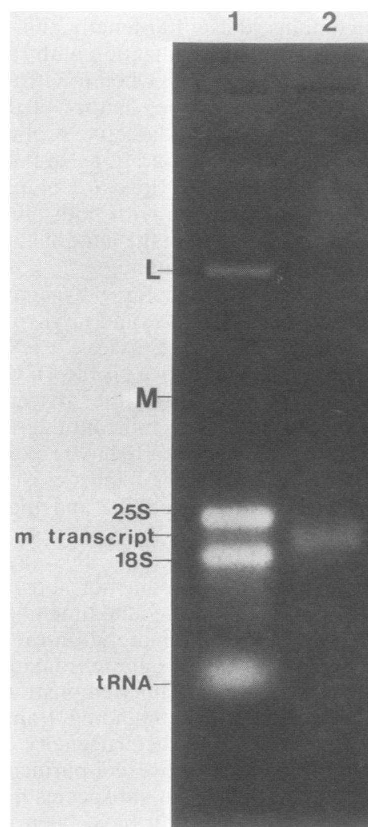

FIG. 3. Agarose (1%) gel electrophoresis of purified m transcript. Lane 1 contains phenol-extracted (26) total RNA from strain A364A × 57 (5 μg); lane 2 contains purified in vitro m transcript (1.1 μg). Electrophoresis was performed in the presence of ethidium bromide (1 μg/ml) for 4 h at 2 V/cm, and RNA species were visualized by UV fluorescence.

to 36 are involved in potential single-stranded loop structures. Bases 65 to 67, which follow the stem structure at the 3′ end of the hairpin, are also susceptible to cleavage. As noted previously (47), oligonucleotides from S1 digestions migrate with fragments 1 to 2 bases shorter in the T1 and

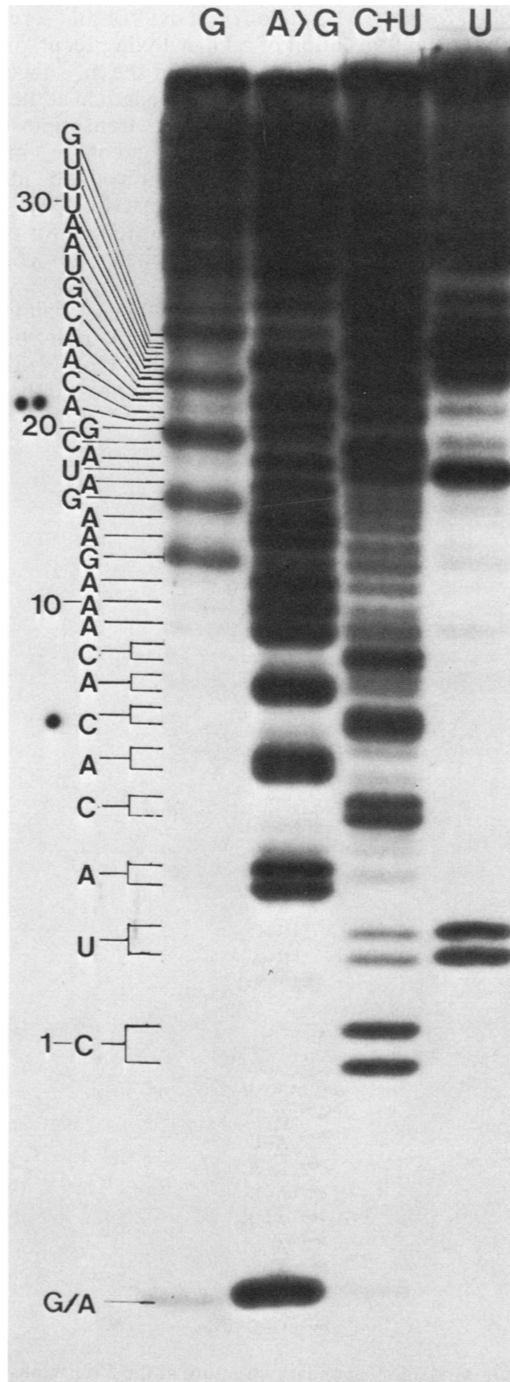

FIG. 4. RNA sequence at the 3′ terminus of m transcript. In vitro m transcript bearing 3′-terminal [5′-^{32}P]pCp was sequenced by the chemical method (39). This 20% polyacrylamide–8 M urea gel was run to retain the [^{32}P]pCp generated by cleavage at the 3′-terminal A or G residue. All bands, except for the extreme 3′ terminus, are doublets generated by the presence of either A or G at the 3′ terminus. Bases 34 to 100 were determined from other gels.

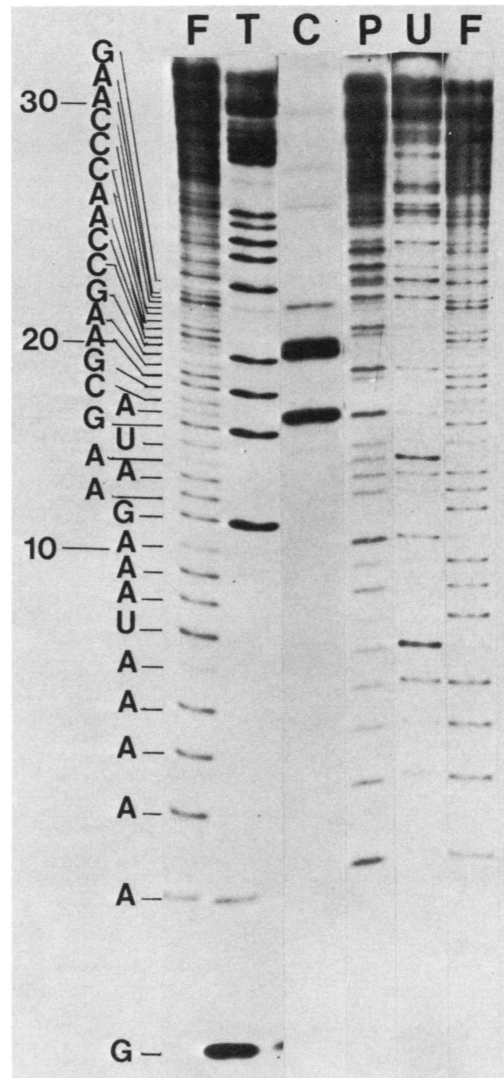

FIG. 5. RNA sequence at the 5′ terminus of m transcript. In vitro m transcript bearing 5′-terminal ^{32}P was partially digested with base-specific RNases and fractionated on a 20% polyacrylamide–8 M urea gel. Digestions were performed with RNases T_1 (T), CL-3 (C), Phy M (P), and U2 (U). F indicates formamide ladder. Three cytosine residues at positions 27 to 29 have been confirmed by other gels.

formamide lanes. The data obtained are consistent with the presence of the predicted secondary structure.

DISCUSSION

We have shown that the two major M dsRNA-specific transcripts produced in vivo are of the same polarity as the full-length m transcript synthesized in vitro by the virion-associated transcriptase activity. The in vivo and in vitro transcripts behave similarly on columns of oligo(dT)-cellulose and poly(U)-Sepharose. Retention of these RNA species on oligo(dT)-cellulose is consistent with the presence of an A-rich tract of at least 30 to 40 residues (36), and thermal elution from columns of poly(U)-Sepharose indicates that this poly(A) tract extends for 70 to 100 AMP residues (37). However, M dsRNA has been claimed to lack runs of AMP

FIG. 6. Partial S1 nuclease digestion of plus strands. M plus strands containing 5′-terminal ^{32}P were partially digested with RNase T_1 and S1 nuclease. Digests were fractionated on (A) 20% or (B) 12% polyacrylamide-8 M urea sequencing gels. F is a formamide digest of 5′-^{32}P-labeled plus strand; C is a control lane. Numbers on the left of each panel indicate the positions of formamide and RNase T_1 fragments; those on the right indicate S1 nuclease fragments.

residues longer than 8 (15). The average poly(A) chain length of yeast mRNA is ca. 50 residues (35).

Bostian et al. (8) have previously reported similar poly(U)-Sepharose binding by the in vivo full-length m and subgenomic m_a transcripts, which they propose is due to 3′-terminal poly(A) tracts. They propose that these may be post-transcriptional additions, although they also note that the poly(A) tract for the m_a species might also be produced by "chattering" of the virion transcriptase at the A,U-rich "bubble" region. Our data suggest that it is not necessary to invoke post-transcriptional polyadenylation or transcriptional infidelity to explain the behavior of the in vivo M dsRNA-specific transcripts on poly(A) affinity columns. In vitro-synthesized, full-length m transcript is not polyadenylated at its 3′ terminus, yet it was eluted from a poly(U)-Sepharose column at the same temperature as the in vivo m and m_a transcripts. The in vivo m species may, in fact, be identical to the full-length in vitro m transcript and may function as a replication intermediate, a template for translation, or both. Bostian et al. (8) have suggested that the m_a species is transcribed from the M-1 region of M dsRNA and serves as a template for the translation of a killer toxin precursor. If its poly(A) tract is template encoded, then the m_a subgenomic transcript may arise by cleavage of full-length m at the 3′ end of the "bubble" region, or the virion transcriptase may terminate transcription at or near the 3′ end of the "bubble" to produce a poly(A) region whose origin would be identical to that present in the full-length m transcript. This A-rich region may then function in a role similar to that for poly(A) tails which are added post-transcriptionally (for example, see references 11 and 56).

S3 dsRNA, a mutant derived by an 1,100-base pair internal deletion of M dsRNA, lacks the internal A,U-rich "bubble" region along with some flanking sequences (26). Neither the separated strands of S3 dsRNA nor the in vitro-synthesized s

FIG. 7. Model for secondary structure at the 5′ terminus of plus strands of M dsRNA. All bases were predicted from the sequence at the 3′ terminus of the M dsRNA minus strand and agree with those found at the 5′ terminus of the m transcript synthesized in vitro. Underlined bases were also confirmed by direct sequencing of genomic plus strands bearing 5′-terminal ^{32}P. Arrows indicate sites of S1 nuclease sensitivity. Asterisks indicate the AUG at positions 14 to 16 that initiates a large open reading frame that extends for at least 54 amino acids (48).

transcript is retained by oligo(dT)-cellulose. S3 dsRNA shares nearly identical terminal sequences with M dsRNA (26; Thiele et al., in preparation). These data support the role of the A,U-rich region of M dsRNA in the binding of transcripts to poly(A) affinity columns. Also, it has been demonstrated that reverse transcription of M dsRNA can be primed by oligo(dT), which apparently base pairs with an oligo(A) tract in the "bubble" region on the plus strand (12; N. Skipper, personal communication).

Such a degree of internal template coding of a poly(A) tract has been demonstrated in few RNA molecules in eucaryotic viruses and cells. Polioviruses (55) and alphaviruses (42), both single-stranded plus RNA viruses, possess a long poly(A) tract at the 3′ terminus of the genomic RNA which is transcribed to create a 5′ poly(U) tract in the minus strand RNA. Vesicular stomatitis virus contains the sequence (3′). . .AUACU$_7$. . .(5′) at the 3′ end of each of five genes. Transcription of each gene by the vesicular stomatitis virus transcriptase proceeds to the U$_7$ tract, where an A$_7$ tract is synthesized, followed by a poly(A) tract presumably produced by a "chattering" mechanism (34, 41, 43). The dicistronic RNA3 of brome mosaic virus contains an oligo(A) tract of 16 to 25 AMP residues (1, 25). Structurally, this oligo(A) serves to separate the two cistrons. However, when RNA3 is translated in vitro, only one protein is synthesized from the 5′-proximal cistron. The 3′-proximal cistron is the coat protein gene and is translated only from a subgenomic mRNA (RNA4). The oligo(A) sequence does not provide a poly(A) tract or poly(A) priming site for any known subgenomic mRNA (2). An oligo(A) sequence of approximately 25 AMP residues has been detected in HeLa cell heterogeneous nuclear RNA which appears to be template encoded (49). This short oligo(A) tract does not appear in cytoplasmic mRNA, and was suggested to serve as a priming site for the post-transcriptional addition of a larger poly(A) tract. We also do not detect a (5′). . .AAUAAA. . .(3′) sequence within the last 20 bases of the in vitro m transcript. Such a sequence is present, however, at positions 159 to 164 bases from the 3′ end of plus strands (48). This sequence is present 11 to 19 bases 5′ to the poly(A) tract of many eucaryotic mRNAs (40) and has been shown to be necessary for polyadenylation of simian virus 40 late mRNAs (24). Certain *S. cerevisiae* mRNAs contain a similar sequence, 5′-UAAAUAAPu-3′, which maps 25 to 40 bases upstream from a polyadenylation site (6).

Studies of in vivo transcripts of other yeast viral dsRNAs have yielded conflicting results. Haylock and Bevan (27) have found that the in vivo full-length transcript of L dsRNA is not polyadenylated and is not bound by oligo(dT)-cellulose. Bostian et al. (8) have found two transcripts of L dsRNA in vivo: l_a, which is subgenomic (2,300 bases) and not polyadenylated, and full-length l, which appears to be polyadenylated by the criterion of retention on columns of poly(U)-Sepharose. There are a number of possible explanations for these results. There may be a difference in the L species present in the strains (21, 23, 45) used in these two studies. It has also been shown that poly(U)-Sepharose will bind RNAs with shorter poly(A) tracts than will oligo(dT)-cellulose (see, e.g., reference 30). Thus, the l in vivo transcript may have either a short 3′-terminal or internal poly(A) tract. We have found that neither the separated strands of L_A dsRNA nor the in vitro-synthesized, full-length l_A transcript is retained by oligo(dT)-cellulose columns. However, the 5′ end of the l_A transcript (in vitro) is relatively A-rich (46a).

The occurrence of either an A or a G residue at the 3′ terminus of the in vitro m transcript may reflect a requirement of full-length transcript termination for a purine residue. The addition of this residue is not template directed, since we have directly determined the 5′-terminal base of M dsRNA minus strand to be a guanine residue by end-group analysis of 5′-^{32}P-labeled minus strand (data not shown); this result is in agreement with a previous report based upon different methodology (14). We observe similar 3′-terminal purine heterogeneity in M dsRNA—to a much lesser degree—in which G residues account for only about 10% of the 3′ termini. We do not find the massive heterogeneity in 3′-terminal regions of M dsRNA seen by others (16) in M dsRNA or in the in vitro m transcript. The appearance of a non-template-encoded purine at the 3′ terminus is also seen in the single-stranded RNA bacteriophages. Removal of the penultimate CMP residue, but not of the 3′-terminal AMP alone, results in the loss of biological and template activity (reviewed in reference 22). In bacteriophage Qβ, terminal adenylation has been shown to be template dependent, and thus not required for any potential "reactivation" of replicase molecules (5). The solubilization of the *S. cerevisiae* virion transcriptase activity has not yet been achieved to perform similar experiments.

Analysis of the 5′ terminus of in vitro m transcript shows that transcription does have a unique start, initiating with a guanosine residue. We have demonstrated similar results for the full-length in vitro l_A transcript (46a). Alternatively, transcription may initiate with a pyrimidine, followed almost immediately by removal of the 5′-terminal base. We have been unable to incorporate radioactivity from [γ-^{32}P]GTP into transcripts. This may be due to a phosphatase activity present in our virion preparations which catalyzes the removal of ^{32}P from [γ-^{32}P]GTP (E. M. Hannig, unpublished data). Transcription in eucaryotic cells, however, generally initiates with a purine residue. We have also presented evidence for the existence of secondary structure at the 5′ terminus of in vitro m transcript which was predicted from sequence data at the 3′ terminus of the minus strand (47). The stem and loop structure depicted in Fig. 7 contains an AUG at positions 14 to 16 which begins a large open reading frame (47). Also present are potential 18S and 5.8S ribosomal RNA binding sites (32). These features may serve to regulate the translation of M-p32, the putative killer toxin precursor.

ACKNOWLEDGMENTS

S1 nuclease was generously provided by Regina Wurst.

E.M.H. and D.J.T. are predoctoral trainees supported by Public Health Service Institutional Research Service Award CA-09069 from the National Cancer Institute. This investigation was supported by Public Health Service grant AI-14843 and by an Alexandrine and Alexander L. Sinsheimer Award (to M.J.L.).

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