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Virion DNA - independent RNA polymerase from Saccharomyces cerevisiae

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ABSTRACT

The "killer" plasmid and a larger double-stranded RNA plasmid of yeast exist in intracellular virion particles. Purification of these particles from a diploid killer strain of yeast (grown into stationary growth on ethanol) resulted in co-purification of a DNA-independent RNA polymerase activity. This activity incorporates and requires all four ribonucleoside triphosphates and will not act on deoxyribonucleoside triphosphates. The reaction requires magnesium, is inhibited by sulfhydryl-oxidizing reagents and high concentrations of monovalent cation, but is insensitive to DNase, α-amanitin, and actinomycin D. Pyrophosphate inhibits the reaction as does ethidium bromide. Exogenous nucleic acids have no effect on the reaction. The product is mostly single-stranded RNA, some of which is released from the enzymatically active virions.

INTRODUCTION

Killer strains of Saccharomyces cerevisiae are those which contain the cytoplasmically-inherited killer plasmid; these strains produce a toxin which specifically kills strains not bearing this plasmid.[1-4] Killer strains contain two major species of double-stranded RNA of varying reported molecular weights. These are denoted M (1.10-1.7 x 10^6 daltons) and L (2.5-3.5 x 10^6 daltons)[5-9] both of which appear to be encapsulated in spherical 160S cytoplasmic virus-like particles.[10,11] The M species of double-stranded RNA appears to be the killer plasmid, while the biological function of the other RNA species is uncertain.[5,6] However, the finding in a wheat germ cell-free protein-synthesizing system that denatured L RNA codes for a protein similar to the major capsid polypeptide present in all the yeast virus-like particles suggests that this capsid protein may be encoded on this RNA species.[12]

Genetic studies indicate that at least 28 chromosomal genes are required for the maintenance of the killer plasmid. These genes include pet18,[7,13,14] 26 mak genes [7,15-18] and spe2.[19] Mutants in none of these genes are com-

pletely defective in the maintenance of L double-stranded RNA, although mak3-1 mutants have decreased amounts of this species.[16] Two chromosomal genes, kex1 and kex2, are also required for toxin production in strains bearing the killer plasmid,[15,20] and at least one more gene, rex1, is required for expression of the resistance phenotype of killer strains.[15] The biochemical function of these host genes in relation to the encapsulated double-stranded RNA species remains unknown.

The role of virion RNA polymerases in the replication and transcription of the genomes of double-stranded RNA viruses has recently been reviewed.[21-23] Virion RNA polymerases have been described in the Reoviridae, including reovirus,[24,25] wound tumor virus,[26] cytoplasmic polyhedrosis virus,[27] bluetongue virus[28,29] and rotavirus,[30] as well as in the double-stranded RNA-containing bacteriophage ϕ6.[31,32] Intracellular double-stranded RNA virions occur in many fungal species.[33] RNA polymerase activity has been detected in the viruses of Penicillium stoloniferum,[34-38] Penicillium chrysogenum,[39] Aspergillus foetidus[40,41] and Allomyces arbuscula.[42] In non-killer strains of Saccharomyces cerevisiae (which lack M double-stranded RNA), virions containing the L species were found to catalyze the incorporation of isotope from [^{3}H]-UTP into molecules with some properties of double-stranded RNA and into single-stranded RNA which could hybridize with denatured L double-stranded RNA.[43,44] In the present work, purification of virions from a killer strain of yeast is shown to result in co-purification of a DNA-independent RNA polymerase. This paper describes the RNA polymerase activity of virions purified from killer strains grown into stationary phase on ethanol. This is the first demonstration that such an activity is purified along with such virions, and that it has properties distinguishing it from other yeast nucleotide-incorporating activities. The single-stranded RNA products of the reaction are described in the following paper.

MATERIALS AND METHODS

Materials. All isotopes were from the New England Nuclear Corp., and included [2,8-^{3}H]-ATP, [5-^{3}H]-CTP, [8-^{3}H]-GTP, [5,6-^{3}H]-UTP, [2-^{14}C]-UTP, and [methyl-^{3}H]-deoxyTTP. Pancreatic ribonuclease and deoxyribonuclease (ribonuclease-free) were from Worthington Biochemical Corp. and nucleoside triphosphates from P-L Biochemicals. Bentonite was from Fisher Scientific Company and prepared as described.[45] Polyethylene glycol (molecular weight 6000) was from Sigma and sodium dextran sulfate (molecular weight 500,000) was from

Pharmacia. All other chemicals were of analytical grade. Buffer A is 50 mM Tris-HCl (pH 7.5), 10 mM $MgSO_4$, 1 mM dithiothreitol, and 0.1 mM EDTA. Buffer G is Buffer A containing 0.15 M NaCl, 0.39 M KCl, and 20% (v/v) glycerol. All reagents were sterilized prior to use by autoclaving or filtration through Millipore filters.

Yeast cells. The diploid killer strain A364A x S7 (a/α adel/+ ade2/+ ural/+ tyrl/+ his7/+ lys2/+ gall/+ [KIL-k]) was grown for 5 days at 28°C in medium containing 1% yeast extract, 2% peptone, 5% ethanol, and was in late stationary growth phase at this time. These growth conditions have been reported to maximize the amount of double-stranded RNA found in non-killer yeast cells.[46]

RNA polymerase assay. Reaction mixtures included 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 20 mM NaCl, 5mM KCl, 0.5 mM each of ATP, CTP and GTP, 20 μM [^{3}H]-UTP (1.06 Ci/mmole) and varying quantities of virions. After incubation at 30°C, reactions were stopped by placing 50 μl onto 2 cm squares marked on a Whatman GF/C glass fiber filter (12.5 cm diameter) presoaked in 10% TCA containing 10 mM sodium pyrophosphate. Filters were then washed twice with 50 ml of cold 10% TCA, 3 times with 50 ml cold water, and once with 20 ml of ethanol, after which they were air dried and radioactivity was measured in a liquid scintillation counter in toluene containing 2,5-diphenyloxazole (4 g/l) and p-bis-[2-(5-phenyloxazoyl)] benzene (50 mg/l). One unit of activity is defined as that amount of enzyme catalyzing the incorporation of one nmole of UMP per minute under these conditions. Protein was assayed as described.[47]

Electrophoretic analysis of RNA. Virion RNA was solubilized by suspension in 0.1M EDTA, 1% sarkosyl containing bentonite (0.1 mg/ml) followed by vortexing with two volumes of water-saturated phenol. RNA in the aqueous phase was subsequently precipitated with 66% ethanol at -20°C and was analyzed by polyacrylamide gel electrophoresis as previously described,[7,19] followed by staining with ethidium bromide (0.5 μg/ml) and photography by ultraviolet-fluorescence.

RESULTS AND DISCUSSION

Purification of virions. A typical purification is described here. All operations were performed at 4°C. Cells (58g) were collected by centrifugation from 4 l of medium, washed in 100 ml of Buffer A containing 0.15 M NaCl, suspended in 58 ml of this buffer, and were lysed in a French Pressure Cell (14,000 psi, one passage). Bentonite (50 mg) was then added, debris was

TABLE 1. Purification of Virions with RNA Polymerase Activity

Fraction	Volume (ml)	Protein (mg)	Activity (U)	Specific Activity (U/mg)
S_{16}	70	5117	10.22	.00200
P_{133}	71	4225	8.64	.00205
PEG	65.9	386	6.70	.0174
KCl	38.7	42.2	6.87	.163
Sucrose	2.7	8.91	2.69	.302

removed by centrifugation (16,000 x g, 30 min) to produce the S_{16} fraction, and virions were collected by centrifugation at 133,000 x g for 90 minutes. The pellet was homogenized in Buffer A containing 0.5 M NaCl (P_{133} fraction) and virions were precipitated in the presence of 4% (w/w) polyethylene glycol for one hour with stirring. After centrifugation (12,000 x g, 10 min) the precipitate was homogenized in Buffer A containing 0.6 M NaCl (PEG fraction). Polyethylene glycol (6.5%w/w) and sodium dextran sulfate (0.2% w/w) were then added. After one hour of stirring and centrifugation (12,000 x g, 10 min), the dextran phase (pellet) was homogenized in 60 ml of Buffer A containing 0.15 M NaCl and 0.15 volume of 3 M KCl was added dropwise while stirring. After 1 hour precipitated dextran and ribosomal contaminants were removed by centrifugation (12,000 x g, 10 min) and the supernatant was dialyzed overnight against 2 l of Buffer G. Virions were then pelleted at 133,000 x g for 90 minutes and were resuspended in Buffer G (KCl fraction). Aliquots (1.5 ml) were then subjected to centrifugation on 35 ml 5-20% sucrose gradients (in Buffer G) at 24,000 rpm for 6 hours in an SW 27 rotor (Beckman). Fractions (1.5 ml) were collected from below and assayed for RNA polymerase. Active fractions (14-17) were pooled, dialyzed overnight against 2 l of Buffer G and were concentrated by ultracentrifugation and resuspension in 2.7 ml Buffer G (Sucrose fraction). The final preparation of virions represented a 151-fold purification of the polymerase activity from the S_{16} with a recovery of 26%.

Figure 1 shows the sedimentation of the polymerase activity in the above purification.

Figure 2 shows a slab gel of RNA extracted from each fraction of the sucrose gradient shown in Figure 1. This electrophoresis was run long enough so that ribosomal RNA, if present, would have run off the gel.

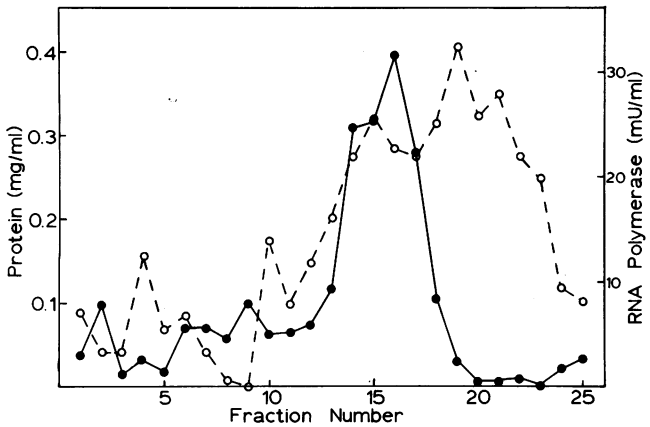

Figure 1. Purification of the virion RNA polymerase activity by sucrose gradient centrifugation. RNA polymerase activity (●) and protein (o) are indicated. In a parallel gradient S. cerevisiae ribosomes (80S) sedimented to fraction number 20. Fraction number 1 is the bottom fraction.

However, gels run for shorter times failed to reveal the presence of ribosomal RNA in the active fractions. Although some double-stranded RNA was seen in all gradient fractions, the highest levels were present in the

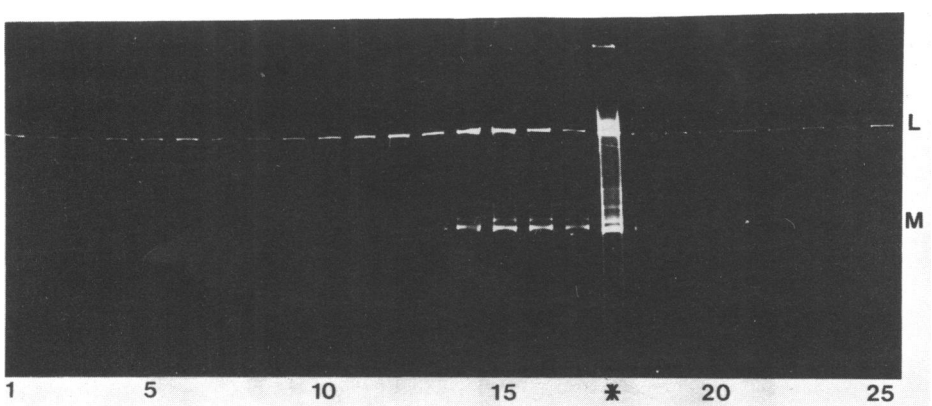

Figure 2. Polyacrylamide gel electrophoresis of RNA from sucrose gradient fractions. RNA was extracted from 0.3 ml of each indicated fraction and 5 µl (of 50 µl) was subjected to electrophresis for 13 hours as described in the text. L and M double-stranded RNA are indicated. Asterisk indicates double-stranded RNA isolated from the same strain.

active fractions (14-17). As previously noted,[11] the more rapidly sedimenting side of the activity peak was relatively enriched for the L species of RNA while the slower sedimenting side was enriched for the M species. The extra bands between L and M have been noted previously to be double-stranded RNA.[7]

Figure 3 shows electron micrographs of particles present in fractions taken from the sucrose gradient. The spherical 40nm diameter virions previously described,[10, 11] were only detected in those fractions (14, 16) which contained the enzyme activity and the highest levels of double-stranded RNA. Some virions excluded the negative stain while others allowed it to

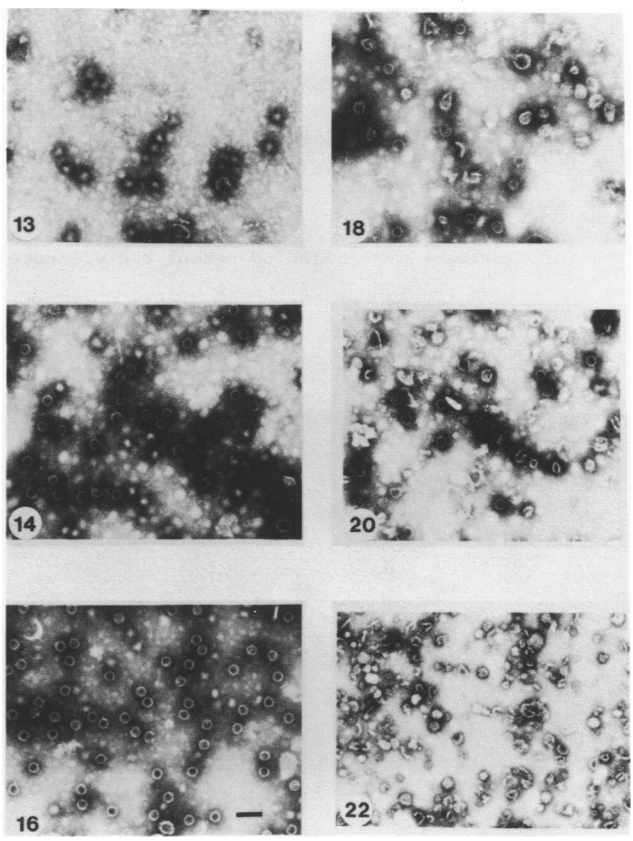

Figure 3. Electron micrographs of sucrose gradient fractions. The indicated fractions were negatively stained with phosphotungstic acid and photographed using a Philips-Norelco EM-300 electron microscope.[46] Micrographs were taken by Ms. Virginia Thomas. The bar indicates 100 nm.

penetrate their cores. Similar particles were seen nowhere else on the gradient, and appeared only in active fractions throughout the purification. In the fractions sedimenting more slowly than polymerase activity, particles were seen which resemble the intact virions but which were broken and not spherical. The P_{133} fraction had relatively few spherical particles of this size, and none were seen in the supernatant from the first 133,000 x g centrifugation.

Polyacrylamide gel electrophoresis of denatured protein[49] from each fraction of the sucrose gradient shown in Figure 1 revealed the same pattern of proteins in the active fractions as that previously described[12] in virions containing L double-stranded RNA. Similar bands were also seen in the fractions (18-20) sedimenting more slowly than the activity, and may be associated with the empty virions seen by electron microscopy in these fractions (Figure 3).

Reaction conditions. Under the standard assay conditions incorporation was linear with time for at least one hour (Figure 4) and the virion particles were the rate-limiting component.

As is seen in Figure 4, the rate of the reaction was proportional to the amount of purified virus added over a wide range of concentrations. The

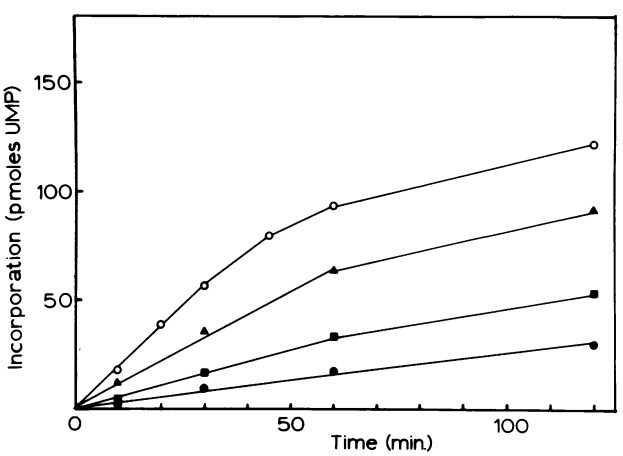

Figure 4. Dependence of virion RNA polymerase reaction on virion concentration and time. Reactions were run under the standard conditions in the presence of varying concentrations of purified virions; 37 µg/ml (●); 74 µg/ml (■); 185 µg/ml (▲); 370 µg/ml (○). At the indicated times aliquots (25 µl) were tested for TCA-precipitable radioactivity. Concentrations are of protein.

addition of bentonite (1.5 mg/ml) resulted in a 50% decrease of the rate of the reaction, but prolonged the linear incorporation of nucleotide with time to at least 5 hours. As seen in Figure 5, the optimal rate of polymerization occurred at pH 7.5. Figure 6 shows that the reaction requires divalent cations.

This reaction requires magnesium which was only partially replaceable by manganese (Figure 6). This preferential stimulation by magnesium was also seen in the virus-like particle RNA polymerases of P. stoloniferum[34,36] as well as those of various reoviridae[25,27,29,30] although an enzyme associated with bacteriophage ϕ6 shows a preference for manganese.[31]

Figure 7 shows the inhibitory effect of monovalent cations on the polymerase reaction. Concentrations of monovalent cation below 20mM failed to inhibit the reaction.

Nucleoside triphosphate requirement. The data in Table 2 demonstrate that the reaction being studied is, indeed, an RNA polymerase and not a ribohomopolymer polymerase. The reaction utilizes each of the four ribonucleoside triphosphates as substrates for polymerization, and the incorporation of each requires the presence of the other three.

The incorporation of ATP, CTP and UTP was relatively less dependent on the presence of GTP than it was on the presence of other triphosphates. This

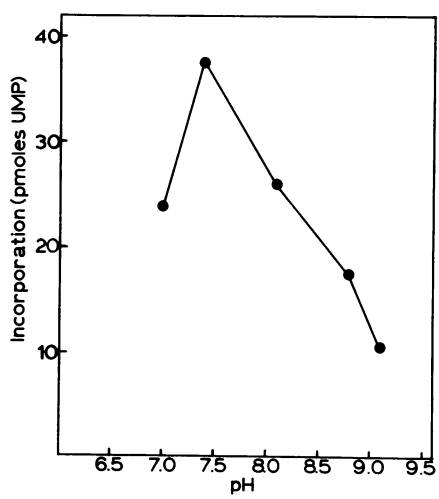

Figure 5. Effect of pH on virion RNA polymerase. Reactions (25 µl) were run as described in the text in the presence of virions containing 2.5 mU of polymerase activity and varying Tris-HCl buffers (50mM).

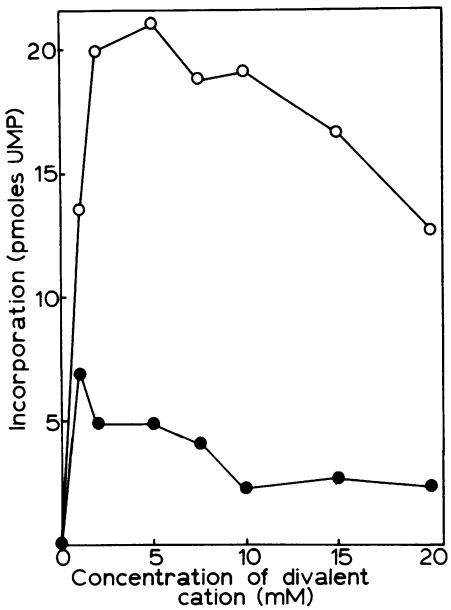

Figure 6. Divalent cation requirement of virion RNA polymerase. Reactions (50 µl) were run as described in the text for 15 minutes in the presence of virions containing 1.5 mU of polymerase activity and varying concentrations of divalent cation (chloride salts); magnesium (○), manganese (●). The reaction in the absence of added divalent cations also included 5 mM EDTA.

does not appear to be due to contamination of the other nucleoside triphosphates with GTP. Thin layer chromatography on PEI cellulose[50] of the other nucleoside triphosphates failed to reveal contamination with GTP, although trace contaminants cannot be excluded. However, the polymerase specific activity was higher when assayed with [^{3}H]-GTP than with the other triphosphates, indicating a lack of gross contamination of the polymerase preparation or assay reagents with GTP. In the polymerase of bacteriophage ϕ6, a similar relative lack of dependence on CTP coupled with an elevated specific activity for incorporation from radioactive CTP was observed;[31] similar results have been found in other viral RNA polymerases.[23] The results rule out the possibility that a ribohomopolymer polymerase is responsible for the nucleotide incorporation observed. Such polymerases include the three poly(A) polymerases of yeast[51] as well as the ribohomopolymer polymerases of double-stranded RNA viruses including the poly(A)[52] and poly(G)[53] polymerases of reovirus and the poly(A) polymerase in double-

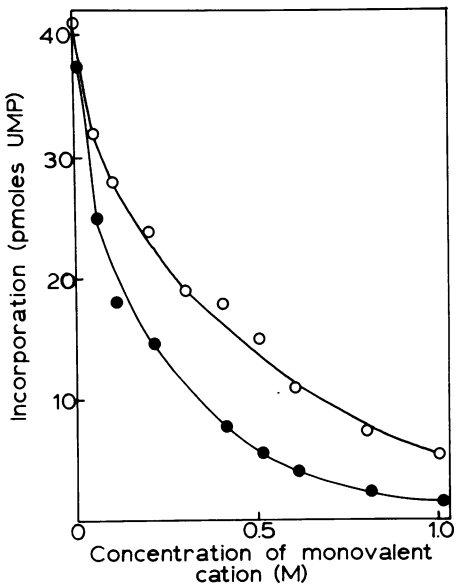

Figure 7. Inhibition of RNA polymerase by monovalent cation. Reactions were run as described in the text for 15 minutes in the presence of virions containing 2.5 mU of polymerase activity and varying concentrations of NaCl (○), or KCl (●).

TABLE 2. Virion RNA polymerase requires all four ribonucleoside triphosphates.

Nucleotide Incorporated	Omission					
	None[a]	ATP	CTP	GTP	UTP	All 3
[^{3}H]ATP	(100)	-	4	42	6	5
[^{3}H]CTP	(100)	2	-	15	2	2
[^{3}H]GTP	(100)	<1	1	-	2	1
[^{3}H]UTP	(100)	2	4	49	-	<1

[a]Reactions (50 µl) were run as described in the text for 15 minutes in the presence of virions containing 2.5 mU of RNA polymerase activity. In the control reactions (no omission) three ribonucleoside triphosphates were present (0.5 mM each) and the fourth was present as the [^{3}H]-ribonucleoside triphosphate (20 µM, 1Ci/mmole). With no omission, 100% incorporation of [^{3}H]ATP was 16.7 pmoles, [^{3}H]CTP was 23.5 pmoles, [^{3}H]GTP was 43.5 pmoles, and [^{3}H]UTP was 37.2 pmoles. All other values are expressed as percent of the complete system.

stranded RNA virions isolated from Allomyces arbuscula.[42]

In reactions run as in Table 2 omitting all ribonucleoside triphosphates but containing 0.5 mM each of deoxy ATP, deoxy CTP and deoxy GTP and 20 μM [^{3}H]-deoxy TTP (1.19 Ci/mmole), no incorporation of radioactivity into TCA-insoluble material was observed, indicating the specificity of the reaction for ribonucleotides. Addition of ribonucleoside triphosphates did not result in incorporation from deoxy TTP, nor could [^{3}H]-deoxy TTP replace [^{3}H]-UTP in the reaction.

Thiol requirement. As is shown in Table 3, the polymerase reaction showed no dependence on the addition of 2-mercaptoethanol. Neither p-hydroxymercuribenzoate nor N-ethylmaleimide inhibited the reaction in the presence of 10mM 2-mercaptoethanol, but both sulfhydryl reagents showed a marked inhibition in the absence of protecting 2-mercaptoethanol. Thus reduced sulfhydryl groups must play some role in the catalysis of polymerization.

Pyrophosphate inhibition. The reaction is markedly inhibited by 5mM pyrophosphate (Table 3). Phosphate, at ten times that concentration, is far

TABLE 3. Properties of RNA Polymerace Reaction

Conditions	% of Control[a]
Control	100
-2-Mercaptoethanol	97
+p-OH Mercuribenzoate (1mM)	103
" -2-Mercaptoethanol	2
+N-Ethylmaleimide (1mM)	104
" -2-Mercaptoethanol	39
+ Sodium pyrophosphate (5mM)	2
+ Sodium phosphate (50mM)	28
+ S-Adenosylmethionine (100mM)	102
+ S-Adenosylhomocysteine (100 mM)	88
+ α-Amanitin (100 μg/ml)	96
+ Actinomycin D (100 μg/ml)	91
+ Ethidium bromide (100 μg/ml)	6
+ Rifampicin (100 μg/ml)	112

[a]Reactions (50 μl) were performed as described in the text for 15 minutes in the presence of virions containing 3 mU of polymerase. The control had no additions or omissions.

less inhibitory (Table 3); this inhibition may, in part, be due to the added sodium (see Figure 7).

No Coupling to Methylation. The virion RNA polymerase reaction is neither stimulated by S-adenosylmethionine nor inhibited by the methylation inhibitor S-adenosylhomocysteine (Table 3). This contrasts with the marked stimulation by S-adenosylmethionine of the RNA polymerase of cytoplasmic polyhedrosis virus[54] and the moderate stimulation seen in reovirus.[55] Results similar to ours have been reported for the virion polymerase of P. stoloniferum.[37]

Inhibitors. Table 3 shows that the virion RNA polymerase is insensitive to high levels of α-amanitin, actinomycin D, and rifampicin. Its resistance to actinomycin D distinguishes this activity from the DNA-dependent RNA polymerases,[56] including the mitochondrial RNA polymerase of yeast which is 80% inhibited by this dose of the drug even in intact mitochondria.[57] Insensitivity to rifampicin distinguishes the activity from typical prokaryotic RNA polymerases.[58] Yeast DNA-dependent RNA polymerases II and III (but not I) are markedly inhibited by the level of α-amanitin used here.[59] As might be expected of an enzyme reaction utilizing a double-stranded nucleic acid as its template, the polymerase activity is markedly inhibited by ethidium bromide. Preincubation of the polymerase preparation for 15 minutes at 30°C in the presence of DNase (0.5 mg/ml) had no effect on the incorporation (98% of control) in a reaction run as in Table 3.

RNA Product. Sucrose gradient centrifugation of the polymerase reaction mixture after 45 minutes of reaction revealed two classes of RNA product: one class (48.0%) cosedimented with the more rapidly sedimenting portion of the virion-enzyme peak and the other (52.0%) sedimented much more slowly (Figure 8). Virion RNA polymerase activity showed approximately the same sedimentation pattern seen in Figure 1 after the reaction.

Similar analysis of product made in a 90-minute reaction showed that only 26.5% of the product cosedimented with the virions and 73.5% was in the more slowly sedimenting peak. The product in all gradient fractions was nearly entirely single-stranded, as judged by nearly complete susceptibility (85-89%) to RNase (1 μg/ml) at 37°C in the presence of 0.8M NaCl. Thus, it appears that at least some of the single-stranded RNA product is released from the virions.

CONCLUSION

Intracellular double-stranded RNA-containing virions from killer

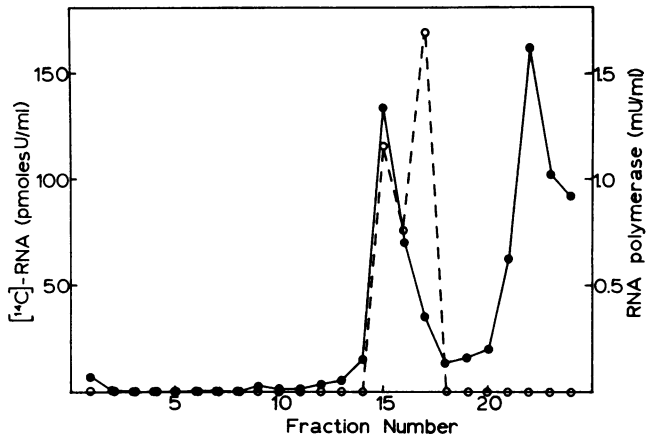

Figure 8. Sucrose gradient analysis of polymerase activity and the product of the reaction. The polymerase reaction (200 µl) was run as in the standard assay, but with [^{14}C]UTP (49 mCi/mmole) in the presence of 14mU of RNA polymerase for 45 minutes. The reaction was then analyzed on a sucrose gradient identical to that in Figure 1. Each fraction was analyzed for [^{14}C]-RNA, i.e., TCA insoluble [^{14}C] (●) and for RNA polymerase by the standard assay, using [^{3}H]-UTP (○), with all scintillation counting done on "double-label" settings. Recovery of [^{14}C]-RNA was quantitative on the sucrose gradient and 40% of the original enzyme activity was recovered.

strains of S. cerevisiae grown into stationary phase on ethanol contain a DNA-independent RNA polymerase activity which co-purifies with the virions. The product of this enzyme-catalyzed reaction is mainly single-standed RNA and is partially released from the catalytically active virus particles. This activity has many properties similar to the UTP-incorporating activity previously described in virions from stationary phase non-killer yeast,[44] but which was not proven to purify along with those particles. The following paper describes the characterization of the products of the reaction, which appear to be asymmetric transcripts of the endogenous double-stranded RNA species in the virions.

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