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Separation and sequence of the 3' termini of M double-stranded RNA from killer yeast

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ABSTRACT

Four subspecies of M double-stranded RNA from a killer strain of Saccharomyces cerevisiae were isolated. Each subspecies was susceptible to heat cleavage, presumably at an internal 190 base pair A,U-rich region, generating two discrete fragments corresponding to each side of the A, U-rich region. Enzymatic and chemical RNA sequence analysis defined the 3'-terminal 175 bases for the larger fragment (M-1) and 231 bases for the smaller fragment (M-2). All four subspecies of M have identical size and 3'-terminal sequences. Potential translation initiation codons are present on the corresponding 5' termini of both fragments, and a possible 18S ribosomal RNA binding site is also present on the 5' terminus of M-1. Stem and loop structures for the 5' and 3' termini of M-1 may function as recognition sites for replication, transcription, and translation.

INTRODUCTION

Killer strains of the yeast Saccharomyces cerevisiae harbor two linear virion-encapsidated cytoplasmically-inherited double-stranded RNA (dsRNA) species, denoted L (4980 base pairs)and M (1830 base pairs). These strains secrete a protein toxin which is lethal to other strains of the same species which do not possess M dsRNA; killer strains are resistant to the toxin (reviewed in 1-3). Naturally occurring non-killer strains lack M dsRNA but usually contain L.

Virions containing dsRNA isolated from both killer and non-killer strains co-purify with a DNA-independent RNA polymerase activity, which catalyzes the formation of single-stranded, full-length, asymmetric transcripts of the dsRNA. These transcripts are of messenger polarity (4-7). Denatured L dsRNA and the l transcript encode the major virion capsid polypeptide in vitro (6,8). Denatured M dsRNA and m transcript encode a 32,000 dalton polypeptide denoted M-p32 which may correspond to a precursor of the toxin protein (7,9,10). Denatured M dsRNA, but not the m transcript, also encodes a 19,000 dalton peptide denoted M-p19 (7). An internal A,U-rich region of M dsRNA of approximately 190 base pairs in

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length (11) can be selectively degraded by treatment with S_1 nuclease under partially denaturing conditions, or by heating, to yield two fragments (7). The larger fragment (M-1) programs the synthesis of M-p32 *in vitro* whereas the smaller (M-2) encodes M-p19 upon denaturation and translation in a rabbit reticulocyte cell-free protein-synthetic reaction (7).

Killer virion M and L dsRNAs purified by agarose gel electrophoresis have been shown to be heterogeneous in sequence just internal to the 3' terminal A residue (12). This has been determined by analysis of oligonucleotides generated by ribonuclease T1 from dsRNA isolated from strains harboring killer virus of type 1 specificity of toxin and resistance functions. Our work also utilizes M dsRNA of type 1 specificity. At least three specificities of killer virus exist in *S. cerevisiae* (13,14). Limited sequence analysis of type 2 killer virus has revealed some sequence homology to type 1 with a lack of terminal heterogeneity in M dsRNA of type 2 (15).

Previously, heterogeneity of M dsRNA has been noted upon polyacrylamide gel electrophoresis (16), which also separates crude preparations of L dsRNA into two species, denoted L and XL (17). Therefore M dsRNA was fractionated into its four subspecies, and their 3'-terminal sequences were determined to see if this was the source of the observed sequence heterogeneity. Each of the four subspecies of M dsRNA was found to be homogeneous in its 3'-terminal sequences and all four had the same pair of termini. The data also indicate the presence of potential translation initiation sites on both 5' termini of M dsRNA, and other unique structural features of possible functional significance.

MATERIALS AND METHODS

Materials. [5'-^{32}P]-pCp (2000-3000 Ci/mmole) was from Amersham Radiochemical Centre. RNA ligase from bacteriophage T_4-infected *Escherichia coli* was from P-L Biochemicals. Polyethyleneimine cellulose plates (CEL-300-PEI) were from Machery-Nagel and Co. (Germany). Ribonucleases T1, Phy M, U2, and ribonuclease from *Bacillus cereus* were from P-L Biochemicals. Pancreatic ribonuclease was obtained from Worthington Biochemical Corp. Chicken liver ribonuclease 3 (CL-3) and formamide (99% ultrapure) were from BRL. Ribonuclease T2 was from Sankyo and tRNA from *E. coli* B was from GIBCO. Urea (electrophoresis purity) was from Bio-Rad Laboratories. Hydrazine was from Eastman Kodak Co. Sodium borohydride and dimethyl sulfate were from Aldrich Chemical Co. All

reagents were sterilized by ultrafiltration or autoclaving.

Isolation of M dsRNA. Cells of the diploid prototrophic killer strain A364A X S7 (4) were grown into late stationary phase (5 days) at 28° C in medium containing 1% yeast extract, 2% peptone, and 5% ethanol. Double-stranded RNA was extracted from whole cells by a modification (5) of the method described (11). L and M dsRNA were separated by preparative horizontal agarose (1.5%) gel electrophoresis and RNA was then ethanol precipitated 3 times, resuspended in sterile glass-distilled water, and stored in aliquots at -20°C.

Isolation of M dsRNA subspecies bearing 3'-terminal [5'-^{32}P]-pCp. [5'-^{32}P]-pCp was linked to the 3'-terminal residues of M dsRNA by a modification of the method described (18). A typical reaction mixture (25-50 µl) contained 50 mM N-2-hydroxymethylpiperazine-N'-2-ethanesulfonic acid (pH 8.0); 10 mM $MgCl_2$; 3 mM dithiothreitol; 50 µM ATP; M dsRNA (0.7-1.3 mg/ml); RNA ligase (500 U/ml); and 0.25 mCi [5'-^{32}P]-pCp. The reaction mixture was incubated on ice for 36-48 hours. Then 25 µl of electrophoresis dye solution (0.08% bromophenol blue, 75% glycerol) was added and the mixture was subjected to electrophoresis on a 2.5 mm thick 5% polyacrylamide gel at 90 volts for 22-24 hours (16). The four subspecies of M dsRNA were located by autoradiography of the wet gel for 10 sec - 2.5 min using Kodak XR-5 X-ray film. RNA bands were excised and eluted as described (19), except magnesium acetate and carrier tRNA were omitted. Eluted RNA was precipitated overnight at -20°C from 0.3 M sodium acetate by the addition of 3 volumes of absolute ethanol followed by centrifugation at 12,000 x g for 1 hour at -15°C in an SS-34 rotor (Sorvall). RNA was redissolved in 0.3 ml 0.3 M sodium acetate, reprecipitated with 3 volumes of ethanol in a dry ice-ethanol bath for 5 min, and centrifuged in a microfuge (15,000 X g, 4°C, 15 min). The pellet was washed with 70% ethanol, vacuum dried, and resuspended in heat cleavage buffer (see below). Agarose gel-purified dsRNA is active as an acceptor of pCp in the reaction catalyzed by RNA ligase, unlike polyacrylamide gel-purified dsRNA.

Heat cleavage of M dsRNA subspecies. Isolated M dsRNA subspecies were lyophilized and dissolved in 50 µl of 40 mM sodium acetate (pH 4.5), 10 mM $ZnSO_4$ and incubated at 75°C for 10 minutes. Then 2.5 µl of 0.5 M EDTA and 25 µl of electrophoresis dye solution were added and electrophoresis was performed on a 5% polyacrylamide gel at 90 volts for 5.5 hours. Fragments resulting from heat cleavage were located by autoradiography, and the two major bands were excised from the gel and eluted as described above

except that carrier E. coli tRNA (10 µg/ml) was included in the elution buffer. Eluted RNA was precepitated with ethanol from 0.3 M sodium acetate three times, vacuum dried, and dissolved in sterile glass-distilled water (100 µl) and stored at -20°C.

3'-Terminal nucleotide analysis of RNA. Approximately 2000-4000 cpm of M-1 or M-2 prepared from isolated subspecies of M dsRNA bearing [5'-^{32}P]-pCp on their 3' termini were lyophilized in the presence of tRNA (5µg) and redissolved in 5 µl of 0.01 M sodium acetate (pH 4.5) containing ribonuclease T2 (500 U/ml). After incubation at 37°C for 30 min, the released 3'-nucleotides were separated by chromatography on polyethyleneimine cellulose plates (20). The 3'-nucleotides were visualized by ultraviolet absorption (254 nm), cut from the plate, and radioactivity in each was determined by liquid scintillation counting.

Chemical sequencing of dsRNA fragments. Heat-generated fragments of M dsRNA bearing [5'-^{32}P]-pCp linked to their 3' termini were sequenced essentially as described (21). After chemical reactions resulting in base-specific incomplete cleavage, the digests were fractionated on gels as described (22).

Enzymatic sequencing of dsRNA fragments. Enzymatic sequencing digestions were carried out as described (22). The enzymes used to partially digest the RNA and their base specificities are as follows: pancreatic ribonuclease, C+U (23); ribonuclease U2, A (23); ribonuclease T1, G (23); ribonuclease Phy M, A+U (24); ribonuclease from B. cereus, C+U (25); and ribonuclease CL-3, C (26). All digestions except CL-3 and ribonuclease from B. cereus were carried out at 50°C for 15 min in the following buffer: 20 mM sodium citrate (pH 5.0); 1 mM EDTA; 7M urea; and 0.025% xylene cyanol and bromophenol blue. Ribonuclease from B. cereus was used under the same conditions but without urea and dyes which were added after 15 min to stop the reaction. Reactions with CL-3 were performed at 37°C for 20 min in 10 mM sodium phosphate (pH 6.5); 7M urea; 0.1 mM spermidine; and 0.025% xylene cyanol and bromophenol blue. Double-stranded RNA was denatured by boiling for 1 min followed by quick chilling on ice prior to addition of ribonucleases. Omission of this step markedly reduced the extent of digestion. Formamide "ladders" were generated as described (23).

RESULTS

Isolation and Fragmentation of M dsRNA subspecies. Fractionation of agarose gel purified M dsRNA bearing [5'-^{32}P]-pCp on its 3' termini on 5% polyacrylamide gels resolved it into four subspecies, denoted M_A-M_D in order of increasing electrophoretic mobility. Figure 1 demonstrates that when M dsRNA fractionated in this manner was subjected to repeated polyacrylamide gel electrophoresis, each of the four subspecies is essentially homogeneous and retains its characteristic electrophoretic mobility. These subspecies are also observed with non-radioactive, non-derivatized M dsRNA, so their existence is not due to an alteration of the RNA in the RNA ligase reaction (data not shown). The four subspecies do not occur in equimolar quantities. The relative ratios seen in Figure 1 were consistently observed in numerous preparations, and are the same as those found in M dsRNA extracted from virions purified as described (4). Agarose gel electrophoresis under denaturing conditions (5) failed to separate these subspecies, implying that their different migration in polyacrylamide gels does not reflect differences in chain length. Identical patterns were found in M dsRNA from 10 subclones of strain A364A X S7.

When each of the four subspecies was independently subjected to heat

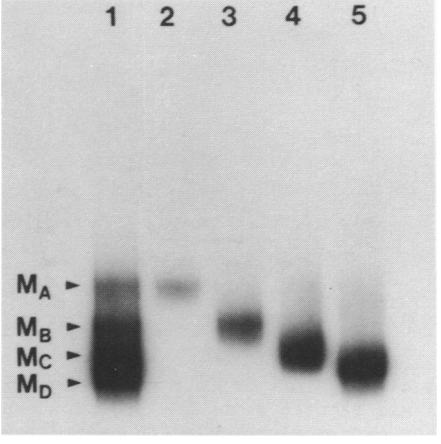

Figure 1. Four subspecies of M dsRNA. Agarose gel electrophoresis was used to purify M dsRNA, bearing 3'-terminal [5'-^{32}P]-pCp. This material was then fractionated into subspecies as described in Materials and Methods. An autoradiogram of this polyacrylamide gel is shown. Lane 1 contains unfractionated M dsRNA; lane 2, M_A; lane 3, M_B; lane 4, M_C; and lane 5, M_D.

cleavage at 75°C, two distinct radioactive fragments resulted. The larger fragment of M_A is denoted M_A-1 and the smaller M_A-2, as previously described (7). These fragments are double-stranded based upon their resistance to the ribonucleases used for RNA sequence analysis unless first denatured at 100°C for 1 min. This criterion was used to establish the dsRNA nature of the genomes of some defective interfering variants of vesicular stomatitis virus (27). The M-1 fragments generated by heat cleavage of all four subspecies co-migrated on polyacrylamide gel electrophoresis, as did the M-2 fragments (Figure 2). Retention of radioactivity on all fragments is consistent with an internal cleavage site in the M dsRNA, as has previously been suggested (7). Recovery of radioactivity in fragments M-1 and M-2 was 31% of that in uncleaved M dsRNA.

. Sequence determination of dsRNA fragments. Enzymatic analysis of the 3' termini of the M_D-1 and M_D-2 dsRNA fragments demonstrated them to have 92.1% and 91.4% 3'-terminal A, respectively. Most extensive

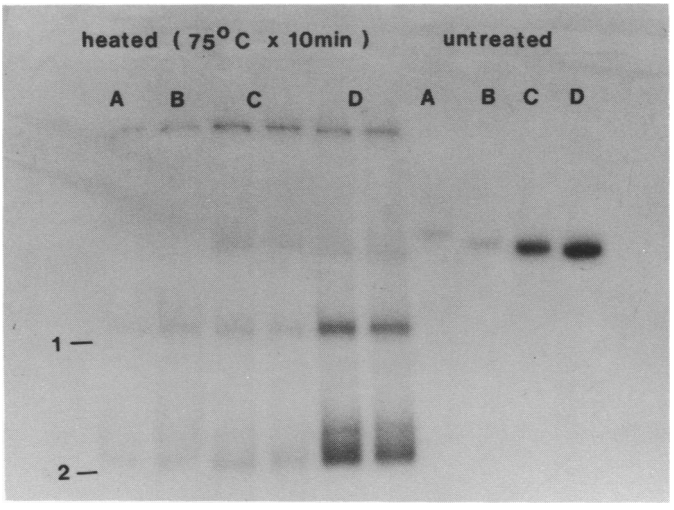

Figure 2. Heat cleavage of the four subspecies of M dsRNA. Agarose gel-purified M dsRNA was reacted with [5'-^{32}P]-pCp, separated into its 4 subspecies, and then each was subjected to heat cleavage as described in Materials and Methods. This figure shows an autoradiogram of a 5% polyacrylamide electrophoretic gel run at 90 volts for 5.5 hours. On the left, A, B, and the duplicated lanes C and D contained heat cleaved M_A, M_B, M_C and M_D. On the right the corresponding untreated dsRNA subspecies are present. On the left the positions of fragments M-1 and M-2 are indicated; in preparative electrophoresis only material at these positions was eluted for further study.

sequence analysis was performed on the fragments of M_D, the most plentiful of the four subspecies of M dsRNA. Representative chemical

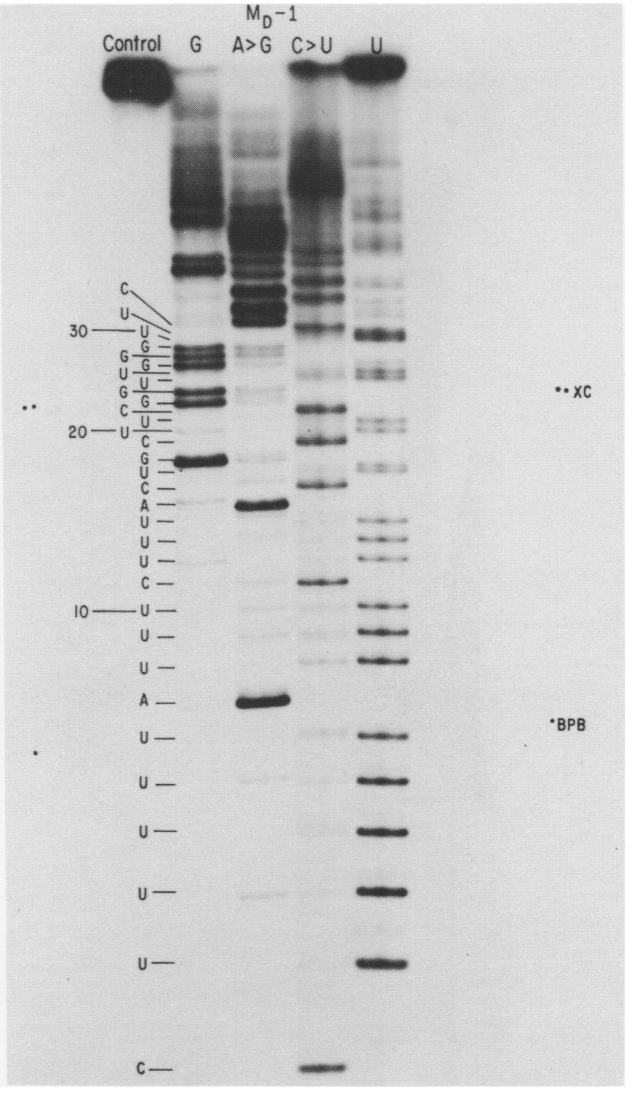

Figure 3. Sequence determination of the 3'-terminus of M_D-1 displaying nucleotides 1 through 32. The pCp generated by cleavage at the 3'-terminal A had run off this sequencing 20% polyacrylamide gel but was seen on other gels. Reactions and Control (undigested sample) were as described (21). BPB and XC indicate the mobilities of bromophenol blue and xylene cyanol, respectively.

sequencing gels for M_D-1 and M_D-2 are shown in Figures 3-6. A unique sequence was generated for each fragment, with M_D-1 being relatively U-rich and M_D-2 relatively C-rich at their extreme 3'

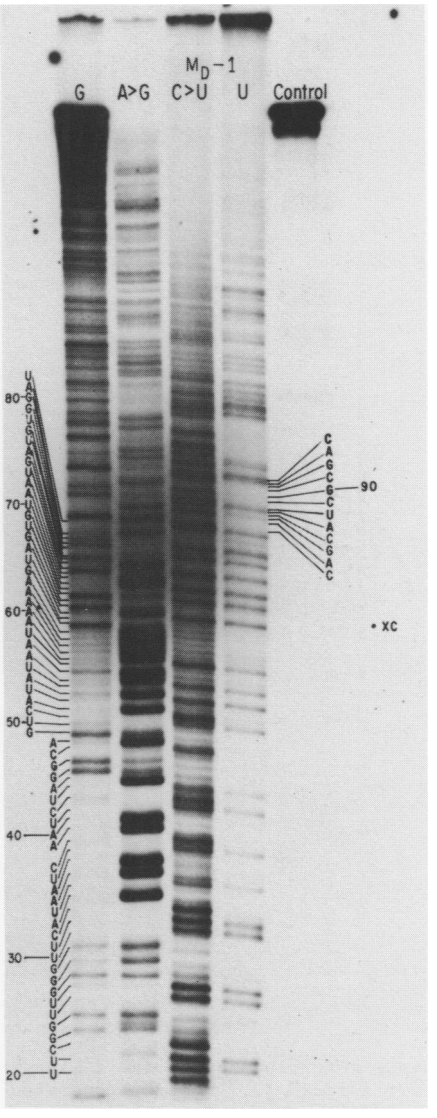

Figure 4. Sequencing gel (8% polyacrylamide) displaying the 3'-terminal sequence for nucleotides 20 through 94 of M_D-1.

termini. Sequence determination for subspecies M_A, M_B, and M_C resulted in the same 3'-terminal sequences observed for M_D. Sequence identity with M_D was shown by the chemical method for M_A-1, 46 nucleotides; M_B-1, 49 nucleotides; M_C-1, 33 nucleotides; M_A-2,

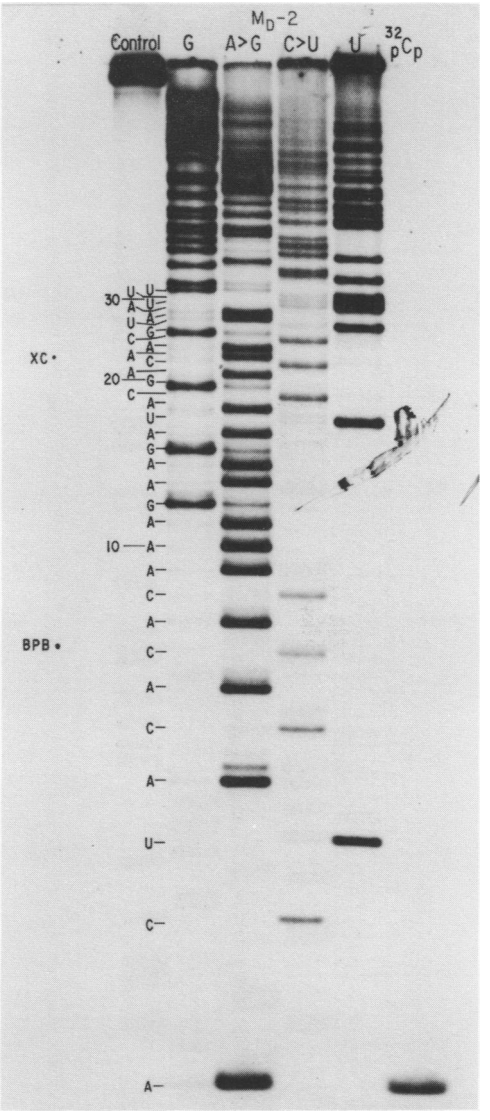

Figure 5. Sequencing gel (20% polyacrylamide) displaying 3'-terminal sequence for M_D-2 for nucleotides 0 through 32. Comigration of the smallest fragment with [5'-^{32}P]-pCp is demonstrated.

42 nucleotides; M_B-2, 57 nucleotides; and M_C-2, 57 nucleotides (sequencing gels not shown). The C-rich and U-rich sequences observed for the two dsRNA fragments of M_A-M_D, correspond to the two classes of 3' termini of M dsRNA proposed previously (12). Figures 7 and 8 present

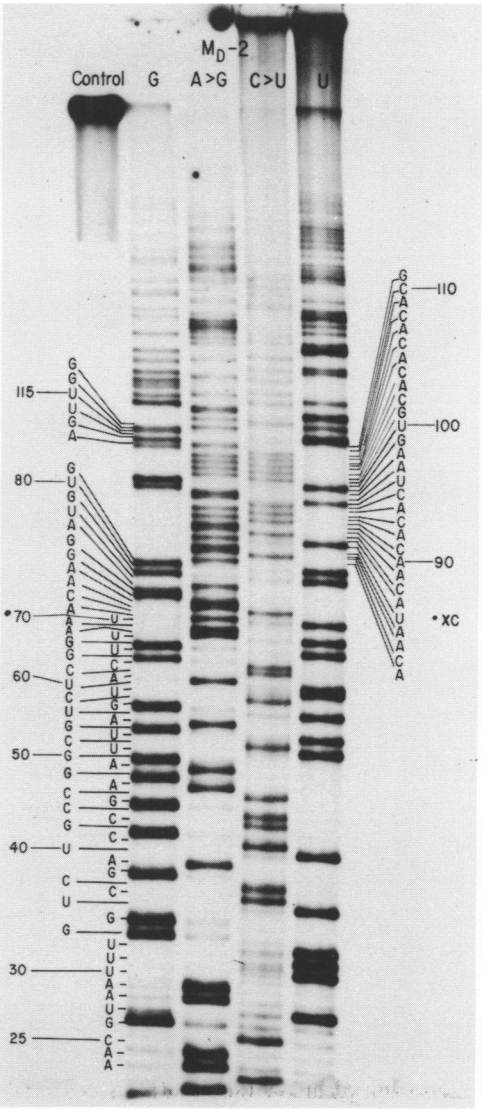

Figure 6. Sequencing gel (8% polyacrylamide) displaying 3'-terminal nucleotide sequence of M_D-2 from nucleotides 23 through 117.

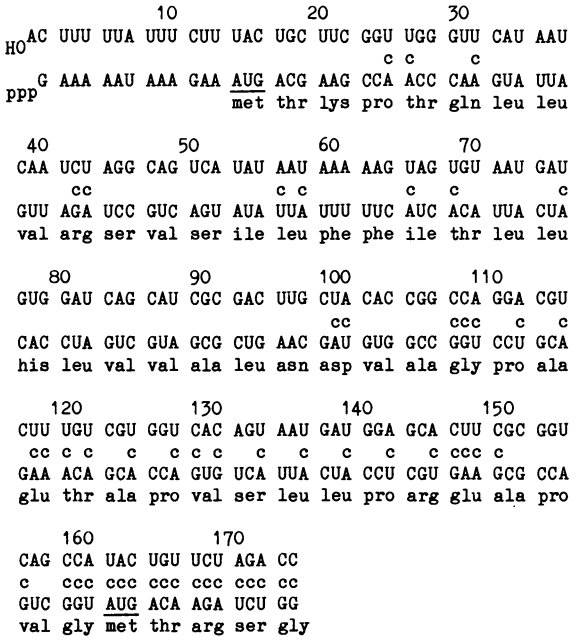

Figure 7. Sequence of the terminal region of M_D-1. The indicated sequence was deduced by both the enzymatic and chemical methods except where indicated: e, only determined by enzymatic digestion; c, only determined by chemical digestion. Only a faint band was seen at U169 by the chemical method, but all other positions were unambiguously determined. The sequence of the complementary 5' terminus was deduced from the 3'-terminal sequence, assuming a 5'-terminal pppG. The putative open reading frame on this sequence is indicated. All AUG sequences on the 5' terminus are indicated by underscoring.

the 3' terminal sequences for M_D-1 (175 nucleotides) and M_D-2 (231 nucleotides). The corresponding 5'-terminal sequences and the amino acids encoded by the largest open reading frames are also indicated. The 5'-termini of M dsRNA have previously been shown to be pppG (28), and the 3' termini have an unpaired A_{OH} (12). The bases are numbered with the 5'-terminal G and the corresponding 3'-subterminal C denoted as number 1. The 5'-terminal sequence deduced for M_D-1 contains a potential translation initiation codon (AUG) at positions 14-16 without any known translational termination signals in that reading frame through position 174. A second AUG at positions 161-163 is in the reading frame established by the first AUG. Of the multiple potential translation initiation codons on the 5' terminus of M_D-2, only the AUG at positions 60-62 is part of

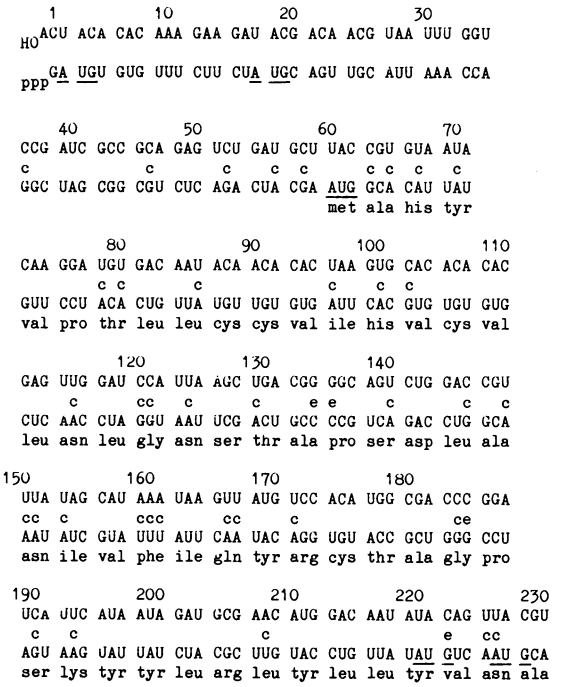

Figure 8. Sequence of the terminal region of M_D-2. The results are displayed as in Figure 7. On the complementary 5' terminus multiple initiation triplets are indicated; only the AUG at nucleotide 60-62 is part of an extended open reading frame. The AUG's at positions 2-4 and 17-19 are in frame with a UAA terminator at position 29-31. The GUG at position 4-6 is in frame with a UGA terminator at position 94-96. The AUG's at 220-222 and 226-228 are in phase with each other.

an extensive reading frame lacking nearby termination signals.

Enzymatic sequence analysis (gels not shown) of M_D-1 and M_D-2 confirmed the chemically-derived sequences, with the majority of nucleotides determined by both methods. In the case of M_D-2, the chemical method failed to yield sharp bands for the two internal G's in the sequence GGGG at positions 133-136, but all four residues were cleaved by the G-specific ribonuclease T1. Otherwise, the chemical method appeared to be easier to interpret due to neighboring base effects resulting in unequal cleavage by the enzymatic method at the same residue in different locations in the RNA. These effects included a relative resistance of bonds between pyrimidines to pancreatic, B. cereus and CL-3 ribonucleases, and an increased susceptibility of pyrimidine-A bonds to pancreatic ribonuclease

(29).

As illustrated for M_D-1 in Figures 3 and 4, occasional sites of apparent sequence heterogeneity were noted on chemical and enzymatic sequence analysis of M_A-1, M_B-1, M_C-1, and M_D-1 but not of the corresponding M-2 fragments. In all cases, the extra bands seen in the M-1 analysis were those corresponding to the M-2 sequence (Figures 5 and 6). We interpret this result as indicating a low level of contamination of polyacrylamide gel purified M-1 by M-2, which had previously migrated through the portion of the preparative gel from which M-1 was eluted. No evidence of other sequence heterogeneity at this level was noted.

DISCUSSION

We have isolated four subspecies of M dsRNA by polyacrylamide gel electrophoresis. These four subspecies are each cleaved by heat treatment to yield fragments with the same electrophoretic mobility. All four subspecies are identical in size based on electrophoresis under denaturing conditions (5), and they are identical in sequence for runs of at least 33 nucleotides from each terminus. Therefore, the subspecies may be stable conformers containing the same primary structure. One possible source of conformational heterogeneity among M dsRNA molecules is the "bubble" seen by electron microscopy, which is present on 50-70% of the molecules (11). Heat cleavage conditions may denature this "bubble" region to the same extent on all sub-species, thus causing them to yield the same fragments. Heat-generated fragments M-1 and M-2 co-migrate on electrophoresis with those generated by S1 nuclease, with sizes estimated at approximately 1,000 and 600 base pairs, respectively (7).

The sequences of both 3' termini of M_A, M_B, M_C, and M_D lack detectable heterogeneity of the type reported for the termini of agarose gel-purified M dsRNA (12,15). This heterogeneity was reported (12,15) mostly at sites internal to the 3' terminal 10-15 nucleotides of M dsRNA. Polyacrylamide gel electrophoretic purification of the [5'-^{32}P]-pCp-bearing M dsRNA separates a class of highly radioactive oligonucleotide fragments from this RNA. These oligonucleotides are heterogeneous in size and sequence (data not shown) and may contribute to the reported heterogeneity of the RNA. We have not attempted to sequence the terminal fragments released from M dsRNA by ribonuclease T1 digestion (12), so we cannot exclude heterogeneity at a low level.

The U-rich and C-rich extreme 3' termini of M_D-1 and M_D-2

probably correspond to the opposite 3' ends of M dsRNA, as previously proposed (12,15). There is agreement between the sequences report here and those nucleotides previously reported to be non-heterogeneous on the 3' termini of M dsRNA. Fragment M_D-1 encodes the M-p32 peptide, the putative precursor of killer toxin protein (7). The 5' terminus of M_D-1 contains two AUG sequences which may be translation initiation codons. Figure 9 shows a single-stranded secondary structure model for this 5' terminus which is energetically stable (30). This sequence, which may be the same as the 5' terminus of the m transcript which also encodes M-p32 (7), has a region just preceding the putative initiator AUG at positions 14-16 where five of six contiguous bases can base pair with the 3'-terminal sequence of the 18S rRNA of S. cerevisiae (31). This type of interaction may function in translation initiation, as has been proposed for prokaryotic messenger RNA (32). In the structure shown in Figure 9, base pairing with 18S rRNA would decrease the stability of the stem-and-loop structure by 4.8 kcal, thus making the putative initiating AUG more accessible for translation. A similar mechanism of translational initiation regulation has been proposed for Reovirus messenger RNA (33). The function of this type of interaction in translation initiation in eukaryotic organisms is unclear at present (34,35).

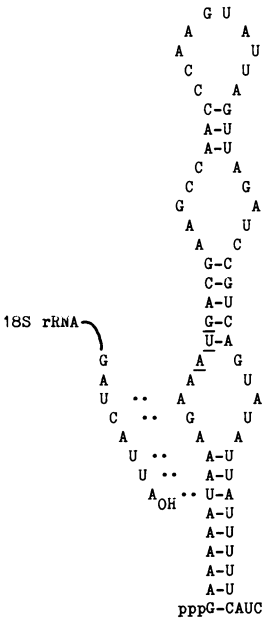

Figure 9. Proposed secondary structure of the 5'-terminal region of M_D contained in M_D-1. The structure of the 5' terminus complementary to the 3' terminal sequence determined is shown. This structure would be stable with $\Delta G(25^{\circ}\ C)=-13$ kcal (30). The stem and loop structure shown involves nucleotides G1-C64. The putatative AUG initiator is underscored and the sites which could base pair with the 3' terminus of yeast 18S rRNA are indicated.

Twenty-eight of the first 41 amino acids encoded on the open reading frame of the 5' terminus of M_D-1 are hydrophobic, with a lysine residue at position 3 of the putative polypeptide. These characteristics are typical of signal polypeptides involved in the biosynthetic transport of exported proteins (36), and may be important in the production of extracellular killer toxin or a precursor polypeptide. The presence of a large open reading frame on the 5' terminus of M_D-2 may represent the region encoding the M-p19 polypeptide which is known to be localized on this RNA fragment (7). Further sequence studies and translation of separated strands of M dsRNA will be required to definitively test the possibilility that both strands of M dsRNA may be codigenic in vitro. No example of a dsRNA genome containing translatable information on both strands has been reported previously. No obvious rRNA binding site or large region of secondary structure is present near the putative initiator AUG on M_D-2.

The 3' terminus of M_D-1 can be folded into single-stranded secondary structure similar to that of the 5' terminus, as is shown in Figure 10. This structure has $\Delta G(25^{\circ}C)=-16.2$ kcal, and contains the sequence $AUUUUUA_{OH}$, which is highly conserved at one 3' terminus in L, M, and S (a deletion mutant of M) dsRNA molecules (12, 15). This sequence has been proposed to be a recognition signal for the capsid-associated transcriptase of the virions of yeast (12). The possible role of the secondary structures shown in Figures 9 and 10 in the configuration of M dsRNA remains unknown.

The structures depicted in Figures 9 and 10 could have three biological functions. Firstly, as discussed above, the potential 18S rRNA binding site on the stem and loop structure of the 5' terminus of M_D-1 or the m transcript might function in the initiation of translation, as has been proposed for other viral (33) and eukaryotic (37) messenger RNAs. Secondly, this structure on one 3' terminus of dsRNA might serve as a transcriptase or replicase recognition site. Similar structures have been proposed as recognition sites for such polymerases of other viruses with multipartite genomes (38,39). Thirdly, the structures could serve as recognition sites for virion capsid protein binding, as has been proposed for alfalfa mosaic virus (40). The limited amount of genetic material present in the yeast viral dsRNA may be reflected in multiple functions for the structures proposed.

The function of the A,U-rich region in M dsRNA is unknown. However,

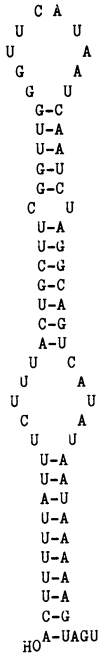

Figure 10. Proposed secondary structure of the 3' terminal region of M_D on M_D-1. This structure, which is stable with $\Delta G(25^{\circ}C)$= -16.2 kcal (30), involves A0-U65.

examples of this type of structure exist in other viruses. For example, similar regions are proposed as intercistronic boundaries in vesicular stomatitis virus (41) brome mosaic virus (42), and alfalfa mosaic virus (39). The A,U-rich region of M dsRNA could represent a transcription termination site in vivo, which is consistent with the presence of subgenomic transcripts of M dsRNA of 1200 nucleotides in length (Bostian, K.A., personal communication). Alternatively, it might represent a site for processing of full-length transcripts such as those made in vitro (5).

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