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Yeast killer dsRNA plasmids are transcribed in vivo to produce full and partial-length plus-stranded RNAs

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ABSTRACT

In vivo transcripts of the L (4.5 kb) and M (1.9 kb) dsRNA plasmids were examined in type I killers of Saccharomyces cerevisiae. Transcripts for both plasmids include full-length (L, M) and partial-length (L\textsubscript{a}, M\textsubscript{a}) single-stranded species. Both L-dsRNA transcripts (L, L\textsubscript{a}) have in vitro mRNA activity for L-P1, previously shown to be identical to ScV-P1, the 88,000 dalton major capsid protein of the virus-like particles containing L- and M-dsRNAs. L, but not L\textsubscript{a}, is bound to poly(U)-sepharose and may be polyadenylated. Other L-dsRNA gene products and their transcripts may exist. For M-dsRNA, both species (m, m\textsubscript{a}) have in vitro mRNA activity for M-P1, the 32,000 dalton pre-protoxin encoded by M-dsRNA. Both m and m\textsubscript{a} are bound to poly(U)-Sepharose and m\textsubscript{a} is probably a 5' terminal fragment of m. A functional model for M\textsubscript{a}-dsRNA killer plasmid structure is presented.

INTRODUCTION

Type I killer strains of Saccharomyces cerevisiae secrete an 11,500 dalton polypeptide toxin (mycocin) that kills sensitive strains by disrupting cytoplasmic membrane functions (1,2). The genetic determinant of toxin production (K\textsubscript{1}+ phenotype) and of resistance to toxin (R\textsubscript{1}+ phenotype) is a cytoplasmically inherited 1.9 kb double-stranded (ds)RNA plasmid (M\textsubscript{1}) that exists in 10-100 copies per cell, along with larger numbers of a 4.5 kb dsRNA plasmid (L). Both are encapsidated in separate virus-like-particles (VLPs) (3) called the ScV-L and ScV-M\textsubscript{1} components of the S. cerevisiae mycovirus, ScV. They are stably maintained by vertical transmission and resemble a primitive form of the Reoviridae in the possession of a particle-associated transcriptase activity that produces, in vitro, full-length, non-complementary, plus-strand copies of the M\textsubscript{1} and L-dsRNAs (4,5,6). As in reovirus (7), these transcripts may participate both in expression and as templates for negative-strand synthesis in dsRNA replication, perhaps accompanied by encapsidation. We have shown (8) that no substantial pools of free dsRNA or capsid exist, suggesting that production of either could control the rate of VLP production.

L-dsRNA encodes the 88,000 dalton major capsid protein (ScV-P1) of both
ScV-L and ScV-M₁ (8,9). If encapsidation is essential for ScV dsRNA maintenance, this explains the apparent dependence of ScV-M on ScV-L, deduced from the inability to find or generate strains containing only ScV-M₁ (e.g.,10).

Our strains (see below) and all known type I killers (with the exception of strain K7) contain a heat-curable major L species (Lₐ) and a heat-stable minor species (L₉ or L₈) (10,11,12). Lₐ, L₉, and Lₐ can exist independently and Lₐ is, by itself, capable of maintaining M₁ (in strain K7) and probably encodes ScV-P₁ (11). The minor capsid proteins (8), ScV-P₂ and ScV-P₃, previously seen in both ScV-L and ScV-M₁ VLP preparations, may be encoded by L₉ or L₉ (11), although these proteins are also candidates for VLP transcriptase activity. The independence of different L species suggests that each encodes all of the plasmid encoded proteins required for its own maintenance. Besides capsid, these might include transcriptase or replicase components since L-species are not necessarily monocistronic.

The complex nuclear and cytoplasmic controls over M-dsRNA maintenance and expression, extensively studied at the genetic level by Wickner (13,14, 15), is a prime model for dsRNA plasmid-nuclear interactions in eukaryotic microorganisms. At least 28 nuclear genetic loci (MAK) are required for maintenance of M₁-dsRNA (13). Only 3 of these are also required for maintenance of Lₐ (11) and none are required for L₉ or L₉. Thus replication of ScV-M₁, ScV-Lₐ and ScV-L₉C may differ substantially.

The M₁-dsRNA encodes a major 32,000 dalton protein (M₁-P₁) believed to be a precursor of ScV-P₅, a glycosylated protoxin (16, 17). We have hypothesized that ScV-P₅ may also be a precursor of the hypothetical resistance determinant. If so, M₁-P₁ may be the only gene product necessary for expression of the killer phenotype and a single M₁-dsRNA mRNA transcript may exist, although it has also been reported (18) that fragmented M₁-dsRNA is translated to give an additional 19,000 dalton product.

Through the efforts of numerous laboratories, the yeast killer system is rapidly becoming the best understood mycoviral system. However very little information exists on the in vivo transcription or replication of mycoviral RNAs. We now demonstrate the existence in killer cells of both full-length and partial-length, single-stranded transcripts of both L and M₁-dsRNA, all of which appear to be plus-stranded. We discuss potential functions and mechanisms of synthesis of these transcripts and propose a model for the functional sequence of M₁-dsRNA.
MATERIALS AND METHODS

Yeast strains and media

The haploid killer strain K12-1 (α ade K+R⁺) and the sensitive strain 3/Al (a K-R⁻) were obtained from E.A. Bevan (Queen Mary College, London, England). The dsRNA-free strain 1556 (α ade mal6, cyh² [cir-o] L-M⁻) was derived by D. Livingston and obtained from R.B. Wickner (Natl. Insts. of Health, Bethesda, Maryland). YEPD and SMD media are described elsewhere (8).

Preparation, purification and size estimation of RNAs

Total cellular RNAs and dsRNA were prepared as previously described (8,16). Individual dsRNA species were prepared from total dsRNA by 2 cycles of preparative agarose slab gel electrophoresis (16). Total cellular RNAs were analyzed by chromatography on CF11 cellulose (50 µg/ml bed volume) (16, 19), by poly(U)-Sepharose chromatography (0.6 mg RNA/ml bed volume) (20,21) and by thermal elution poly(U)-Sepharose chromatography (21,22). RNA markers used to size RNAs fractionated by formaldehyde/agarose gel electrophoresis were: denatured L-dsRNA (4.5 kb), 26S yeast ribosomal RNA (3.0 kb), denatured M₁-dsRNA (1.9 kb) and 17S yeast ribosomal RNA (1.5 kb). These defined a linear relationship between log [length (kb)] and mobility (cm).

DNase and RNase hydrolysis

RNA preparations were treated with DNase or RNase, with or without 0.4 M NaCl, as previously described (8). Analytical scale reactions contained up to 5 µg of RNA in a total volume of 40 µl. The phenol phases were re-extracted twice to ensure quantitative recovery of nucleic acids, and yeast tRNA was added to 50 µg/µl for ethanol precipitation.

Cell-free protein synthesis and immunoprecipitation

Total cellular RNAs and dsRNAs (native and denatured) were translated in a wheat-germ or reticulocyte (23) cell-free protein synthesis system using [³²P]methionine according to published procedures (16). Translation products were analyzed by immunoprecipitation, electrophoresis and fluorography as described previously (9,16). The mRNA activities for synthesis of the corresponding immunoprecipitated proteins were determined by densitometry of gel autoradiograms using a Joyce-Loebl recording densitometer. Under the conditions employed, the densitometry signal for these proteins was linearly proportional to the concentration of added RNA. Preparation of antisera to capsid (ScV-P1) and toxin (ScV-P4) in New Zealand white rabbits, purification of the immunoglobulin fraction, and their characterization have been previously described (16). Immuncompetition assays were as described elsewhere (16).

Hybridization selection

RNAs complementary to L- and M₁-dsRNA were selected from total cellular RNAs by hybridization to denatured dsRNA covalently attached to nitrocellulose by a modification of the procedure of Alwine et al (24) for the attachment of dsRNA (17). Selected RNAs were identified by RNA blot hybridization analysis and by their translation in an in vitro protein synthesis system.

RNA blot hybridization analysis

Formaldehyde gel electrophoresis and blot hybridization of RNA were performed as described elsewhere (25). Autoradiographic exposures were made with Kodak XAR-5 film using Dupont Cronex Lightning-Plus intensifier screens. Autoradiograms were optically quantitated using a Joyce-Loebl recording densitometer. At appropriate autoradiographic exposure, densitometry signal was linearly proportional to RNA content, as previously demonstrated by reconstruction experiments using acid phosphatase mRNA (25). DNA was labeled by nick translation (26) with [³²P]dCTP (Amersham/Searle). dsRNA was kinase-labeled after alkaline hydrolysis at 95°C for 90 min in 0.05 M Tris, pH 9.5, 1 mM EDTA. Kinase reactions (1-10 µg dsRNA) were at 37°C for 45 min in 100 µl
of the hydrolysis buffer containing, in addition, 10 mM MgCl₂, 5 mM DTT, 5 units of T4 polynucleotide kinase and 1 mCi of crude γ[^32P]ATP (ICN). Unincorporated label was removed by multiple ethanol precipitations of the RNA.

RESULTS

Complexity of products of translation of denatured M₁- and L-dsRNA

In previous publications we demonstrated that denatured dsRNAs will serve as translation templates in wheat-germ cell-free protein synthesizing systems, programming the synthesis of authentic ScV proteins (9,16,17). The major and largest product of L-dsRNA was L-P1 (88,000 daltons), shown by peptide mapping to be identical to ScV-P1 capsid (9). Two minor components, L-P2a (85,000 daltons) and L-P4 (77,000 daltons, previously called L-P2; 9) were also precipitated with anti-capsid and were shown to be truncated forms of ScV-P1 produced by premature termination of translation (unpublished observations). Many minor components of smaller size were also produced. The products of M₁-dsRNA were a major 32,000 dalton preprotoxin (16,17) and a minor protein of 30,000 daltons. The reticulocyte cell-free translation system (23) has a much lower background of endogenous products and is known for its low incidence of premature chain termination. In this system denatured M₁-dsRNA gave a single product, the 32,000 dalton preprotoxin (Fig. 1A, lane f) (16,17). However, denatured L-dsRNA still gave a complex mixture of products (Fig. 1A lanes c,e,h). Since none of these were formed from native dsRNA (Fig. 1A, lane b,g), they are derived from denatured dsRNA, rather than from contaminating single-stranded mRNAs. The largest products, L-P1, P2 and P3, comigrated with ScV-P1, P2 and P3. Only L-P1 and L-P4 were precipitated with anti-capsid IgG (data not shown). L-P2 and L-P3 have not been further identified but are candidates for unique VLP components encoded by L-dsRNAs. The nature of the many smaller products is obscure, although the presence of L-P4, apparently a truncated form of L-P1, suggests that translation of denatured L-dsRNA in the reticulocyte system may be particularly inefficient, producing a series of related protein fragments, in contrast to translation of denatured M₁-dsRNA.

In the above experiments and elsewhere, we have utilized the insensitivity of dsRNA to pancreatic RNase at high osmolarity to confirm the strandedness of RNA species prior to denaturation. In 400 mM salt (but not in 20 mM salt) RNA or messenger activity surviving RNase treatment is due to dsRNA, while that destroyed in 400 mM salt is due to single-stranded RNA. As shown in Fig. 1A, lane d, hydrolysis of K12-1 dsRNA with RNase in 400 mM salt, prior to denaturation, had no effect on mRNA activity.
In vivo mRNA for L-P1 and M₁-P1 can be detected in total cellular RNAs

Three yeast strains were used to examine the in vivo transcripts of L and M₁-dsRNA: 1556 (K⁻R⁻), 3/A1 (K⁻R⁻), and K12-1 (K⁺R⁺). Strain 1556 cells contain no detectable dsRNA. Strain 3/A1 contains only L-dsRNA, which comprises about 0.4% of the total cellular RNA (9). Strain K12-1 possesses both L- and M₁-dsRNA at a mass ratio of L- to M₁-dsRNA of 3.5 to 1: L- and M₁-dsRNAs represent 0.03 and 0.009% of the total RNA, respectively, in a molar ratio of 1:0.69 (Table 1). The major L species in both strains 3/A1 and K12-1 is Lₐ (11). The minor species have not been identified.

Native total RNA from these strains was translated in a wheat-germ system. Immunoprecipitation of the products with anti-capsid IgG produced clearly visible bands of L-P1, L-P2a and L-P4, together with a high background of non-specifically bound labelled protein, from RNA of strains 3/A1 and K12-1 (Fig. 1B, lanes b,d). The authenticity of the specifically precipitated proteins was confirmed by their absence in immunoprecipitates with pre-immune or anti-toxin IgGs (Fig. 1B, lanes a,c and Fig. 1C) and in the products of translation of RNA from strain 1556 (Fig. 1B, lanes e,f). The weaker L-P1 mRNA activity for strain K12-1 is due to its lower L-dsRNA content. Anti-toxin IgG precipitated M₁-P1 from the products of translation of K12-1 total RNA (Fig. 1C, lane b). The antigenicity of the reaction products was confirmed by immunocompetition with purified toxin (Fig. 1B, lane c). Thus mRNA activity for M₁-P1 exists in cells of this killer species. No M₁-P1 mRNA activity was detectable in 3/A1 or 1556 total RNAs (Fig. 1C lanes d-h).

Although we have previously demonstrated that isolated dsRNAs only function as translation templates after thorough denaturation, and the products described here were produced from undenatured RNA fractions, we felt it important to establish that the templates assayed in these complex RNA mixtures were single-stranded transcripts rather than the dsRNAs themselves. To demonstrate the translational inactivity of dsRNA in total RNA translations, purified dsRNA from strain K12-1 was added to total RNA from strain 1556 or K12-1 at 0.1% or 1% of the total RNA, and the translation products of the undenatured RNAs analyzed by immunoprecipitation. Equivalent mRNA activities were observed for transketolase (TK), enolase (EA), glyceraldehyde-3-phosphate dehydrogenase (GPDH) and alcohol dehydrogenase (ADH) in native total RNAs from both strains, with or without the addition of dsRNA; mRNA activities for L-P1 and M₁-P1 in K12-1 RNA were not enhanced by the addition of K12-1 dsRNA and no L-P1 or M₁-P1 mRNA activity was detected when K12-1 dsRNA was added to native total 1556 RNA (data not shown).
The single-stranded nature of the RNAs was also demonstrated by their behavior upon chromatography on CF11 cellulose and by their RNase sensitivity at high salt concentration. Fractionation of K12-1 total RNA on analytical columns of CF11 cellulose separated a peak of unbound RNA from a peak of bound L- and M₁-dsRNA. Hydrolysis of these RNA fractions with RNase in 400 mM salt demonstrated the unbound RNA to be single-stranded and not contaminated with dsRNA (data not shown). All of the mRNA activities for L-P1 and M₁-P1 were found in this single-stranded RNA peak together with the TK, EA, GPDH and ADH mRNAs (data not shown). In unfractionated total K12-1 RNA these mRNA activities were sensitive to RNase in 400 mM salt (data not shown).

Because killer dsRNA transcripts are of such low abundance, their products, among the products of translation of native total RNA, could only be seen above a high background of nonspecifically bound, labelled protein even

Figure 1. Comparison of Translation Products of Denatured Total dsRNAs, Native Total in vivo RNAs and HySt in vivo RNA's

| (A) Purified dsRNA species were translated in a reticulocyte system using [³⁵S]L-methionine, with or without prior denaturation, as indicated. The RNA added in lane d was hydrolyzed with RNase in 400 mM salt prior to denaturation. Products were fractionated on a 10-15% SDS polyacrylamide gel and detected by fluorography. |
|---|---|---|
| Lane | Added dsRNA | Denatured |
| a | none | -- |
| b | K12-1 total | No |
| c | " | Yes |
| d | " | " |
| e | Purified L(K12-1) | " |
| f | Purified M₁(K12-1) | " |
| g | Purified L(K3/Al) | No |
| h | " | Yes |

| (B) Native total cellular RNA preparations were translated in a wheat-germ system, using [³⁵S]L-methionine. Translation products were immunoprecipitated with the indicated IgGs, and analyzed as in A. |
|---|---|---|
| RNA from Strain |
| K12-1 3/Al 1556 IgG |
| (lane) (lane) (lane) |
| a | c | e | pre-immune |
| b | d | f | anti-capsid |

| (C) Native total cellular RNA preparations were translated and analyzed as in part B, using anti-toxin IgG. Where indicated (+ Ag), 1 µg of pure Toxin was present during immunoprecipitation. |
|---|---|---|
| RNA from Strain |
| K12-1 3/Al 1556 IgG |
| (lane) (lane) (lane) |
| a | d | g | pre-immune |
| b | e | h | anti-toxin |
| c | f | - | anti-toxin+Ag |

| (D) Native total RNAs from strain 3/Al or K12-1 cells were fractionated by hybridization to denatured, total strain K12-1 dsRNA bound to nitrocellulose. The hybrid selected (HySt) RNAs were translated in a wheat germ system and the products analyzed as in Part C. |
|---|---|---|
| Total RNA's Hyst RNA's |
| 3/Al K12-1 3/Al K12-1 IgG |
| (lane)(lane)(lane) |
| a | d | h | l | pre-immune |
| b | e | i | m | anti-capsid |
| c | f | j | n | anti-toxin |
| g | k | o | anti-toxin+Ag |
Table 1. Estimated Cellular Contents of Killer RNA Species in Strain K12-1

<table>
<thead>
<tr>
<th>Species</th>
<th>% Mass</th>
<th>Molar Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>0.03</td>
<td>100</td>
</tr>
<tr>
<td>l</td>
<td>0.0003</td>
<td>2</td>
</tr>
<tr>
<td>l_a</td>
<td>0.0007</td>
<td>9</td>
</tr>
<tr>
<td>M_1</td>
<td>0.0090</td>
<td>69; 100</td>
</tr>
<tr>
<td>m</td>
<td>0.0061</td>
<td>9; 14</td>
</tr>
<tr>
<td>m_a</td>
<td>0.0047</td>
<td>11; 17</td>
</tr>
</tbody>
</table>

Data for % of total cellular RNA (% mass) are derived from steady-state incorporation levels of $[^{32}P]P_{i04}$ for L- and M$_1$-dsRNAs. Data for the transcripts (l, l_a, m, m_a) are derived from hybridization signals, relative to L and M, given with appropriate probes (see text). Molar ratios are derived assuming the following base contents: L, 9,000; l, 4,500; l_a, 2,300; M_1, 3,800; m, 1,900 and m_a, 1,200.

after enrichment by immunoprecipitation with anti-capsid or anti-toxin IgGs (Fig. 1B,C), precluding visualization of products other than M_1-P1 and L-P1. dsRNA transcripts were, therefore, selected from total native RNAs by hybridization to denatured, total K12-1 dsRNA, bound to nitrocellulose. The background due to endogenous mRNAs present in the wheat-germ extract obscured direct detection of translation products of hybrid-selected RNAs (data not shown), however, immunoprecipitation with anti-capsid IgG now showed L-P1, L-P2a and L-P4 very clearly (Fig. 1D, lane i), and anti-toxin now gave a single band of M_1-P1 (Fig. 1D, lane n).

Physical detection of partial transcripts of M_1- and L-dsRNA (Fig. 2)

We used a modification of the "Northern" blot procedure (24) to physically detect transcripts of the L- and M_1-dsRNAs. Denatured total cellular RNA, with and without prior treatment with RNase, was electrophoresed on formaldehyde/agarose gels, transferred to nitrocellulose, and hybridized with the following probes: (A), purified L-dsRNA from strain 3/Al, partially alkaline hydrolyzed, and labelled with $[^{32}P]$ATP using T4 polynucleotide kinase (complementary to any region of either strand of L-dsRNA); (B), M_1-dsRNA, fractionated from K12-1 total dsRNA (16), labelled as for probe A (complementary to any region of M_1-dsRNA); and, (C), a partial cDNA clone of M_1-dsRNA, nick-translated with $[^{32}P]$dCTP. This partial cDNA clone (UIB7) was prepared by oligo(dT)-primed cDNA synthesis of a poly(A)$^+$ fraction of K12-1 total RNA, enriched for M_1-dsRNA transcripts (see below) and cloned by GC tail insertion into the Pst I site of pBR322 (Bostian, Villa-Komaroff and Tipper, unpublished observations). It is about 890 bp in length and should be complementary to any species overlapping the 45% region of M_1-cDNA cloned.

Probe A reacted specifically with l, the 4.5 kb single-stranded form of L-dsRNA, in a denatured mixture of K12-1 L and M_1-dsRNA (Fig. 2A, lane a). Prior to denaturation, the L-dsRNA was resistant to RNase in 400 mM salt but sensitive in 20 mM salt (Fig. 2A, lanes b,c). In both K12-1 and 3/Al total
RNA, probe A detected a strong band with the mobility of full length 1, and a minor band 1a, with a mobility of 2.3 kb (Fig. 2A, lanes d,f). Probe A, and all other dsRNA probes synthesized, bound slightly to the 17S and 26S ribosomal RNA (rRNA) species present in total K12-1 and 3A1 RNAs. The reaction is distinct at this exposure (Fig. 2A, lanes d,f) because the rRNA concentrations are at least 10^4 of that of 1a. They can also be seen in the total RNA from strain 1556 cells which, as expected, contained neither 1 nor 1a species (Fig. 2A, lane h). The rRNAs and 1a were sensitive to RNase in 400 mM salt (Fig. 2A, lanes e,g,i), so were apparently single-stranded, while 1 was at least 95% resistant, so must be derived from L-dsRNA. A trace of full-length 1 was
retarded by poly(U)-sepharose (Fig. 2A, lane k) while most of it and all of the 1a species failed to bind (Fig. 2A, lane j).

The M₁-dsRNA probe (B) reacted specifically with m, the 1.9 kb single-stranded form of M₁-dsRNA, in denatured strain K12-1 L + M₁-dsRNAs (Fig. 2B, lane a). Prior to denaturation, the M₁-dsRNA had the expected resistance to RNase in 400 mM salt and sensitivity in 20 mM salt (Fig. 2B, lanes b,c). The dsRNAs applied to the gel had been electrophoretically purified, and the faint smear of reactive species of high mobility is incompletely denatured M₁-dsRNA. It is RNase resistant in 400 mM salt (Fig. 2B, lanes a and b). Probe B also detected RNase-resistant, full-length m in total K12-1 RNA, together with a fainter band of an RNase-sensitive, 1.2 kb species, mₐ (Fig. 2B, lanes d,e). Both species were absent from 3/A1 and 1556 total RNAs (Fig. 2B, lanes f-i) and while most m failed to bind to poly(U)-Sepharose, all mₐ and an approximately equivalent amount of m did bind (Fig. 2B, lanes j,k). Enrichment of mₐ by poly(U)-Sepharose chromatography enhanced its detectability (Fig. 2B, lane k).

Use of M₁-cDNA probe (C) gave exactly the same result as the M₁-dsRNA probe (B) except that the background due to reaction with rRNA species was absent (Fig. 2C). Thus, clone UIB7 does indeed contain M₁-cDNA. Since all of the M₁-dsRNA transcripts detectable with probe B also reacted with the cDNA probe, they must all overlap substantially the region cloned in UIB7. In addition, it was consistently observed that the ratio of the hybridization signals given by m and mₐ (measured by densitometry of autoradiographs) with the cDNA probe was 2/3 that given with probe (B) (compare lanes k in Fig. 2B,C). Thus, a higher fraction of mₐ than m is complementary to the cDNA probe. Since this ratio is equal to the length ratio of mₐ to m (1.2 to 1.9), all of the cDNA sequence may be contained within mₐ, suggesting that mₐ was the template for cDNA synthesis (see below).

Hybrid-Selected in vivo RNAs include partial and full-length transcripts

Hybrid-selected RNAs were also analyzed by Northern blot hybridization for the presence of L and M₁-dsRNA transcripts. Hybridization with the M₁-cDNA probe C revealed both m and mₐ species, in approximately equal amounts, in hybrid-selected K12-1 total RNA (Fig. 3, lane d). RNA selected from an artificial mixture of 1556 total RNA (99%) and K12-1 dsRNA (1%) contained only a trace of m (Fig. 3, lane e), even though this mixture contained 25 fold more dsRNA than does K12-1 total RNA (Table 1). Thus, almost all of the full-length m detected in K12-1 selected RNA is single stranded, as indicated by its sensitivity, like that of the selected mₐ species, to RNase in 400 mM.
FIGURE 3. Northern Blot Analysis of Hybrid-Selected RNAs

The indicated RNA samples were denatured and analyzed as in Fig. 2, using either L-dsRNA probe (A) or M1-cDNA probe (C). Hybrid-selected (HySt) RNAs were those analyzed in Fig. 1D. The HySt sample in lane f was hydrolyzed with RNase in 400 mM salt prior to denaturation.

<table>
<thead>
<tr>
<th>PROBE RNA (M)(L) (lane)</th>
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<tbody>
<tr>
<td>a g K12-1 total in vivo</td>
<td></td>
</tr>
<tr>
<td>b K12-1 total, poly(U) bound</td>
<td></td>
</tr>
<tr>
<td>h K12-1 total, poly(U) unbound</td>
<td></td>
</tr>
<tr>
<td>c i 1556, total in vivo</td>
<td></td>
</tr>
<tr>
<td>d j,k K12-1 HySt</td>
<td></td>
</tr>
<tr>
<td>e l 1556 total plus K12-1 dsRNA: HySt</td>
<td></td>
</tr>
<tr>
<td>f K12-1 HySt (+ RNase)</td>
<td></td>
</tr>
</tbody>
</table>

Salt prior to denaturation (Fig. 3, lane f). Controls again demonstrated that some m and all m_a in K12-1 total RNA was bound to poly(U)-Sepharose (Fig. 3, lane b) and that 1556 total RNA contained neither (Fig. 3, lanes c).

Detection of single-stranded L species from hybridization-selected K12-1 dsRNA was more difficult because of their low concentration and the ten-fold lower specific activity of the kinase-labeled L-dsRNA probe (A). However, we were able to detect both l and l_a species above a high background (Fig. 3, lanes j,k). The hybridization signal given by l was about 50% of that given by l_a. Controls again demonstrated that l_a failed to bind to poly(U)-Sepharose (Fig. 3, lane h), and that both were absent from strain 1556 RNA (Fig. 3, lane i). The failure to detect these species in the artificial mixture of 1556 total RNA and K12-1 dsRNA (Fig. 3A, lane l) indicated that the selected l is not significantly contaminated by L-dsRNA.

The RNAs selected in this experiment were also translated in vitro and the products analyzed by immunoprecipitation. While RNAs selected from K12-1 total RNA showed production of L-P1, L-P2a, L-P4 and M1-P1 as before (Fig. 1D), no specific products were detectable from the products of translation of the selected artificial mixture of 1556 RNA plus K12-1 dsRNA (data not shown). We believe, therefore, that none of the products seen from selected K12-1 RNA were derived from dsRNA, either in the RNA selected, or from the species bound to the nitrocellulose selective agent.

Fractionation of killer RNAs by poly(U)-Sepharose chromatography

We examined the chromatographic behavior of the dsRNA transcripts on poly(U)-Sepharose, using native total K12-1 RNA and the chromatographic condi-
Figure 4. Fractionation of L-dsRNA and its Transcripts on Poly(U)-Sepharose

Native total RNAs from cells of strain K12-1 were fractionated on poly(U)-Sepharose. Following the flow through (Ft) and wash (Wh) fraction, additional fractions were eluted under loading conditions before elution with 90% formamide (front at fraction 18). Fractions were analyzed by Northern blot hybridization, with and without prior RNase hydrolysis in 400 mM salt, using L-dsRNA probe A, as in Fig. 2. mRNA activities were determined by translation and immunoprecipitation with anti-capsid IgG, as in Fig. 1B. Insert A shows the relevant section of the Northern blot autoradiograms for the 1 and la species. Two lanes are shown for each fraction, without (left) or with (right) RNase treatment. The histogram shows the results of densitometry of the L-P1 band from the fluorogram of translation products (closed bar), and of densitometry of the northern blot for RNase-resistant 1 (open bar, L-dsRNA) and for RNase-sensitive 1 and la (closed bars, full-length and partial-length transcripts, respectively). Insert B shows a control of the 1 species region of a Northern blot autoradiogram of the poly(U)-Sepharose fractions of a mixture of native total in vivo RNA from strain 1556 cells (99%) and total K12-1 dsRNA (1%). Again, two lanes are shown for each fraction, without (left) or with (right) RNase treatment.

The data are presented in Fig. 4 and 5. Most of the dsRNA reproducibly eluted in the flow through and wash fractions of the column with a small portion detectable at
Figure 5. Fractionation of M₁-dsRNA and its Transcripts on Poly(U)-Sepharose

A Northern blot of the same poly(U)-Sepharose fractions analyzed in Fig 4 was probed with M₁-cDNA probe (C) (Fig. 2). Insert A shows the relevant sections of the Northern blot autoradiogram for m and m₃ species. Details as in Fig. 4, except that M₁-P₁ activity (closed bar) was determined by immunoprecipitation of translation products with anti-toxin IgG as in Fig. 1 C. Insert B shows a control of the m species region of a Northern blot autoradiograph of the poly(U)-Sepharose fractions of a mixture of total 1556 RNA and K12-1 dsRNA (as in Fig. 4), probed with M₁-cDNA. Again, two lanes are shown for each fraction, without (left) or with (right) RNase treatment.

the front of the elution buffer (frac. 18). Poly(A) RNA eluted in fractions 22-25. As before, all of the dsRNA species were resistant to RNase in 400 mM salt, and the other RNA species were sensitive (data not shown).

More than 95% of the 1 species eluted in the flow-through (Ft) fraction (Fig. 4A) with only a trace of 1 in the wash (Wh) prior to elution. None of the 1 component in these fractions was detectably sensitive to RNase prior to denaturation and this component was, therefore, L-dsRNA. All of the 1a species eluted in the Ft and Wh fractions was sensitive to RNase in 400 mM salt and was, therefore, single-stranded. A residual amount of RNase-resistant 1 was also found in fraction 18. Small amounts of 1 eluted in fractions 22-25,
together with the poly(A) RNA and most of it was RNase sensitive and, therefore, single-stranded. In a control experiment utilizing the mixture of 1556 total RNA and K12-1 dsRNA, L-dsRNA behaved like that in K12-1 total RNA: 95% was found in the flow-through, 5% in fraction 18, and traces only in the fractions 20-26 containing poly(A)$^+$ RNA (Fig. 4, insert B).

The ratio of L-P1 mRNA activity to single-stranded l content in fractions 18-25 was constant and equal to the ratio of L-P1 mRNA activity to l$^a$ content in the Ft and Wh fractions (Fig. 4). Since fractions 22-25 were free of detectable l$^a$, and the Wh fraction was free of detectable l, both l and l$^a$ are plus strand transcripts, encoding L-P1 with similar in vitro mRNA efficiencies. l$^a$ is not polyadenylated. Detection of l in the Ft fraction was not possible because of the large amounts of l present, but the data suggest that all L-P1 mRNA activity in this fraction was derived from l$^a$. All single-stranded l detected is bound to poly(U)-Sepharose and appears, therefore, to be polyadenylated.

The same poly(U)-Sepharose fractions analyzed for L-dsRNA transcripts and L-P1 mRNA activity (Fig. 4) were assayed for M$_1$-dsRNA transcripts and M$_1$-P1 mRNA activity (Fig. 5). 95% of m eluted in the flow through (Ft) fraction and fraction 18 and all of this was RNase resistant and was, therefore, M$_1$-dsRNA.

Significant amounts of RNase-resistant M$_1$-dsRNA eluted with RNase-sensitive (single-stranded) m and m$^a$ species, which peaked in fractions 22-24, a little after the peak of total polyadenylated RNA. Fractionation of the artificial mixture of 1556 total RNA and K12-1 dsRNA gave the same result (Fig. 5 insert B). Moreover, when the bound fractions derived from this artificial mixture were refractionated on poly(U)-Sepharose, the bulk of the RNA was still bound, but the small amount of M$_1$-dsRNA accompanying it was now found in the flow through fraction. Conversely, refractionation of the originally un-bound fraction resulted in a small amount of M$_1$-dsRNA adhering to the column, eluting with polyadenylated species (not shown). We believe, therefore, that bound M$_1$-dsRNA is a non-specifically bound fraction of the total M$_1$-dsRNA.

M$_1$-P1 mRNA activity relative to single stranded m plus m$^a$ content varied twenty-fold between fractions 24 and 26, in inverse ratio to the concentration of total poly(A)$^+$ RNA (Fig. 5). The lack of correlation between M$_1$-dsRNA transcript concentration and M$_1$-P1 mRNA activity in these fractions is probably due to an inhibitory effect of total RNA on this mRNA activity, since addition of RNA from cells of strain 1556 caused a similar inhibition (data not shown), as did rRNA (see below). Fractionation of total K12-1 RNA by thermal elution poly(U)-Sepharose chromatography avoided this inhibition (Fig.
Figure 6. Thermal Elution of $M_1$-dsRNA Transcripts from Poly(U)-Sepharose in 25% Formamide

Native total RNAs from cells of strain K12-1 were fractionated by thermal elution poly(U)-Sepharose chromatography with 25% formamide at the indicated temperatures. Individual fractions, with or without prior RNase hydrolysis in 400 mM salt, were analyzed by Northern blot hybridization, using the $M_1$-cDNA probe (C), as in Fig. 2. The insert shows the $m$ and $m_a$ region of the Northern blot autoradiogram. Lanes on left (20°-60°) are fractions without RNase treatment, and lanes on right with RNase treatment. The histogram shows concentrations of RNase-resistant $m$ (open bar, $M_1$-dsRNA), RNase-sensitive $m$ and $m_a$ (closed bars, full-length and partial-length transcripts, respectively), and $M_1$-P1 mRNA activity, determined as in Fig. 5, and presented on the same scale.

b), since both the $m$ and $m_a$ species were separated from the bulk of poly(A)$^+$ RNA. $M_1$-P1 mRNA activity was now proportional to single-stranded $m$ plus $m_a$ content (Fig. 6). This procedure has been used to size poly(A) tracts in poly(A)-containing RNA (22). The bulk of $M_1$- and L-dsRNAs again eluted in the flow through fraction (data not shown), while a small amount of $M_1$-dsRNA eluted together with the bound, RNase-sensitive (single-stranded) $m$ and $m_a$ species (Fig. 6). These peaked in the 45° to 60°C fractions, substantially after the bulk of polyadenylated RNA, which eluted in the 30° to 45°C fractions (data not shown) as previously described for yeast mRNA (21). These
Native total RNAs from cells of strain K12-1 were electrophoretically fractionated and assayed for $M_1$-dsRNA transcripts $m$ and $m_a$, by Northern blot analysis as in Fig. 2, using the $M_1$-cDNA probe (C).

(A) Concentrations of $m$ (▲) and $m_a$ (●) and $M_1$-P1 mRNA activity (■) were determined as in Fig. 6.

(B) The absorbancy profile at 254 nm ($A_{254}$) is shown for the same fractions, illustrating the elution positions of the peaks of 17S and 26S rRNA species.

Elution positions correspond to poly(A) sequence lengths of 30 to 70 for the bulk of the bound RNA, and of 70 to 100 for the $m$ and $m_a$ species (22). $m_a$, enriched by preparative gel electrophoresis, has $M_1$-P1 mRNA activity.

The $m$ and $m_a$ species in native total K12-1 RNA were fractionated by agarose gel electrophoresis (27). Electrophoresis under non-denaturing conditions resulted in elution of individual RNA species as very broad bands, presumably due to aggregation. Nevertheless, complete separation of $m_a$ from later eluting $m$ and $M_1$-dsRNA species occurred, and the fraction where $m_a$ first appeared contained RNA of the expected 1.2 kb size (Fig. 7). $M_1$-P1 mRNA activity in these fractions paralleled $m_a$ content, except where the relatively massive amounts of co-eluted ribosomal RNA inhibited translation (Fig. 7). Since $m_a$ has $M_1$-P1 mRNA activity, this transcript must cover the ca 890 bp $M_1$-P1 gene.

Estimation of killer dsRNA transcript concentration

From the data of Fig. 4 and 5 (and similar experiments), we have estimated the relative concentrations of dsRNAs and their transcripts in K12-1 cells. Concentrations and relative molarities were calculated from previously derived data for total dsRNA concentration (Table 1). Assuming that all species react equally with the dsRNA probes, the averaged data represent relative
concentrations by mass. Because of the enhanced signal given by \( m_a \) with the cDNA probe (C), \( m_a \) concentrations measured using this probe were corrected by a factor of 2/3 (see above) (Table 1). The derived relative concentrations of 1 to \( l_a \) and of \( m \) to \( m_a \) are consistent with the relative hybridization signals seen after hybrid selection (Fig. 3), assuming that selection was equally efficient for each species. Concentrations of dsRNA transcripts are about 1000 fold lower than the mRNA concentrations for glycolytic enzymes (28). While the mass ratio of \( m \) to 1 was close to 2, the ratio of concentrations of these species, relative to their dsRNA templates, was 7. The relatively low \( 1 \) and \( l_a \) concentrations suggests that control of these concentrations, by transcription rate or stability, may be rate limiting for L-P1 and VLP production and expression of the killer phenotype.

**DISCUSSION**

Because of the composition of probe A and the low concentrations of 1 and \( l_a \) (Table 1), it is probable that both species are transcripts of \( L_A \) and that transcripts of minor \( L \) species were not detected. This is consistent with the provisional identification of \( L_A \) as the species encoding L-P1 (11) and the L-P1 mRNA activities of both 1 and \( l_a \). Confirmation will require the use of cDNA probes for the different \( L \) species. The virtual identity of the results obtained with probes B and C are consistent with the existence of a single \( M_1 \) species. Since the \( m_a \), \( l_a \), 1 and possibly also the \( m \) species detected here have mRNA activity, they are, by definition, plus-stranded. If cells contained compartmentalized complementary single-stranded dsRNA transcripts, the majority, at least, of these would reanneal during isolation of total cellular RNA. Only the excess plus or minus strand would be isolated as single stranded RNA. While our data do not, therefore, eliminate the possible existence of lower concentrations of minus-stranded transcripts in vivo, the plus-strandedness of the killer RNA transcripts detected is consistent with their being products of the transcriptase activity detected in VLPs (4,5,6), and is consistent with a reovirus-like replication mechanism (7) with temporal separation of plus and minus strand synthesis. Transcription of both \( L \) and \( M_1 \)-dsRNAs initiates in VLP preparations at AU-rich termini which share 5 and 7 base sequences presumed to be the transcription recognition signal (32, 35). The termini of \( M_1 \) and \( L \)-dsRNAs distal from the site of transcription initiation are both GC rich (32), but lack significant homology. This implies the involvement of different recognition sequences and proteins in the initiation of plus and minus strand synthesis, also consistent with a reo-virus-like
mechanism. It is not known whether these proteins, other than L-P1 capsid, are encoded by L species or by nuclear genes such as MAK 3 and 10(11). Since 1ₐ encodes L-P1 and mₐ encodes M₁-P1, they may be responsible for expression of these dsRNA gene products in vivo. It is also possible that both full and partial-length dsRNA transcripts serve as translation templates in vivo. Different secondary structures or protein binding properties could expose different ribosome binding sites, leading to differential expression of common sequences. For example, this may lead to the production of an as yet unidentified M₁-dsRNA gene product that is the determinant of resistance to toxin. Alternatively, it is possible that capsid protein binding or secondary structure prevents translation of m and 1 in vivo and that m and 1 are solely replicative intermediates. If so, their transcription rates and half-lives are sufficiently long for their concentrations to be 14 and 2%, respectively, of their dsRNA templates (Table 1), implying that initiation of negative-strand replication on these templates may be rate-limiting for dsRNA synthesis. The hybrid-selected mixture of 1 and 1ₐ transcripts of L-dsRNA had mRNA activity for L-P1 capsid and apparently for L-P2 and L-P3 also (unpublished observations). While 1ₐ is barely large enough to encode L-P1, it could easily encode L-P2 or P3. If 1ₐ is a 5' terminal fragment of 1, then the L-P1 gene must initiate close to this 5' terminus. Since 1ₐ is approximately half the size of 1, it is potentially a mixture of two half transcripts encoding two different proteins. It will be important to investigate the relationship between L-P1, P2 and P3, ScV-P1, P2 and P3 and the different L-dsRNA species.

The 1.2 kb mₐ transcript is probably a fragment of a plus-stranded, full-length m transcript covering the 0.89 kb M₄-P1 gene. Hypothetical models for the structure of M₁-dsRNA are shown in Fig. 8. Since the M₁-P1 gene lies within the 1 kb S1 nuclease fragment of M₄-dsRNA (18), it seems likely that mₐ represents a 5' terminal fragment of m, initiating at the normal terminal site and terminating at the AU-rich "bubble". mₐ could be produced by cleavage of m, possibly followed by polyadenylation, or alternatively, VLP transcriptase may "slip" or "chatter" at an oligo U region at the start of the denaturation bubble, producing poly(A) and eventually leading to termination of transcription, essentially as postulated for VSV (36). The upper and lower models predict lengths for ma (including a 90 bp poly(A) tail) of 1.25 and 1.15 kb, respectively, either consistent with our observations.

All of the mₐ and most of the m species bind to poly(U)-Sepharose with an affinity corresponding to a poly(A) length of about 90, well above the
Fig. 8. Models of $M_1$-dsRNA

Two alternates are presented. In each, sequence A is the 5' untranslated region of the plus (upper) strand transcript; B is the $M_1$-P1 gene with the functional sequence Leader-Toxin-Resistance determinant (1.6, 11.7 and 18.7 K daltons of protein, 50,320 and 500 bp RNA, respectively) (17); C plus D is the 3' untranslated region. $M_1$-dsRNA (1850 bp) contains a 190 bp AU-rich denaturation "bubble" sequence (29) which is removed by S1 nuclease leaving 1000 and 600 bp dsRNA fragments (18). The AU-rich plus strand transcription initiation site (32,35) and the $M_1$-P1 gene lie in the 1000 bp fragment (18). Plasmid S3 is derived from $M_1$ by an internal deletion which encompasses the "bubble" and leaves 500 and 220 bp terminal fragments (29). Translation of S3, presumably initiating at the normal ribosome binding site for $M_1$-P1, produces S-P1, an 8K dalton polypeptide (220 bp) including the leader of $M_1$-P1, and an N-terminal antigenic fragment of the toxin (17). S-P1 presumably terminates at the first in phase stop codon beyond the deletion, placing the A/B junction no more than 220 bp upstream of the deletion. It is not known whether the A/B junction lies in the 500 bp or 220 bp fragment of $M_1$ in S3, so both alternatives are shown. Segment sizes, based on the information summarized above, are only approximations. In order to fit the S1 nuclease fragment sizes (28), the bubble sequence, where transcription of $m_1$ is postulated to terminate (see text), is placed at the end of the $M_1$-P1 gene in the upper model and 175 bp downstream in the lower. The 5' end of the plus strand of $M_1$-dsRNA contains a probable ribosome binding site at bases 7-12, an AUG at 11-16 and an open reading frame to the end of the 175 bp region sequenced (30). This fits the lower model well ($A = 13$ bp) and is consistent with the known proximity of translation initiation sites in reovirus mRNA's to their 5' termini (31).

The terminal regions of $M_1$ are retained in all suppressive deletion mutants (29,32) and are assumed to contain sequences essential for initiation of transcription (region A) and replication (region D). Nucleation of capsid binding presumably accompanies nascent dsRNA synthesis in region D, which is also assumed to contain regions for the control of $M_1$ replication by MAK (13) and SK1 (33) gene products. This region, therefore, is also likely to be the site of plasmid mutants (KIL-, sd, b, d) (4,15) affecting the control of $M_1$-dsRNA maintenance. Sites for the processing of $M_1$-P1 and of nuclear mutations (kex, sec) affecting this processing (17) are also indicated, as are the sites of the neutral (KIL-n) and pH-suicide (KIL-pH) plasmid mutations (34) which are hypothesized to affect the toxin and resistance determinant peptide sequences of $M_1$-P1, respectively.
average for yeast mRNA, and an oligo (dT)-primed cDNA was derived from this poly(U)-bound mixture. No oligo(A) region longer than about 8 has been detected in \( M_1 \)-dsRNA (37), even though the low melting temperature of the internal 190 bp "bubble" suggests a very high AU content (29). While binding of \( m \) to poly(U)-Sepharose might be attributable to the transcribed bubble sequence, we have been unable to test this by fractionation of denatured \( M_1 \)-dsRNA, since it reanneals too rapidly under the conditions employed for poly(U)-Sepharose chromatography. Binding of \( m_a \) to the column could only be due to the bubble sequence if it were included in this transcript. If multiple, short oligo(A) regions are responsible for the very tight binding to poly(U) observed, the same oligo(A) regions could act as minimally efficient templates for oligo(dT)-primed cDNA synthesis from either \( m \) or \( m_a \). Post-transcriptional polyadenylation of these transcripts is a more conventional explanation of both findings. If \( m_a \) is polyadenylated, then synthesis of \( l_a \) must differ, since \( l_a \) is clearly not polyadenylated. L-dsRNA contains no denaturation bubble (18,29) and it seems likely, therefore, that \( l \) is polyadenylated. Using liquid phase hybridization between denatured, labelled, in vivo RNA and denatured L, Haylock and Bevan (38) found approximately full-length species accounting for about 0.15% of non-polyadenylated polysomal RNA. No translational analysis was reported and its relationship to the L-transcripts observed in this study is unknown. Further structural analysis of our dsRNA transcripts will clearly be necessary.

A discrete class of yeast transcripts with a poly(A) size of about 90 is found in yeast (20) and since the killer transcripts are presumably polyadenylated in the cytoplasm, this size class may be characteristic of such products. If \( l \) and \( m \) are replication intermediates, adenylation and deadenylation may be steps in the dsRNA replication cycle. These might not only account for the unpaired 3' terminal A residues found in mature L and \( M_1 \) dsRNAs (32), but also provide targets for control of \( M_1 \)-dsRNA replication by the products of \( MAK \) genes. These products and those of the \( SKI \) genes, defects in which serve to suppress some \( mak \) phenotype (33), might serve cellular functions in controlling the poly(A) lengths of yeast transcripts.

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