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Structural and functional analysis of separated strands of killer double-stranded RNA of yeast

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ABSTRACT

The two strands of the M double-stranded RNA species from a killer strain of Saccharomyces cerevisiae have been separated, and the 3'-terminal sequences of these strands have been determined. The positive strand programs the synthesis of the putative killer toxin precursor (M-p32) in a rabbit reticulocyte in vitro translation system. Only the negative strand hybridizes to the positive polarity transcript (m) synthesized in vitro by the virion-associated transcriptase activity. Secondary structural analysis of the extreme 3'-terminus of the negative strand using S_1 nuclease is consistent with the presence of a large stem and loop structure previously proposed on the basis of RNA sequence data. This structure, and a similar structure at the corresponding 5'-terminus of the positive strand, may have functional significance in vivo.

INTRODUCTION

Certain strains of the yeast Saccharomyces cerevisiae, termed killers, secrete a protein toxin which is lethal to other strains, but to which killers are resistant. Toxin production and resistance are genetically encoded by an 1830 base-pair linear double-stranded (ds) RNA species denoted M. All killers and many non-killer strains also harbor a 4980 base-pair dsRNA, denoted L. Both M and L dsRNA are cytoplasmically inherited and are encapsidated in intracellular virions (reviewed 1,2).

Virus particles isolated from cell extracts of killer and non-killer strains co-purify with a virion-associated transcriptase activity which catalyzes the synthesis of full-length single-stranded, asymmetric transcripts of L and M dsRNA (3-5). These transcripts are of positive polarity since they encode the major polypeptides which the denatured dsRNA species program in a cell-free protein synthesis system. Denatured L dsRNA and transcript l encode the major capsid polypeptide in vitro (5,6), and denatured M dsRNA and transcript m encode M-p32, a putative toxin precursor (7,8). In addition, M-p19, a polypeptide of unknown function, has been synthesized in lesser quantities in a rabbit reticulocyte cell-free

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translation reaction upon addition of denatured M dsRNA or a purified fragment of M derived from selective S_1 nuclease cleavage (8).

Under partially denaturing conditions M dsRNA has been shown to be cleaved specifically at an internal readily-denaturable region (9) by S_1 nuclease or by high temperature (8,10) to yield two fragments denoted M-1 (1,000 base pairs) and M-2 (600 base pairs). This technique has facilitated localization of the toxin-coding sequence on the M-1 fragment of the M dsRNA molecule (8) and has been used to sequence the 3'-termini of M dsRNA (10). RNA sequence data revealed the presence of potential translation initiation codons and open reading frames on both deduced 5'-terminal sequences, as well as potential stem and loop structures on termini of the genomic fragment which encodes M-p32 (10). Both open reading frames initiate with AUG, the only functional initiation codon in yeast (11).

In order to facilitate determination of the strand of M dsRNA codigenic for M-p32, we have developed a method for strand separation of this dsRNA molecule. We have identified the positive strand by in vitro translation, and present evidence for the existence of a 3'-terminal stem and loop structure in the negative strand.

MATERIALS AND METHODS

Materials. Ribonucleases T_1 and Phy M were from P-L Biochemicals. Pancreatic ribonuclease was from Worthington Biochemical Corp. Chicken liver ribonuclease 3 (CL-3) and formamide (99% ultra pure) were from Bethesda Research Laboratories. Dimethyl sulfoxide was from J.T. Baker Chemical Co. RNA ligase from bacteriophage T_4-infected Escherichia coli was from P-L Biochemicals. Nitrocellulose (BA-85) was from Schleicher and Schuell, Inc. The rabbit reticulocyte lysate (lot 16), [^{35}S]-methionine (1420 Ci/mmole), [^{14}C]-methyl-labeled protein mixture and [5'-^{32}P]-pCp (2000-3000 Ci/mmole) were from Amersham Radiochemical Centre. Ribosomal RNA was purified from Saccharomyces carlsbergensis Y379-5D as previously described (4). Autoclaved glass-distilled water was used, and all reagents were filter or autoclave sterilized.

Isolation of M dsRNA and attachment of 3'-terminal [5'-^{32}P]-pCp.

Cells of the diploid prototrophic killer (Type 1 killer toxin specificity) A364A x S7 (3) were grown to late stationary phase (5 days) at 28°C in medium containing 1% yeast extract, 2% peptone, and 5%

ethanol. Double-stranded RNA was extracted and purified by a modification (4) of the method described (9). [5'-^{32}P]-pCp was linked to the 3'-termini of M dsRNA using RNA ligase by a modification (10) of the method described (12). The M dsRNA preparations bearing 3'-terminal [5'-^{32}P]-pCp were further purified by 5% polyacrylamide gel electrophoresis (10) to yield essentially pure subspecies M_A-M_D where indicated.

As previously described (10), M dsRNA purified by agarose gel electrophoresis contains four subspecies of dsRNA separable on polyacrylamide gel electrophoresis, in addition to heterogeneous oligonucleotides. The four subspecies have the same electrophoretic mobility under denaturing conditions, are cleaved to fragments of the same size at 75°C, and have the same nucleotide sequences at their 3'-termini (10) and thus may represent "conformers" of a single nucleic acid species. In this communication, experiments were performed with agarose gel-purified M dsRNA (except where noted). Separated strands of M dsRNA and of the M_D subspecies purified by polyacrylamide gel electrophoresis (10) gave identical results upon 3'-terminal sequence analysis.

Strand separation of M dsRNA. M dsRNA (with or without 3'-terminal [5'-^{32}P]-pCp) was vacuum dried and resuspended in 20-100 µl of strand separation buffer containing 30% dimethylsulfoxide, 1 mM EDTA, 0.05% xylene cyanol and bromophenol blue (13). Samples (10 µl) were heated at 90°C for 2 minutes, and chilled on ice for 2 minutes. Samples were then loaded onto a 5% polyacrylamide slab gel (1.5mm x 100mm x 300mm) for strand separation as described for DNA (13). Immediately after loading the samples, electrophoresis was carried out at 1000 volts (constant voltage) for 10 minutes. The voltage was then lowered to 700 volts and electrophoresis was continued for a total of 5 hours. The separated strands were located either by autoradiography of the wet gel, or by staining the gel in ethidium bromide (0.5 µg ml) and visualization with intermediate wavelength (302 nm) ultraviolet light. Bands corresponding to the two strands of M dsRNA (denoted fast and slow strands) were excised and eluted as described (10) except carrier tRNA was omitted in non-radioactive RNA preparations. Eluted RNA was precipitated overnight at -20°C from 0.3 M sodium acetate by the addition of 3 volumes of absolute ethanol. RNA was pelleted at 12,000 x g for 1 hr. at -15°C in an SS-34 rotor (Sorvall). RNA was dried in vacuo, redissolved in 100 µl 0.3 M sodium

acetate, and reprecipitated at -20°C overnight with 3 volumes of absolute ethanol. RNA was pelleted by centrifugation in a microfuge (15,000 x g, 15', 4°C) and the pellet was washed with 70% ethanol, vacuum dried, resuspended in 50-200 µl water, and stored at -70°C.

Preparation of m transcript. Killer virions purified from strain A364A x S7 were used as a source of virion transcriptase to catalyze the synthesis of l and m transcripts (in the presence of non-radioactive or [α-^{32}P]-UTP) as described (3). RNA was extracted from the reaction mixture (3) and purified by 1.5% horizontal agarose gel electrophoresis.

Electrophoretic analysis of RNA. Separated strands of M dsRNA were analyzed on native (1.5%) horizontal agarose gels run for 16-20 hrs at 25 volts. Denaturing (1% agarose-formaldehyde) electrophoresis was performed as described (14) at 70 volts for 5 hours. Gels were dried and exposed to X-ray film.

Blot hybridization. Transfer of RNA from a 1% agarose-formaldehyde gel to nitrocellulose sheets was performed as described (15). The [^{32}P]-transcript (m) was dried in vacuo and resuspended in 200 µl of hybridization solution containing 50% formamide, 5 x SSC, 20 mM sodium-phosphate (pH 7.0), and 0.1% SDS, and pre-incubated at 65°C for 5 min. This probe was then added to the nitrocellulose filter with bound RNA (pre-warmed in hybridization solution at 65°C for 15 min), mixed, and incubated at 65°C for 18 hrs. Following hybridization the nitrocellulose sheet was washed as follows: 2 times with 2 x SSC, 0.1% SDS at room temperature for 15 minutes; once in 1 x SSC, 0.1% SDS at 65°C for 15 min; and twice in 0.25 x SSC, 0.1% SDS at 65°C for 15 min. The filter was the blotted dry and exposed to X-ray film at -70°C with an intensifying screen (Dupont).

Enzymatic sequencing of the ssRNA molecules. Enzymatic RNA sequencing reactions were carried out as described (10,16) except denaturation by heating at 100°C was omitted. Formamide ladders were generated as described (17), and the digests were fractionated on sequencing gels as described (16). The enzymes used and their specificities are as previously cited (10). All were used to partially digest RNA samples by cleavage 3' to the indicated bases: pancreatic ribonuclease, C+U; ribonuclease T_1, G; ribonuclease Phy M, A+U; and ribonuclease CL-3, C. These enzymes are also sensitive to the effects of neighboring residues, which result in unequal cleavage at the same nucleotide occurring in different locations in the RNA. These effects include a relative resistance of bands between

pyrimidines to pancreatic and CL-3 ribonucleases, and an increased susceptibility of pyrimidine-A bonds to pancreatic ribonuclease (25).

In vitro translation analysis. RNA was translated in vitro using the rabbit reticulocyte lysate system. RNA samples were suspended in water (3 µl). M dsRNA was denatured by boiling for 3 min and 20 µl of a mixture of reticulocyte lysate: [^{35}S]-methionine in volumetric proportions of 4 parts lysate: 1 part [^{35}S]-methionine were added. Reactions were incubated at 30°C for 90 min and terminated by freezing at -20°C. Samples (4-6 µl) were digested with pancreatic ribonuclease (200 µg/ml, 30 min at 37°C) and analyzed on 10% SDS-polyacrylamide gels (18) run at 100 volts for 3.5 hrs. Radioactive polypeptides were detected by fluorography (19).

Analysis of the secondary structure of ssRNA. Analysis of the separated strands of M dsRNA utilizing S_1 nuclease was carried out as described (20). In these experiments an aliquot of the separated strands of M_D subspecies of dsRNA containing 3'-terminal [5'-^{32}P]-pCp and 10 µg E. coli B tRNA was vacuum dried and resuspended in 10 µl of the appropriate buffer. Digestions with S_1 nuclease were performed in a buffer containing 40 mM sodium acetate (pH 4.5), 10 mM $ZnSO_4$, and 0-400 mM NaCl. Digestions with ribonuclease T_1 were performed in a buffer containing 20 mM sodium citrate (pH 5.0), 1 mM EDTA, 7 M urea, and 0.025% xylene cyanol and bromophenol blue. Samples were preincubated for 10 min at 37°C followed by addition of S_1 nuclease at an enzyme-to-substrate ratio of 0.03 U/µg RNA or ribonuclease T_1 at an enzyme-to-substrate ratio of 0.05 - 0.1 U/µg RNA. Samples were then incubated for 5-10 min at 37°C, and S_1 nuclease digestions were terminated by the addition of EDTA to 50 mM and 10 µl of a solution containing 10 M urea, 0.05% xylene cyanol and bromophenol blue, and chilled on ice. Ribonuclease T_1 reactions were terminated by chilling on ice. Formamide "ladders" were generated as described (17). The digestion products were fractionated on sequencing gels as described (16).

RESULTS

Strand Separation of M dsRNA. The strands of M dsRNA were separated and resolved by electrophoresis on a 5% polyacrylamide gel after brief heat denaturation in 30% dimethylsulfoxide, a known nucleic acid helix destabilizing agent (13,21). Figure 1 demonstrates the resolution of two species of RNA, denoted fast and slow strands, according to their relative

Figure 1. Strand separation of M dsRNA. M dsRNA was dissolved in 10 µl of strand separation buffer, heated, and subjected to electrophoresis on a 5% polyacrylamide gel as described in Materials and Methods. The gel was stained in ethidium bromide (0.5 µg/ml) and photographed under ultraviolet illumination. Lane 1 contains untreated M dsRNA; lane 2 contains strand-separated M dsRNA. Note that untreated M dsRNA is separated into subspecies in lane 1 (10).

mobility. Although the mechanism responsible for the separation of the strands is not clear, once denatured, each strand of the dsRNA may assume a characteristic, stable conformation, which may result in the observed differential migration. The faster migration of M dsRNA (Figure 1) resembles the characteristic more rapid migration of dsDNA relative to denatured separated strands (22). The fast and slow strands were determined to be single-stranded RNA by their susceptability to the sequence-specific ribonucleases without prior denaturation (Figures 3 and 4), a treatment essential for enzymatic sequencing of double-stranded RNA (10,23). When the strand separation procedure was performed on M dsRNA bearing 3'-terminal [5'-^{32}P]-pCp, approximately equivalent amounts of radioactivity were recovered in the fast and slow strands. Additional radioactivity was found in oligonucleotides migrating slightly slower than [5'-^{32}P]-pCp in this electrophoretic system. Analysis of these oligonucleotides on a 20% polyacrylamide-urea gel revealed them to be heterogeneous in size (data not shown).

Agarose gel electrophoresis of fast and slow strands eluted from the

polyacrylamide gel demonstrated that under native conditions the fast strand co-migrated with the full-length, single-stranded, asymmetric transcript (m) of M dsRNA (Figure 2). The slow strand migrated slightly more slowly under these conditions of electrophoresis (Figure 2). However, both strands co-migrated with denatured M dsRNA in denaturing (agarose-formaldehyde) gel electrophoresis (data not shown).

3'-terminal sequence analysis of separated strands. The fast and slow strands of M dsRNA bearing 3'-terminal [5'-^{32}P]-pCp were subjected to enzymatic RNA sequencing digestions (16). Sequencing data (Figures 3 and 4) demonstrate that the 3'-termini of the separated strands represent unique sequences which correspond to the sequences previously suggested to represent the two 3'-termini of M dsRNA (10,24).

Filter hybridization. In order to determine the relationship between the m transcript and the strands of M dsRNA a blot hybridization was

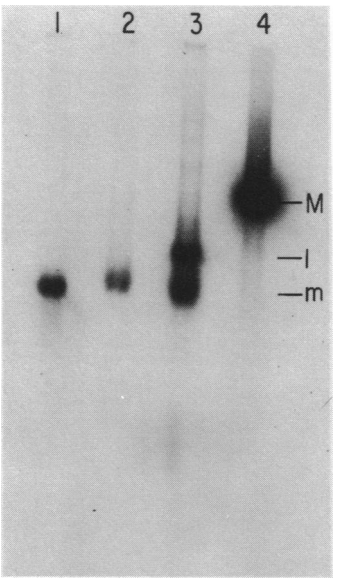

Figure 2. Agarose gel electrophoresis of purified separated strands. Separated fast and slow strands of M dsRNA were prepared from RNA bearing 3'-terminal [5'-^{32}P]-pCp. Electrophoresis of the strands was performed on a horizontal 1.5% agarose gel at 20 volts for 16 hrs as described in Materials and Methods. This figure shows an autoradiogram of the gel: fast strand (lane 1); slow strand (lane 2); [^{32}P]-transcripts I and m synthesized in a reaction catalyzed by virions purified from strain S7xA364A (lane 3); and M dsRNA containing 3'-terminal [5'-^{32}P]-pCp (lane 4).

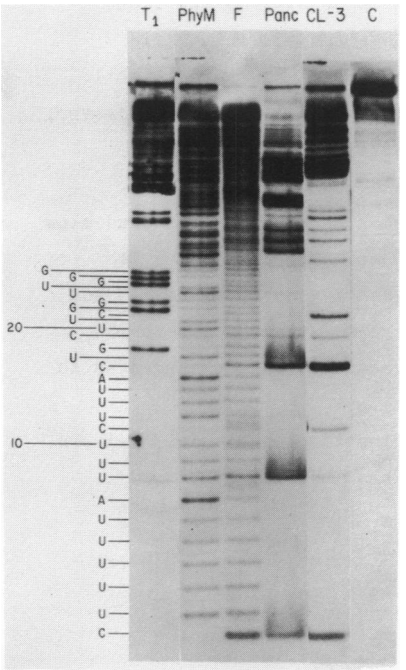

Figure 3. RNA sequence at the 3'-terminus of the slow strand. Slow strand bearing 3'-terminal [5'-^{32}P]-pCp was partially digested with the indicated ribonucleases and the products were fractionated on a 20% polyacrylamide-urea sequencing gel, as described in Materials and Methods. Digestions were performed using ribonucleases T_1, Phy M, pancreatic (A), and CL-3. F indicates formamide ladder, and C, control incubated under ribonuclease T_1 reaction conditions without added enzyme. The sequence indicated is that for the 3'-terminus present on the M-1 fragment of all four subspecies of M dsRNA (10). The 3'-terminal A in this sequence is also present, but has migrated off the end of this gel. The lanes shown are all from the same autoradiographic exposure of the same gel.

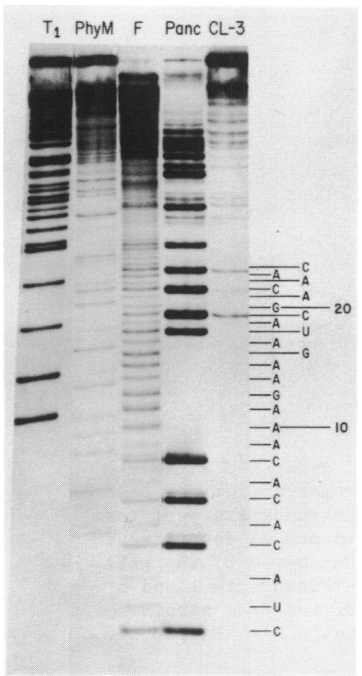

Figure 4. RNA sequence at the 3'-terminus of the fast strand. Digestion products were fractionated on a 20% polyacrylamide-urea gel as in Figure 3. The sequence indicated is that of the 3'-terminus of the M-2 fragments of all four subspecies of M dsRNA (10). The 3'-terminal A band has migrated off this gel. The lanes shown are all from the same autoradiographic exposure of the same gel.

carried out. Following strand separation and elution, the strands were electrophoretically resolved on an agarose-formaldehyde gel, transferred to a nitrocellulose sheet, and probed with [^{32}P]-m transcript synthesized *in vitro*. As demonstrated in Figure 5, m transcript hybridized to M dsRNA and to the slow strand, but not to the fast strand, to L dsRNA, or to the 18S and 25S rRNAs of *S. carlsbergensis*. The slow strand, by virtue of its complementarity to the message-sense transcript m, is the genomic negative strand. Lack of detectable hybridization of m transcript to the fast strand indicates that this strand may be of positive polarity. This prediction was verified by *in vitro* translation analysis of the separated strands (*vide infra*).

In vitro translation analysis. The result of translation of the separated strands of M dsRNA in a rabbit reticulocyte lysate (Figure 6) shows that the fast strand codes for the putative killer toxin precursor, M-p32, as do denatured M dsRNA and transcript m (8). Therefore, the fast strand is denoted the positive strand. The slow strand (negative strand) does not program the synthesis of any new polypeptides. Under these conditions, this lot of rabbit reticulocyte lysate catalyzed the synthesis of only trace quantities of M-p19 in reactions programmed by denatured M dsRNA; this is not visible on the exposure shown in Figure 6. No M-p19 was detected among the translation products of either separated strand.

Secondary Structural Analysis of Separated Strands. The usefulness of the single-strand specific S_1 nuclease to probe nucleic acid secondary structure is well established (20,25,26). This technique was used to examine the 3'-termini of the strands of M dsRNA for the presence of stem and loop structures previously proposed based on RNA sequence data (10), as

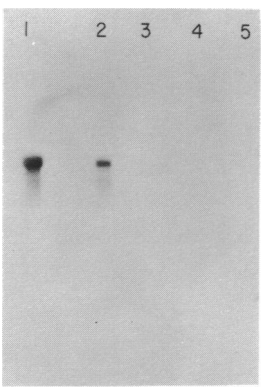

Figure 5. Blot hybridization analysis of the fast and slow strands. RNA was subjected to electrophoresis on a 1% agarose-formaldehyde gel at 70 volts for 5 hours, transferred to nitrocellulose, and hybridized with [^{32}P]-m transcript. Lane 1, M dsRNA; lane 2, slow strand; lane 3, fast strand; lane 4, L dsRNA; lane 5, *S. carlsbergensis* 25S and 18S rRNA.

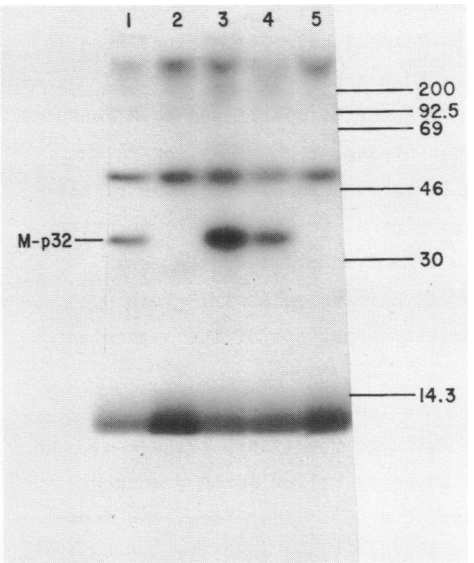

Figure 6. In vitro translation analysis of the separated strands of M dsRNA. Reticulocyte lysate reactions were performed as described in Materials and Methods. Aliquots were digested with ribonuclease A and analyzed by fluorography of a 10% SDS-polyacrylamide gel. The added RNA templates were as follows: lane 1, fast strand (44 µg/ml); lane 2, slow strand (44 µg/ml); lane 3, denatured M dsRNA (36 µg/ml); lane 4, m transcript (22 µg/ml); lane 5, no added RNA. Positions of [^{14}C]-methylated protein molecular weight standards are indicated.

shown in Figure 7. Figure 8 demonstrates that the extreme 3'-terminus of the negative strand, corresponding to nucleotide positions 1-16 (see Figure 7), is moderately sensitive to S_1 nuclease. This region is characterized by an internal loop and a potential double-stranded region in which most base-pairing involves A and U residues. Transient "breathing" of these relatively unstable base interactions may confer some sensitivity

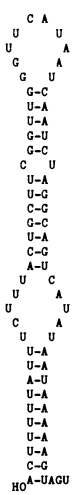

Figure 7. Proposed secondary structure of the 3'-terminal region of the negative strand (10). The 3'-terminal A is denoted nucleotide number 0, since it extends beyond the 5'-terminal G of the complementary strand (24).

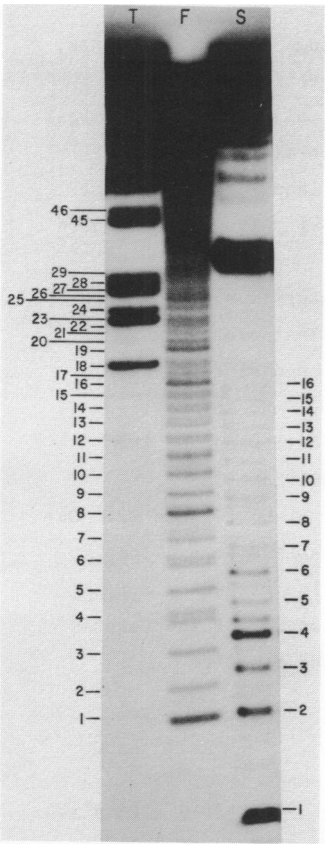

Figure 8. Partial S_1 nuclease digestion of native negative strand. Negative strand bearing 3'-terminal [5'-^{32}P]-pCp and 10 µg *E. coli* B tRNA were dissolved in S_1 nuclease buffer or ribonuclease T_1 buffer and digested as described in Materials and Methods. Digestion products were fractionated on a 20% polyacrylamide-urea gel. The numbers on the left indicate the positions of oligonucleotides generated by ribonuclease T_1 or formamide digestion. The numbers on the right refer to oligonucleotides generated by S_1 nuclease digestion. The differential migration of the fragments is explained in Results. T, ribonuclease T_1; F, formamide ladder; and S, S_1 nuclease digestion.

to S_1 nuclease at this region. No such sensitivity was observed for nucleotide positions 17-29. This region contains 5 G-C base pairs among 12 base pairs proposed (Figure 7) (10), and is thus a more stable duplex structure. The single base-pair mismatch in this region (bases 22 and 43) is not recognized and cleaved by S_1 nuclease, consistent with the inability of S_1 nuclease to cleave such mismatches in either RNA (26) or DNA (27). Beginning at nucleotide position 30, a region of extreme S_1 nuclease susceptibility occurs. Data presented in Figure 9 localized this region of sensitivity to positions 30-37, which corresponds to 8 of 10 bases in the hairpin loop proposed for this sequence (Figure 7).

Partial digestion products generated from ribonuclease T_1 digestion migrated more slowly than those generated by S_1 nuclease

digestion (Figure 8). This migration difference is a result of S_1 nuclease cleavage yielding oligonucleotides containing 3'-hydroxyl groups, whereas formamide hydrolysis and ribonuclease T_1 digestion yield oligonucleotides with 3'-phosphates (20). On a 20% polyacrylamide-urea gel (Figure 8) the oligonucleotides from S_1 digestions co-migrate with fragments 1-2 bases shorter in the T_1 and formamide lanes. This differential migration is not as pronounced on the 12% polyacrylamide-urea gel (Figure 9).

The S_1 nuclease structural analysis for the 3'-terminus of the negative strand was sodium ion concentration-independent over a range of 0-400 mM sodium (data not shown). A similar ion-independence of RNA secondary structure has previously been reported (20). No apparent secondary structural features exist at the extreme 3'-terminus of the positive strand (Figure 9). The first region of significant S_1 nuclease cleavage corresponds to nucleotide postions 81-86 (Figure 9). Our calculations (28) do not reveal the presence of a stable stem and loop structure at this region. Trace quantities of negative strand contaminating the positive strand preparation may account for the faint lower bands observed upon S_1 nuclease digestion of the positive strand.

DISCUSSION

We have modified a procedure previously used for the strand separation of double-stranded DNA fragments (13) to separate the strands of M dsRNA. Enzymatic RNA sequencing of the 3'-termini of positive and negative strands confirmed previous assertions that the relatively C-rich and U-rich sequences correspond to the individual 3'-termini of M dsRNA (10,24). Since the slow strand exclusively hybridized to the positive polarity transcript (m), and the fast strand programmed the synthesis of M-p32, strand polarity is established.

The negative strand did not encode any polypeptides under these translation conditions, despite the sizeable open reading frame deduced for the 5'-terminus of this strand based on sequence analysis (10). This fails to identify a strand of M dsRNA which encodes M-p19, a peptide produced in variable quantities in translations utilizing denatured M dsRNA as template (8).

Analysis of secondary structure by partial S_1 nuclease digestion supported the presence of a stem-and-loop structure at the 3'-terminus of

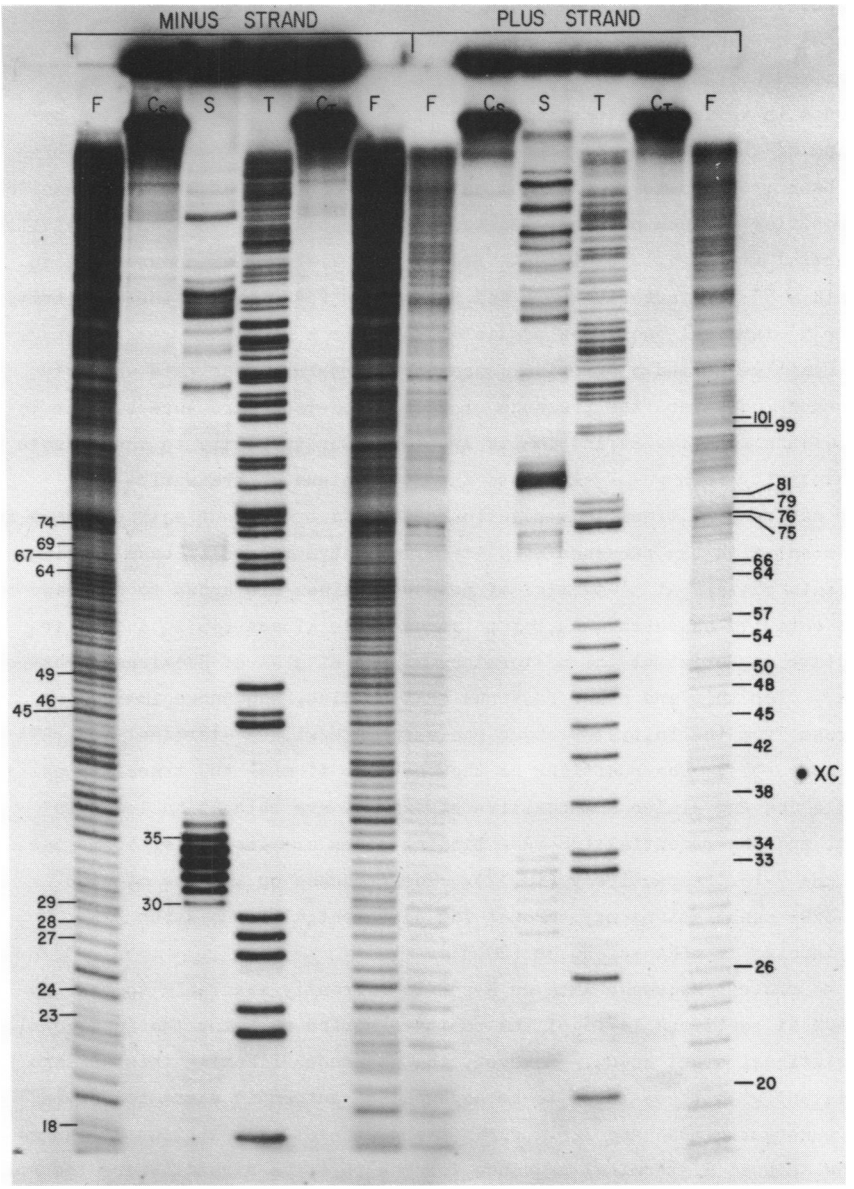

Figure 9. Partial S_1 nuclease digestion of positive and negative strands. Enzymatic digestions and formamide ladders were performed as described in Materials and Methods, and analyzed on a 12% polyacrylamide-urea gel. Lanes are labeled as in Figure 8, except Cs is a control for S_1 nuclease digestion and Ct is a control for ribonuclease T_1 digestion.

the genomic negative strand. Such 3'-terminal structures have been proposed to function as transcriptase or replicase recognition sites in several RNA virus systems (23,30,31). A similar A,U-rich 3'-terminal sequence in vesicular stomatitis virus is protected from methylation by binding of the putative transcriptase protein, NS (32). Smaller hairpins have been proposed to be replication recognition sites at the 3'-termini of the positive strands of other yeast dsRNA species (33). Sequence analysis of partial transcripts of L dsRNA from type 1 killers has shown these to contain a 5'-terminus rich in A and U residues (34), indicating similarity to the 5'-terminal sequences predicted here for M dsRNA.

Positive strand 5'-terminal secondary structural analysis was not performed. However, the presence of a stem-and-loop structure similar to that of the negative strand is likely (10). Our inability to incorporate radioactivity from [γ-^{32}]-ATP onto the 5'-terminus of transcript m under native conditions in a reaction catalyzed by polynucleotide kinase is consistent with the presence of 5'-terminal intra-molecular base pairing. Base interactions at 5'-termini of polynucleotides are known to decrease their activity as substrates for polynucleotide kinase (35). A similar structure may exist at the 5'-terminus of the s1 mRNA of Reovirus serotype 2 (36). For this and other reovirus mRNA species, sequences immediately upstream from the initiator codon can associate with 3'-terminal 18S rRNA sequences (37). The positions of the reovirus s1 mRNA AUG translational start codon and killer RNA positive strand AUG are both at nucleotides 14-16, and the potential 18S rRNA binding sites are located at positions 7-10 and 7-12, respectively (10,37). The presence or absence of such mRNA-rRNA associations may account for differential translation efficiencies of mRNA molecules (20,37).

No protein sequence data on M-p32 is currently available to confirm the AUG at positions 14-16 of the positive strand as being the functional translational start codon. However, the sequences flanking this AUG are consistent with those found to be adjacent to authentic start codons in most eukaryotic mRNA species. Purines are present at positions -3 and +4 in the deduced 5'-terminal sequence for the positive strand (where the A in AUG is +1) which is in agreement with consensus nucleotides found in most eukaryotic mRNAs (38). In addition, the sequence preceding the putative translational start codon is rich in A residues (10 A residues among 13 nucleotides), a characteristic of many yeast mRNA species (39). The open reading frame on the negative strand (10) lacks many of these features,

with a pyrimidine at position -3, a purine at +4, and only 4 A residues among the 13 preceding the AUG at positions 60-62.

The method for strand separation of M dsRNA described here may be useful for analysis of other dsRNA viral genomes. By slight modifications of the dimethylsulfoxide concentration we have successfully separated strands of L dsRNA from S. cerevisiae (unpublished results). Thus the technique is not uniquely applicable to M dsRNA. This method of strand separation is an alternative to a method utilizing agarose gel electrophoresis which has been applied to reovirus dsRNA (40).

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