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_a, m_a) single-stranded species. Both L-dsRNA transcripts (l, l_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 M₁-dsRNAs. l , but not l_a , is bound to poly(U)-sepharose and may be polyadenylated. Other L-dsRNA gene products and their transcripts may exist. For M₁-dsRNA, both species (m, m_a) have in vitro mRNA activity for M₁-P1, the 32,000 dalton pre-prototoxin encoded by M₁-dsRNA. Both m and m_a are bound to poly(U)-Sephareose and m_a is probably a 5' terminal fragment of m . A functional model for M₁-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_1^+ phenotype) and of resistance to toxin (R_1^+ phenotype) is a cytoplasmically inherited 1.9 kb double-stranded (ds)RNA plasmid (M_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-M₁ 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 M₁ 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 (L_A) and a heat-stable minor species (L_B or L_C) (10,11,12). L_A, L_B, and L_C can exist independently and L_A 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 L_B or L_C (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 L_A (11) and none are required for L_B or L_C. Thus replication of ScV-M₁, ScV-L_A and ScV-L_{B/C} 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/A1 (α K^-R^-) were obtained from E.A. Bevan (Queen Mary College, London, England). The dsRNA-free strain 1556 (a ade mal6, cyh^r [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 μ g/ml bed volume) (16, 19), by poly(U)-Sephacrose chromatography (0.6 mg RNA/ml bed volume) (20,21) and by thermal elution poly(U)-Sephacrose 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 M_1 -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 μ g of RNA in a total volume of 40 μ l. The phenol phases were re-extracted twice to ensure quantitative recovery of nucleic acids, and yeast tRNA was added to 50 μ g/ μ l 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 [35 S] 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). Immunocompetition assays were as described elsewhere (16).

Hybridization selection

RNAs complementary to L- and M_1 -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 [32 P]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 μ g dsRNA) were at 37°C for 45 min in 100 μ l

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 L_A (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 M ₁ (K12-1)	"
g	Purified L ₁ (3/A1)	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			IgG
K12-1 (lane)	3/A1 (lane)	1556 (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			IgG
K12-1 (lane)	3/A1 (lane)	1556 (lane)	
a	d	g	pre-immune
b	e	h	anti-toxin
c	f	-	anti-toxin+Ag

(D) Native total RNAs from strain 3/A1 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		IgG
3/A1 (lane)	K12-1 (lane)	3/A1 (lane)	K12-1 (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

Species	% Mass	Relative Molar Ratios	
L	0.03	100	-
l	0.0003	2	-
l _a	0.0007	9	-
M ₁	0.0090	69	100
m	0.00061	9	14
ma	0.00047	11	17

Data for % of total cellular RNA (% mass) are derived from steady-state incorporation levels of [³²P]-PO₄ for L- and M₁-dsRNAs. Data for the transcripts (l, l_a, m, m_a) are derived from hybridization signals, relative to L and M₁, 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; M₁, 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 M₁-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 M₁-P1 (Fig. 1D, lane n).

Physical detection of partial transcripts of M₁- and L-dsRNA (Fig. 2)

We used a modification of the "Northern" blot procedure (24) to physically detect transcripts of the L- and M₁-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/A1, partially alkaline hydrolyzed, and labelled with [³²P]ATP using T4 polynucleotidyl kinase (complementary to any region of either strand of L-dsRNA); (B), M₁-dsRNA, fractionated from K12-1 total dsRNA (16), labelled as for probe A (complementary to any region of M₁-dsRNA); and, (C), a partial cDNA clone of M₁-dsRNA, nick-translated with [³²P]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 M₁-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 M₁-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 M₁-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/A1 total

Figure 2. Northern Blot Analysis of Denatured Total in vivo RNAs

Total dsRNA (lanes a-c), or total cellular RNA (lanes d-h) from cells of the indicated strains, with or without prior hydrolysis with RNase at the indicated salt concentrations, were denatured, fractionated by 1.5% agarose/formaldehyde gel electrophoresis, blotted onto nitrocellulose and probed as indicated. Samples in lanes j (unbound) and k (bound) were K12-1 total RNA fractionated on poly(U)-Sepharose prior to denaturation.

Probe	Lane	Strain	RNase	Salt
(A) kinase-labeled L-dsRNA from strain 3A/1	a	K12-1	0	
	b	K12-1	+	400 mM
	c	K12-1	+	20 mM
(B) kinase-labeled M ₁ -dsRNA from strain K12-1 (containing about 2% residual L-dsRNA)	d	K12-1	0	
	e	K12-1	+	400 mM
	f	3A/1	0	
	g	3A/1	+	400 mM
(C) nick-translated M ₁ -cDNA from clone UIB7	h	1556	0	
	i	1556	+	400 mM

RNA, probe A detected a strong band with the mobility of full length l, and a minor band la, 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 3A/1 RNAs. The reaction is distinct at this exposure (Fig. 2A, lanes d,f) because the rRNA concentrations are at least 10⁴ of that of la. They can also be seen in the total RNA from strain 1556 cells which, as expected, contained neither l nor la species (Fig. 2A, lane h). The rRNAs and la were sensitive to RNase in 400 mM salt (Fig. 2A, lanes e,g,i), so were apparently single-stranded, while l was at least 95% resistant, so must be derived from L-dsRNA. A trace of full-length l 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 M_1 -dsRNA probe (B) reacted specifically with m, the 1.9 kb single-stranded form of M_1 -dsRNA, in denatured strain K12-1 L + M_1 -dsRNAs (Fig. 2B, lane a). Prior to denaturation, the M_1 -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 M_1 -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, m_a (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)-Sephadex, all m_a and an approximately equivalent amount of m did bind (Fig. 2B, lanes j,k). Enrichment of m_a by poly(U)-Sephadex chromatography enhanced its detectability (Fig. 2B, lane k).

Use of M_1 -cDNA probe (C) gave exactly the same result as the M_1 -dsRNA probe (B) except that the background due to reaction with rRNA species was absent (Fig. 2C). Thus, clone UIB7 does indeed contain M_1 -cDNA. Since all of the M_1 -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 m_a (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 m_a than m is complementary to the cDNA probe. Since this ratio is equal to the length ratio of m_a to m (1.2 to 1.9), all of the cDNA sequence may be contained within m_a , suggesting that m_a 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 M_1 -dsRNA transcripts. Hybridization with the M_1 -cDNA probe C revealed both m and m_a 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 m_a 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.

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

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 by to poly(U)-Seph-
arose (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)-Seph-
arose (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)-Seph- arose chromatography

We examined the chromatographic behavior of the dsRNA transcripts on poly(U)-Seph-
arose, using native total K12-1 RNA and the chromatographic condi-

Figure 4. Fractionation of L-dsRNA and its Transcripts on Poly(U)-Sephadex

Native total RNAs from cells of strain K12-1 were fractionated on poly(U)-Sephadex. 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 l and l_a 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 l (open bar, L-dsRNA) and for RNase-sensitive l and l_a (closed bars, full-length and partial-length transcripts, respectively). Insert B shows a control of the l species region of a Northern blot autoradiogram of the poly(U)-Sephadex 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.

tions normally employed for the purification of poly(A) RNA. 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 M_1 -dsRNA and its Transcripts on Poly(U)-Sephadex

A Northern blot of the same poly(U)-Sephadex fractions analyzed in Fig. 4 was probed with M_1 -cDNA probe (C) (Fig. 2). Insert A shows the relevant sections of the Northern blot autoradiogram for *m* and *m_a* species. Details as in Fig. 4, except that M_1 -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 autoradiogram of the poly(U)-Sephadex fractions of a mixture of total 1556 RNA and K12-1 dsRNA (as in Fig. 4), probed with M_1 -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 *l* species eluted in the flow-through (Ft) fraction (Fig. 4A) with only a trace of *l* in the wash (Wh) prior to elution. None of the *l* component in these fractions was detectably sensitive to RNase prior to denaturation and this component was, therefore, L-dsRNA. All of the *la* 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 *l* was also found in fraction 18. Small amounts of *l* 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 a polyadenylated.

The same poly(U)-Sepharose fractions analyzed for L-dsRNA transcripts and L-P1 mRNA activity (Fig. 4) were assayed for M₁-dsRNA transcripts and M₁-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, M₁-dsRNA.

Significant amounts of RNase-resistant M₁-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 M₁-dsRNA accompanying it was now found in the flow through fraction. Conversely, refractionation of the originally unbound fraction resulted in a small amount of M₁-dsRNA adhering to the column, eluting with polyadenylated species (not shown). We believe, therefore, that bound M₁-dsRNA is a non-specifically bound fraction of the total M₁-dsRNA.

M₁-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 M₁-dsRNA transcript concentration and M₁-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)-Sephacrose in 25% Formamide

Native total RNAs from cells of strain K12-1 were fractionated by thermal elution poly(U)-Sephacrose 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

Figure 7. Preparative Gel Electrophoresis of Native Total RNA from Strain K12-1

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 (Δ) and m_a ($\bullet$) and M_1 -P1 mRNA activity ($\blacksquare$) 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 nondenaturing 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 l 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 l was close to 2, the ratio of concentrations of these species, relative to their dsRNA templates, was 7. The relatively low l 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 l 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 l 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 , l 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 l_a encodes L-P1 and m_a encodes M_1 -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 M_1 -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 l in vivo and that m and l 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 l and l_a transcripts of L-dsRNA had mRNA activity for L-P1 capsid and apparently for L-P2 and L-P3 also (unpublished observations). While l_a is barely large enough to encode L-P1, it could easily encode L-P2 or P3. If l_a is a 5' terminal fragment of l , then the L-P1 gene must initiate close to this 5' terminus. Since l_a is approximately half the size of l , 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 m_a transcript is probably a fragment of a plus-stranded, full-length m transcript covering the 0.89 kb M_1 -P1 gene. Hypothetical models for the structure of M_1 -dsRNA are shown in Fig. 8. Since the M_1 -P1 gene lies within the 1 kb S1 nuclease fragment of M_1 -dsRNA (18), it seems likely that m_a represents a 5' terminal fragment of m , initiating at the normal terminal site and terminating at the AU-rich "bubble". m_a 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 m_a (including a 90 bp poly(A) tail) of 1.25 and 1.15 kb, respectively, either consistent with our observations.

All of the m_a 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 M₁-dsRNA

Two alternatives are presented. In each, sequence A is the 5' untranslated region of the plus (upper) strand transcript; B is the M₁-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. M₁-dsRNA (1850 bp) contains a 190 bp AU-rich "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 M₁-P1 gene lie in the 1000 bp fragment (18). Plasmid S3 is derived from M₁ 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 M₁-P1, produces S-P1, an 8K dalton polypeptide (220 bp) including the leader of M₁-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 M₁ 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 m₂ is postulated to terminate (see text), is placed at the end of the M₁-P1 gene in the upper model and 175 bp downstream in the lower. The 5' end of the plus strand of M₁-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 M₁ 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 M₁ replication by MAK (13) and SKI (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 M₁-dsRNA maintenance. Sites for the processing of M₁-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 M₁-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)-Sephacrose 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)-Sephacrose 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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