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Multiple L Double-Stranded RNA Species of *Saccharomyces cerevisiae*: Evidence for Separate Encapsidation

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The L double-stranded (ds) RNA component of *Saccharomyces cerevisiae* may contain up to three dsRNA species, each with a distinct sequence but with identical molecular weights. These dsRNAs have been separated from each other by denaturation and polyacrylamide gel electrophoresis. The 3′ terminal sequences of the major species, L_A dsRNA, were determined. Secondary structural analysis supported the presence of two stem and loop structures at the 3′ terminus of the L_A positive strand. In strain T132B NK-3, both the L_A and L_C species are virion encapsidated. Two distinct classes of virions were purified from this strain, each with a different RNA polymerase activity and with distinct protein components. The heavy virions harbored L_A dsRNA, whereas the L_C dsRNA species copurified with the light virion peak. Thus, L_A and L_C dsRNAs, when present in the same cell, may be separately encapsidated.

The killer virus of the yeast *Saccharomyces cerevisiae* is a model system in which to study the interaction of a multipartite double-stranded (ds) RNA viral genome with its eucaryotic host (for reviews, see references 9, 11, 36, 38). Killer strains harbor two linear, virion-encapsidated, cytoplasmically inherited dsRNA components denoted M (1,830 base pairs) and L (4,980 base pairs). Killer toxin and resistance are genetically encoded on M dsRNA (2, 15). Two killer specificities (K_1, K_2), based on their spectra of killing, are associated with the presence of M_1 or M_2 dsRNA (reviewed in reference 36). In vitro translation studies have demonstrated that denatured M_1 dsRNA encodes M-p32, a presumptive precursor of killer toxin (3, 4, 28, 34). The L dsRNA component programs the synthesis of the major virion protein in which both L and M are encapsidated (5, 18). L dsRNA may also be present in non-killer strains. M dsRNA depends upon the presence of L for its encapsidation (5) and possibly for other factors required for expression and maintenance. In addition, M dsRNA depends upon multiple nuclear genes (*mak* for *ma*intenance of *k*iller; *kex* for *k*iller *ex*pression) for its replication and expression (reviewed in reference 38).

At least three other genetic elements affect the killer phenomenon. The cytoplasmically inherited element [HOK] (*h*elper *o*f *k*iller) enables a replication-defective mutant of M_1 dsRNA to replicate (31, 40). [EXL] (*exc*luder of [KIL-k_2]) interferes with the replication of M_2 dsRNA (exclusion) but has no effect on M_1 dsRNA (37). This is distinct from exclusion of M_2 dsRNA by M_1, which occurs independently of the presence of [EXL] (37). In addition, [NEX] ([KIL-k_2] *n*on-*ex*cludable) prevents the exclusion of M_2 dsRNA by [EXL], but does not prevent the M_1-mediated exclusion of M_2 (37). These three genetic elements all depend on the nuclear genes *MAK3*, *MAK10*, and *PET18* for their maintenance or replication (37).

The analysis of L dsRNA preparations from strains harboring the [HOK], [NEX], and [EXL] phenotypes, and their heat-cured derivatives, indicates that there are three distinct L dsRNAs (L_A, L_B, L_C) which have different RNase T_1 fingerprint patterns (27). Field et al. (14) also have found evidence of multiple species of L dsRNA. These have been resolved into closely adjacent bands by native agarose gel electrophoresis (13). L_A may coexist in the same cell with L_B or L_C. The L_B and L_C species share sequence homology at approximately the 50% level, and the virion proteins from strains which harbor L_B or L_C alone are similar to each other. However, these proteins are different from the virion proteins of strains containing L_A (27). When the [HOK], [NEX], or [EXL] genetic elements are eliminated from strains by growth at elevated temperatures or by the presence of the mutant *mak3* genotype, the L_A dsRNA species is simultaneously lost (13, 26, 27, 37, 40). Therefore, the [HOK], [NEX], and [EXL] traits may be carried on the L_A dsRNA. The relationship of the L_B or L_C dsRNA species to the killer phenomenon is unknown.

This multiplicity of L dsRNA species complicates evaluation of previous experiments. The existence of these multiple L dsRNAs with identical molecular weights but distinct RNA sequences may be responsible for the previously observed sequence heterogeneity of L dsRNA (10). In this report, we show that L dsRNA preparations from strains which harbor two L dsRNA species can be physically separated by gel electrophoresis into L_A and L_B or L_C dsRNAs. We have determined the 3′-terminal primary and secondary structural features of L_A, the most abundant L species in many yeast strains, and we show that transcription in vitro of L_A dsRNA results in an accurate copy of the 5′ terminus of the plus strand of the genome. Two classes of virions can be purified from a strain which harbors two L dsRNA components. These two types of virions contain distinct virion-associated RNA polymerase activities, and each harbors a distinct L dsRNA species. These results indicate that the two species are separately encapsidated when present in the same cell.

MATERIALS AND METHODS

***S. cerevisiae* strains and cell growth.** Strains of *S. cerevisiae* used in these experiments are listed in Table 1. Cultures used for dsRNA isolation or the purification of virions were grown to late stationary phase (5 days) at 28°C, 150 rpm, in medium containing 1% yeast extract, 2% peptone, and 5%

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TABLE 1. Strains of *S. cerevisiae* and dsRNA content

Strain	Genotype[a]	dsRNA	Reference
A364A × S7	a/α *ade1/+ ade2/+ tyr1/+ lys2/+ ura1/+ his7/+ gal1/gal1* [KIL-k_1] [HOK] [NEX]	L_A, L_B, M	35
1480	a *mkt1-1 lys1* [HOK] [NEX] [KIL-o]	L_A, L_B	37
S140	a *mkt1-1 lys1* [HOK-o(39°C)] [NEX-o(39°C)] [KIL-o]	L_B	26
S161	a *lys11 mkt1* [HOK-o(39°C)] [EXL-o(39°C)] [KIL-o] [NEX-o]	L_C	26
T132B NK-3	a *ade2-1 his4-864* [KIL-s3]	L_A, L_C, S_3	16; This work

[a] Cytoplasmic genotypes indicated in brackets are according to Sommer and Wickner (27). The genotype and dsRNA content of strain A364A × S7 are based upon those of the parental strains A364A ([KIL-k_1] [HOK] [EXL]) and S7 ([KIL-o][HOK][EXL]) as described by Wickner (39). These genotypes differ from those reported by Field et al. (14).

ethanol. Strain T132B NK-3 was grown in this medium supplemented with 0.4 g of adenine sulfate per liter.

Purification of dsRNA and strand separation. dsRNA was extracted from whole cells and purified by a modification (33) of the method of Fried and Fink (16). L dsRNA purified by electrophoresis on agarose gels was subjected to strand separation as described previously (28), except that electrophoresis was initiated at 1,000 V for 20 min. Bands corresponding to the separated strands were located by staining the gel in ethidium bromide (0.5 μg/ml) and visualization with UV light (302 nm). Single-stranded RNA was eluted as described previously (29) and repurified by a second cycle of denaturation and electrophoresis on an identical polyacrylamide gel.

Agarose-formaldehyde gel electrophoresis and blot hybridization. Electrophoresis under denaturing conditions (0.8% agarose-formaldehyde) was performed as described previously (21) at 60 V for 5 h. Gels were either dried and exposed to X-ray film, or the RNA was transferred to nitrocellulose sheets (1). Hybridization and filter washing were carried out as described previously (28).

Purification of dsRNA-containing virions and in vitro transcription. Virions were purified from strain T132B NK-3 by a modification of the method of Welsh et al. (35). The P'_{100} fraction was purified on a 10 to 40% sucrose gradient centrifuged at 24,000 rpm for 12 h (4°C) in an SW27 rotor (Beckman Instruments, Inc.). These virions were used as a source of virion transcriptase to catalyze the synthesis of l_A transcript in vitro (in the presence or absence of [α^{32}P]UTP) as described by Welsh et al. (35). RNA was extracted from virions or the transcription reaction mixture by the addition of disodium EDTA to 10 mM followed by extraction with an equal volume of 90% phenol. The aqueous phase was extracted with an equal volume of chloroform-isoamylalcohol (24:1) and precipitated by the addition of sodium acetate to 0.3 M and 2 volumes of ethanol. The transcripts were then resolved into s and l species by electrophoresis on agarose gels, as described previously (28).

Incorporation of ^{32}P onto 5′ and 3′ termini of RNA. Radioactive [5′-^{32}P]pCp (cytidine 3′,5′-[^{32}P]bisphosphate) was attached to the 3′ termini of L dsRNA species in a reaction catalyzed by bacteriophage T4 RNA ligase as described previously (29). Agarose gel-purified l_A transcript (28) was modified at the 5′ terminus with ^{32}P after treatment with alkaline phosphatase as described by Efstratiadis et al. (12). After extraction with an equal volume of phenol, chloroform-isoamylalcohol (24:1) extraction, and ethanol precipitation, the l_A transcript was subjected to electrophoresis on a 5% polyacrylamide gel as performed for strand separation and eluted as described previously (28).

Primary and secondary structure analysis of RNA. The strands of L_A dsRNA bearing 3′-terminal [5′-^{32}P]pCp were analyzed for secondary structure by partial digestion with the single-strand-specific S1 nuclease as described previously (28). The sequence of RNA was determined by the chemical method of Peattie (24) or by the enzymatic method as described by Thiele et al. (29). Analysis of the 5′- and 3′-terminal nucleotides was performed by the method of Volckaert and Fiers (32).

RESULTS

Separation and identification of the L dsRNA species. The demonstration that L_A, L_B, and L_C dsRNAs represent molecules with discrete RNase T_1 fingerprints (and thus different sequences) predicts that the single-stranded form of these molecules may assume unique secondary structures. We find that these molecules comigrate in denaturing (agarose-formaldehyde) gel electrophoresis (data not shown). However, upon denaturation in 30% dimethyl sulfoxide and electrophoresis on a native 5% polyacrylamide strand separation gel, the strands of L_A, L_B, and L_C dsRNAs are separated and have characteristic migrations (Fig. 1). In Fig. 1 (lanes 1 and 2), the predominant RNA species represent the strands of L_A, which is present at approximately five times the copy number of L_B or L_C in the strains tested (27). The faster migrating doublet in lanes 1 and 2 represents the strands of L_B dsRNA (from strains A364A × S7 and 1480), which is in agreement with the L dsRNA content of strain 1480 as determined by RNase T_1 fingerprinting (27). The separated strands of L dsRNA from strains bearing only L_B (lane 3, strain S140) or only L_C (lane 4, strain S161) migrate quite differently from the L_A dsRNA strands, verifying our assignment of the L dsRNA species by this analysis. The strands of L_B and L_C dsRNAs migrate close together in this system, possibly due to a significant degree of sequence homology (27). The undenatured forms of the L dsRNAs, regardless of species type, comigrate in this electrophoretic system, as indicated by the arrow in Fig. 1. The L dsRNA fraction which remains double-stranded in this analysis may be separated into its component strands by a second round of strand separation.

Identification of L_A dsRNA in these strains was confirmed by blot hybridization analysis (Fig. 2, lanes 1 and 2). Preparations from those strains lacking L_A dsRNA by strand separation gel analysis (S140, S161) and by RNase T1 fingerprint analysis (27) did not hybridize with the L_A probe (lanes 3 and

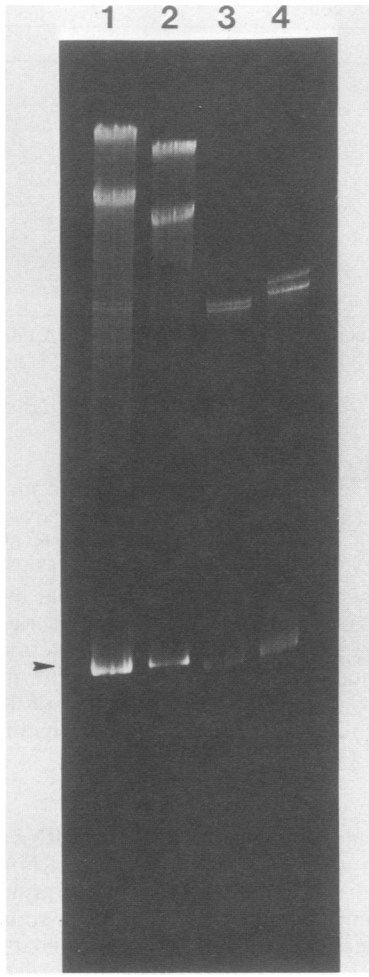

FIG. 1. Separation of L_A, L_B, and L_C dsRNAs by strand separation gel electrophoresis. Agarose gel-purified L dsRNA preparations (1 μg) were denatured and subjected to electrophoresis on a 5% polyacrylamide strand separation gel, as described in the text. L dsRNA preparations are as follows: lane 1, L_A, L_B (strain A364A × S7); lane 2, L_A, L_B (strain 1480); lane 3, L_B (strain S140); lane 4, L_C (strain S161). The arrow indicates the position of native L dsRNA. The gel was stained with 0.5 μg of ethidium bromide per ml and photographed under UV illumination (302 nm).

4). The virions isolated from strain A364A × S7 harbor L_A by this analysis (lane 5), and did not contain detectable levels of L_B or L_C dsRNA as determined by a strand separation gel electrophoretic analysis (data not shown). In blot hybridizations of denatured dsRNA preparations, material larger than L dsRNA hybridizing to radioactive L_A dsRNA or l_A transcript was observed (see Fig. 2 and 9). The significance of this result is unknown.

Sequence analysis of L_A dsRNA and the l_A transcript. The polarity of the L_A strands was determined after purification of the separated strands of the L_A dsRNA through two rounds of denaturation and polyacrylamide gel electrophoresis. After subjecting the separated strands to denaturing (agarose-formaldehyde) gel electrophoresis, they were transferred to nitrocellulose. RNA bound to the nitrocellulose filter was hybridized with [^{32}P]UMP-l_A transcript synthesized in vitro by the virion-associated RNA polymerase. Hybridization of the positive polarity l_A transcript to the L_A slow strand, but not the L_A fast strand, identified the L_A fast strand as the plus strand (data not shown). Strand polarity of L_A dsRNA was corroborated by sequencing studies of the l_A transcript (see below). Strand polarity has not yet been determined for L_B and L_C dsRNA.

The agarose gel-purified preparation of L dsRNA from strain A364A × S7 was modified at its 3′ termini with [5′-^{32}P]pCp by using bacteriophage T4 RNA ligase. After phenol extraction and ethanol precipitation, the strands of L_A dsRNA were separated and purified by two rounds of electrophoresis on 5% polyacrylamide strand separation gels. The sequence of the eluted plus and minus strands of L_A was determined (24). A 3′-terminal nucleotide analysis demonstrated that 87% of the total radioactivity was in adenosine for each strand. Figure 3 shows the 3′-terminal sequence of the L_A minus strand for 112 nucleotides, and the corresponding predicted 5′-terminal sequence of L_A positive strand. The 5′-terminal G residue on the L_A dsRNA plus strand is deduced from the data of Bruenn and Keitz (8). An AUG triplet, located at positions 30 to 32, begins a potential open reading frame for translation which continues for as far as we have sequenced. The amino acid sequence corresponding to this reading frame is also indicated. This reading frame may code for a portion of the major virion capsid protein (5, 7, 18), or another product such as a transcriptase. No evidence for the function of this reading frame in vivo has been demonstrated. The sequence determined here for the 3′ terminus of the minus strand of L_A is almost identical to that reported for one T_1 RNase-generated oligonucleotide derived from an L dsRNA preparation of unknown composition (6).

The predicted 5′-terminal sequence of the full-length l_A transcript (33; Fig. 3) has been confirmed by direct 5′-

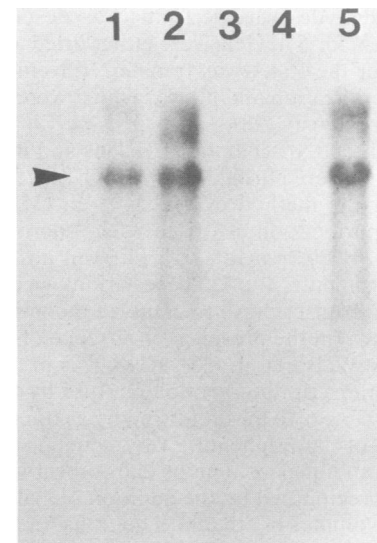

FIG. 2. Blot hybridization analysis of L dsRNA species. L dsRNA preparations were subjected to electrophoresis on a denaturing (0.8% agarose-formaldehyde) gel and transferred to nitrocellulose as described in the text. The RNA bound to nitrocellulose was hybridized with positive strands of L_A bearing [5′-^{32}P]pCp (65,000 cpm) on their 3′ termini at 65°C for 26.5 h. The filter was washed and exposed to X-ray film. The lanes contained equivalent amounts (1 μg) of L dsRNA preparations from: lane 1, strain A364A × S7 (L_A, L_B); lane 2, strain 1480 (L_A, L_B); lane 3, strain S140 (L_B); lane 4, strain S161 (L_C); and lane 5, virions purified from strain A364A × S7. The arrow indicates the position of L_A, L_B, and L_C dsRNA in this electrophoretic system.

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                10                  20                  30
HO ACU UUU UAA AAA UUU AAG UAU AUU GAG GGG UAC GAU UCU
   GA AAA AUU UUU AAA UUC AUA UAA CUC CCC AUG CUA AGA
PPP                                          MET LEU ARG

 40                  50                  60                  70
AAA CAA UGA UUU UUG AGA GUU CUA UUU AGC AGA CUA GAU
UUU GUU ACU AAA AAC UCU CAA GAU AAA UCG UCU GAU CUA
PHE VAL THR LYS ASN SER GLN ASP LYS SER SER ASP LEU

 80                  90                  100                 110
AAG AGA UAA ACA AGA CUA GCG CCU UGA AAC AAC G
UUC UCU AUU UGU UCU GAU CGC GGA ACU UUG UUG C
PHE SER ILE CYS SER ASP ARG GLY THR LEU LEU
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FIG. 3. 3′-Terminal sequence of the L_A dsRNA negative strand, and the deduced 5′-terminal sequence of the L_A positive strand. Separated strands of L_A dsRNA bearing 3′-terminal [5′-^{32}P]pCp were purified as described in the text. After chemical sequencing reactions (24), the digests were fractionated on 8, 12, and 20% polyacrylamide-urea sequencing gels. The top line represents the 3′-terminal sequence of the L_A negative strand; the second line represents the deduced 5′-terminal sequence of the L_A positive strand. Underlined nucleotides are those which have been confirmed by direct 5′-terminal sequence analysis of the l_A transcript. Amino acids encoded by these triplets are indicated on the third line.

terminal sequence determination of this transcript. Figure 4 shows the 5′-terminal sequence analysis of l_A and demonstrates that transcription in vitro probably initiates at the 5′ terminus of the plus strand of L_A dsRNA. We cannot rule out initiation with uridine and subsequent cleavage of this residue. The l_A transcript, like the m transcript of M dsRNA (16a), has a 5′ terminus rich in A residues, with only two G residues in the first 32 nucleotides. These features are characteristic of a large number of yeast mRNA species (17).

The 3′ terminus of the L_A plus strand has been sequenced for 113 nucleotides (Fig. 5). This sequence allows the formation of two stem and loop structures. The stem and loop structure proposed for nucleotide positions 5 through 19 is similar, although not identical, to that predicted by Brennan et al. (6) from the sequence of an oligonucleotide derived from an unknown species of L dsRNA. The structure proposed here is considerably more stable (ΔG [25°C] = −14 kcal [−58.6 kJ]) (30). This sequence predicts the presence of an additional stem and loop structure at positions 29 through 55, which has a calculated stability of ΔG (25°C) = −17.6 kcal (−73.7 kJ).

Secondary structure analysis. Analysis of secondary structural features by the use of the single-strand-specific S1 nuclease (22, 28, 41) supports the presence of the predicted stem and loop structures. Partial cleavage of the positive strand of L_A dsRNA with S1 nuclease resulted in sensitivity at positions 1 to 6, 11 to 14, and 20 to 28 (Fig. 6A). These sites (indicated by arrows in Fig. 5) occur in single-stranded regions in the proposed structure. This preference is also true for the susceptible nucleotide positions 20 to 28 and 40 to 44 (Fig. 6B). This sensitivity to S1 nuclease did not occur at regions proposed to be double stranded. We have previously demonstrated the existence of stem and loop structures at the 3′ and 5′ termini of the M_1 dsRNA minus and plus strands, respectively (16a, 28). The likely presence of these structures at the 3′ terminus of l_A transcript may account for the inefficient acceptor activity of this molecule for [5′-^{32}P]pCp in the reaction catalyzed by RNA ligase (E. M. Hannig, unpublished data). No stable stem and loop structures could be formed from the sequence at the 3′ terminus of the minus strand, and this region exhibited a high degree of S1 nuclease sensitivity. This is consistent with this region existing primarily as a single-stranded form in isolated minus strands (data not shown). Similar secondary structure may be present at the 5′ terminus of the l_A transcript.

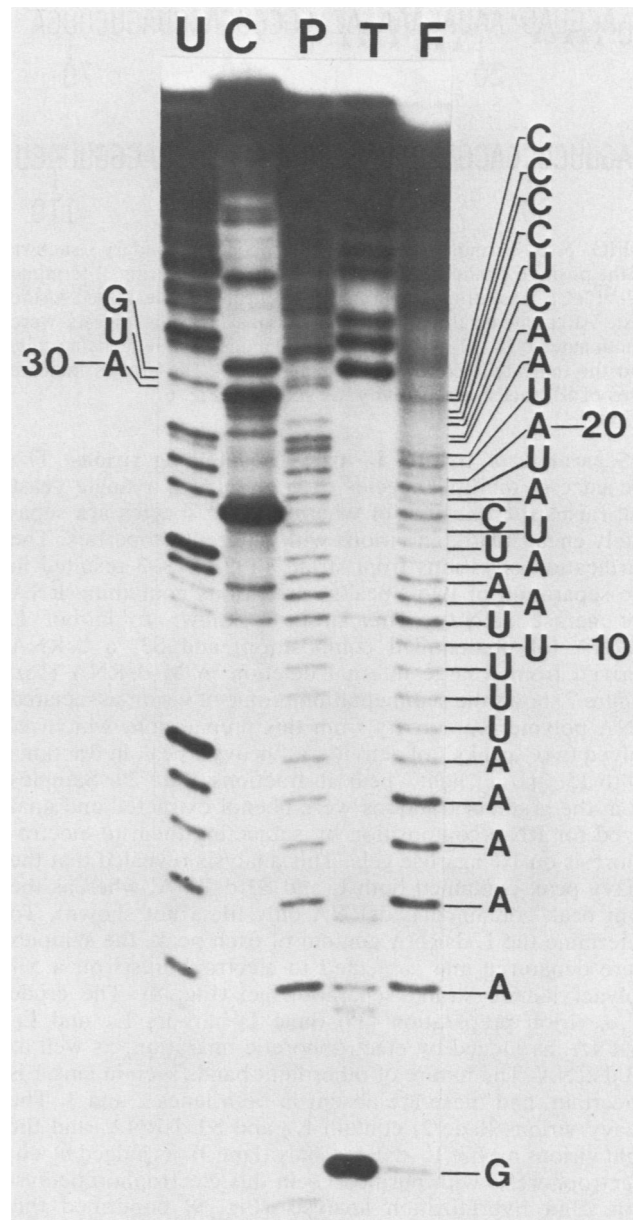

FIG. 4. 5′-Terminal enzymatic sequence analysis of the l_A transcript. The l_A transcript was purified and modified at the 5′ terminus with ^{32}P as described in the text. Enzymatic sequencing digestions using base-specific RNases were carried out as described by Thiele et al. (29), except that RNase U_2 digestion was at pH 3.5. Digests were fractionated on a 20% polyacrylamide-urea gel. Lane U, U_2 (A-specific); lane C, CL-3 (C-specific); lane P, Phy M (A + U-specific); lane T, T_1 (G-specific); lane F, formamide ladder.

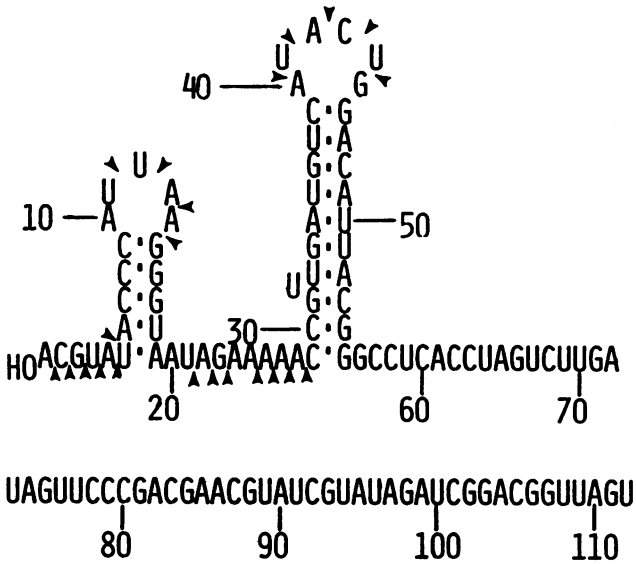

FIG. 5. 3′-Terminal sequence and proposed secondary structure of the positive strand of L_A dsRNA. L_A dsRNA bearing 3′-terminal [5′-^{32}P]pCp was strand separated and purified as described in the text. After chemical sequencing reactions (24), the digests were fractionated on 8, 12, and 20% polyacrylamide-urea sequencing gels, and the indicated sequence was determined. The arrows indicate sites of S1 nuclease sensitivity, as shown in Fig. 6.

Separation of L_A and L_C dsRNA-containing virions. The presence of multiple species of L dsRNA in a single yeast cell raises the question of whether these species are separately encapsidated in virions with different properties. The purification of virions from strain T132B NK-3 resulted in the separation of two "peaks" of virions containing RNA polymerase activity. This strain is known to harbor L dsRNA (of unidentified composition) and S3, a dsRNA derived from a large internal deletion in M dsRNA (16). Figure 7 shows the sedimentation profile of virion-associated RNA polymerase activity from this preparation, which resolved two "peaks" of activity, a "heavy" peak in fractions 10 to 15, and a "light" peak in fractions 26 to 29. Samples from the gradient fractions were phenol extracted and analyzed for RNA composition by subjecting them to electrophoresis on 1% agarose gels. This analysis revealed that the heavy peak contained both L and S3 dsRNA, whereas the light peak contained L dsRNA only (data not shown). To determine the L dsRNA content of each peak, the samples were denatured and subjected to electrophoresis on a 5% polyacrylamide strand separation gel (Fig. 8). The crude P'_{100} virion preparation (35) (lane 1) harbors L_A and L_C dsRNA, as judged by electrophoretic migration, as well as S3 dsRNA. The nature of other faint bands seen in lane 1 is uncertain, and these are absent in both lanes 2 and 3. The heavy virions (lane 2) contain L_A and S3 dsRNA, and the light virions harbor L_C dsRNA only (lane 3), as judged by coelectrophoresis with purified L_C in this electrophoretic system. Blot hybridization analysis (Fig. 9) confirmed this finding. The crude virion preparation (lane 2) and the heavy virions (lane 3) hybridized to the purified, ^{32}P-labeled L_A probe, as did the L_A dsRNA control in lane 1. An equivalent amount of RNA from the light virions failed to hybridize significantly to the L_A probe (lane 4). The trace level of hybridization seen in lane 4 might be due to low levels of L_A dsRNA in light virions or to slight contamination of the light virion preparation by heavy virions or by L_A dsRNA. These data indicate that the L_A and L_C dsRNAs (when present in the same cell) may be separately encapsidated in distinct virions. Sommer and Wickner (27) observed two peaks of RNA polymerase activity in virion preparations from four different strains. The RNA components in each peak were not characterized; they may be similar to those we have isolated from T132B NK-3. Two peaks of RNA polymerase activity have also been observed in killer strains (M. J. Leibowitz, unpublished data).

The characteristics of the RNA polymerase activity associated with each peak are quite different. Under standard reaction conditions (35), the heavy virion polymerase activity catalyzes the incorporation of UMP in a linear fashion for at least 3 h. The RNA polymerase activity associated with light virions catalyzes the incorporation of UMP in a linear manner for only 15 min. The cessation of incorporation by the light virion polymerase does not simply reflect instability

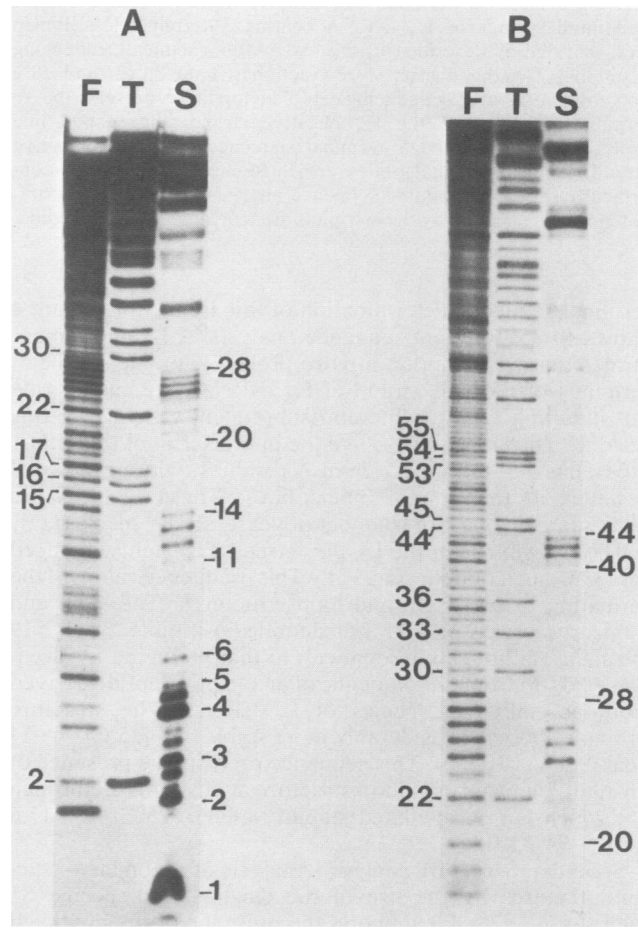

FIG. 6. Secondary structural analysis of the L_A positive strand 3′ terminus. The L_A dsRNA positive strand bearing 3′-terminal [5′-^{32}P]pCp was subjected to partial digestion with the single-strand-specific S1 nuclease as described in the text. Digestion products were electrophoretically fractionated on either a 20 (panel A) or a 12% (panel B) polyacrylamide-urea gel. The numbers on the left in each panel represent the length of oligonucleotides generated by RNase T_1 digestion or formamide hydrolysis. The numbers on the right in each panel refer to the length of oligonucleotides generated by S1 nuclease digestion. F represents a formamide ladder; T represents RNase T_1 digestion; and S represents the S1 nuclease digestion. The doublets in panel A may result from the 3′ phosphatase activity of S1 nuclease (22).

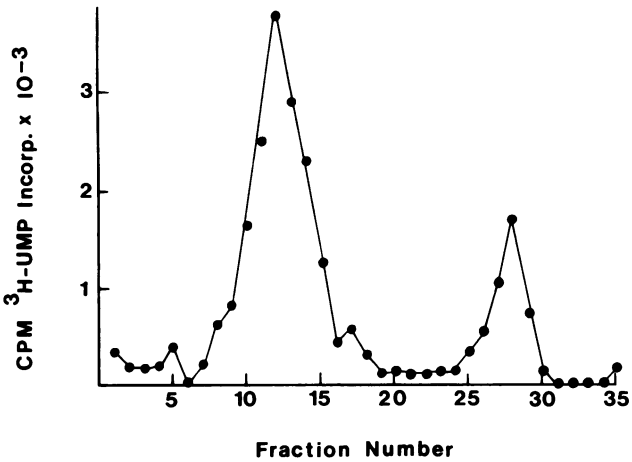

FIG. 7. Sucrose gradient centrifugation of virions purified from strain T132B NK-3. Virions were purified through the P'_{100} step according to the method of Welsh et al. (35) and subjected to sucrose gradient centrifugation as described in the text. Fractions were collected from the bottom and assayed for RNA polymerase activity under the standard conditions of Welsh et al. (35). Fractions 10 to 15 (heavy peak) and 26 to 29 (light peak) were separately pooled and concentrated for further analysis.

at 28°C, since the loss of activity only occurred if ribonucleoside triphosphates were present (data not shown). The heavy virion RNA polymerase activity exhibits a reaction optimum at pH 7.5 and is inhibited to approximately 20% of the control level by 200 mM KCl. The light virion RNA polymerase activity exhibited a pH optimum of 8.1 to 8.4 and retained approximately 80% of its activity in 1 M KCl (data not shown). The heavy peak has properties similar, if not identical, to the virions from the killer strain A364A × S7 characterized by Welsh et al. (35). We find that both the heavy virions from strain T132B NK-3 and those virions purified from strain A364A × S7 as described by Welsh et al. (35) harbor only the L_A species of L dsRNA.

The protein compositions of the two virion types described here are very different (Fig. 10). The L_C dsRNA-containing virions lack the 81-kilodalton (kd) major capsid polypeptide previously observed to be associated with dsRNA-containing virions in yeast (lane 1) (5, 18). Instead, the light virions contain smaller polypeptides of 76, 68, and 65 kd (lane 3), similar to the polypeptide composition observed for virions purified from strains which harbor only L_C or L_B dsRNA (27). The heavy virion fraction contained the 81-kd major capsid polypeptide and low levels of lower-molecular-weight polypeptides (lane 2), as described by Sommer and Wickner (27). We cannot exclude low levels of other subcellular particles and breakdown as being partially responsible for the multiple protein species found in the light virion fraction, although the absence of the 81-kd peptide implies relative freedom from heavy virion contamination.

DISCUSSION

We have demonstrated that the multiple L dsRNA species detected by selective curing and analysis of RNase T_1-generated oligonucleotides (14, 27) can be separated and analyzed by gel electrophoresis. The RNase T_1 fingerprint analyses of Sommer and Wickner (27) suggest that the L_A, L_B, and L_C dsRNAs have distinct nucleotide sequences. These sequence differences may account for the separability of these dsRNA species by subjecting the denatured strands to electrophoresis on a native polyacrylamide gel. The basis for strand separation by native gel electrophoresis, although uncertain, is thought to be due to each single-stranded nucleic acid assuming a characteristic, stable conformation (25). Since different sequences would likely lead to distinct conformations, the ability to separate the strands of these molecules from each other, and from those of the other L dsRNA species, is consistent with this mechanism. Small differences in mobility of the strands of L_A dsRNA isolated from different strains, for example, A364A × S7 versus 1480 in Fig. 1, may reflect small sequence changes, as suggested by the fingerprint analyses of Sommer and Wickner (27).

The two 3′-terminal sequences of L_A dsRNA, the most abundant of the L dsRNA species, have been determined by direct chemical sequencing. The minus strands of L_A and M dsRNA share the sequence 3′-HO-ACUUUUUA, with very little other sequence homology at their termini. The 5′ terminus of the L_A positive strand, unlike that of M dsRNA (16a, 28), does not form apparent stem and loop structures. Like M dsRNA, the 5′ terminus of the positive strand does

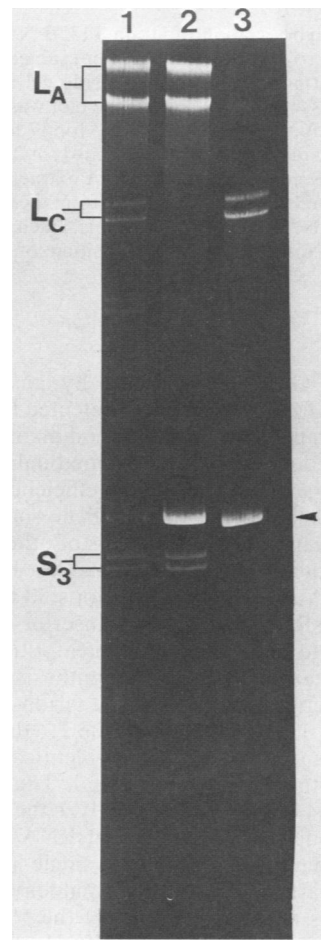

FIG. 8. Double-stranded RNA components of heavy and light virions from strain T132B NK-3. Virion dsRNA was extracted, denatured, and subjected to electrophoresis on a 5% polyacrylamide strand separation gel as described in the text. Lane 1 contains RNA from the crude virion preparation (2 μg); lane 2 contains RNA from the heavy virions (2 μg); and lane 3 contains RNA from the light virions (2 μg). The position of undenatured L dsRNA is indicated by an arrow. The gel was stained with 0.5 μg of ethidium bromide per ml and photographed under UV illumination (302 nm).

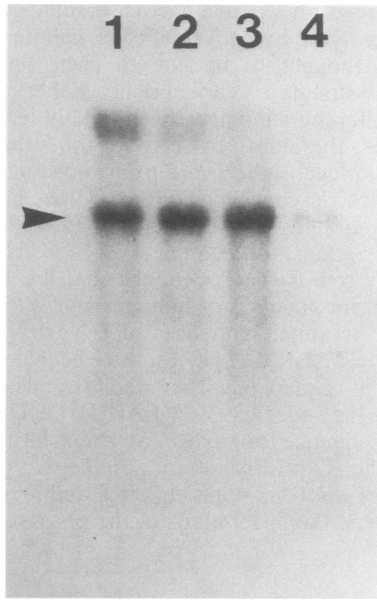

FIG. 9. Blot hybridization of strain T132B NK-3 virion dsRNA with L_A dsRNA probe. RNA samples were subjected to electrophoresis in a denaturing agarose-formaldehyde gel and transferred to nitrocellulose. RNA on the nitrocellulose blot was hybridized to [5′-^{32}P]pCp-L_A positive strand (90,000 cpm) for 17 h at 65°C, washed, and exposed to X-ray film. Lane 1 contains L dsRNA (L_A, L_B) from strain A364A × S7; lane 2 contains RNA extracted from the crude virion preparation; lane 3 contains RNA from the heavy virions, and lane 4 contains RNA from the light virions. Each lane contains 1 μg of RNA. The arrow indicates the position of L_A, L_B, and L_C dsRNAs in this electrophoretic system.

contain an AUG triplet followed by an extended open reading frame. If the AUG triplets detected for M and L_A are functional as translation initiation codons in vivo, different accessibility of these sites to ribosome binding may regulate their relative translation initiation efficiencies. Differential accessibility of initiator codons has been suggested to play a role in the regulation of the synthesis of other proteins, such as mouse and rabbit α and β globins (23). Flanking nucleotides near the AUG codon at positions 30 to 32 of the plus strand of L_A dsRNA and the l_A transcript do not resemble those adjacent to most functional eucaryotic initiators (19).

We have determined that the synthesis of the positive polarity l transcript catalyzed by the virion-associated RNA polymerase (7, 33) is directed by the L_A dsRNA template. The 5′ terminus of this transcript is identical to the deduced 5′ terminus of the L_A positive strand. The virions isolated from strain A364A × S7 used to catalyze the synthesis of this RNA harbor only the L_A and M dsRNA species. The l_A transcript, shown to be a full-length single-stranded RNA as judged by denaturing (agarose-formaldehyde) gel electrophoresis (33), is an accurate copy of the 5′ terminus of the L_A dsRNA positive strand. Difficulty in attachment of [5′-^{32}P]pCp to the 3′ terminus of full-length l_A may be due to the presence of secondary structural features at this location (6; E. M. Hannig, unpublished data).

The two classes of virion-associated RNA polymerase activity found in extracts of strain T132B NK-3 are similar to those observed by Sommer and Wickner (27) and have previously been observed in our laboratory in extracts of killer strains (M. J. Leibowitz, unpublished data). The protein composition of the heavy virion peak (harboring L_A dsRNA) is very similar to that of virion proteins previously described in strains which harbor L_A dsRNA (5, 18, 27). These virions contain an 81-kd major capsid polypeptide. The lighter virion peak (containing only L_C dsRNA) lacks this 81-kd major capsid polypeptide (27). Instead, these virions have a polypeptide composition similar to that of virions from strains which harbor only L_B or L_C dsRNA (27). The recovery of the light virion RNA polymerase activity varied greatly among preparations from the same strain, and this activity was unstable upon storage of purified virions at −20°C, consistent with the suggestion of instability of virions purified from L_B- or L_C-containing strains (27).

Two similar peaks of virion-associated RNA polymerase activity have been described in various dsRNA-containing strains (27). Although eliminating L_A from a strain appears to correlate with decreased levels of heavy virions, an absolute correspondence of L subspecies and virion gradient profiles was not observed (27). Low levels of heavy virions have been reported in strains lacking L_A, as have low levels of light virions in strains only containing detectable L_A dsRNA (27). In summary, it appears that L_A is preferentially encapsidated in heavy virions and the other L species in light virions, although some incorporation into both virion types in strains harboring only a single L species cannot yet be excluded.

The multiple L dsRNA species present in many yeast strains comigrate in several electrophoretic systems but are resolvable, as described here. These species have also been resolved on agarose gels run under somewhat different conditions (13). Several strains harbor two unique L dsRNA species which differ in sequence. Preliminary sequence analysis of L_B and L_C dsRNAs purified in this fashion

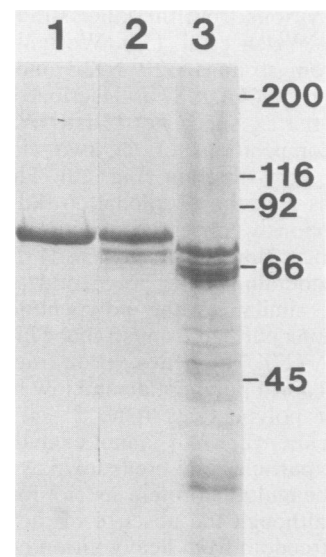

FIG. 10. Polpeptide components of heavy and light virions from strain T132B NK-3. Virion polypeptides from heavy and light virions and from virions isolated from strain A364A × S7 were subjected to electrophoresis on a 7.5% sodium dodecyl sulfate-polyacrylamide gel as described previously (20). Lane 1, virions purified from A364A × S7; lane 2, heavy virions; lane 3, light virions. The gel was stained with Coomassie brilliant blue. The positions of the molecular weight standards are indicated: ovalbumin (45 kd); bovine serum albumin (66 kd); phosphyorylase B (92.5 kd); β-galactosidase (116 kd); myosin (200 kd).

reveals that the 3′ termini of these molecules, although very similar to each other, are different from the 3′ termini of L_A dsRNA (unpublished data). Another source of heterogeneity in sequence analysis of dsRNA molecules is the presence of oligonucleotides which are very efficient acceptors for [5′-^{32}P]pCp in agarose gel-purified L_A, L_B, L_C, M, and S3 dsRNAs (data not shown). The removal of these oligonucleotides by polyacrylamide gel electrophoresis results in unambiguous sequences for M (28, 29), L_A (this work), and S3 dsRNA (D. J. Thiele et al., manuscript in preparation).

The role, if any, of the L_B and L_C dsRNAs in the killer system is unknown. Clearly, future studies concerning the structure and genetic information encoded on the L dsRNA components will require the purification of individual species of L dsRNA.

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