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Transcription of killer virion double-stranded RNA in vitro

J.Douglas Welsh and Michael J.Leibowitz

Department of Microbiology, College of Medicine and Dentistry of New Jersey, Rutgers Medical School, Piscataway, NJ 08854, USA

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ABSTRACT

Intracellular virions of stationary phase ethanol-grown cells of a killer strain of Saccharomyces cerevisiae contain encapsulated M (1.1 x 10^6 dalton) and L (3.2 x 10^6 dalton) double-stranded RNA plasmids. These virions also contain RNA polymerase activity which catalyzes the synthesis of full-length, single-stranded, asymmetric transcripts (denoted m and l) of the virion double-stranded RNAs. Product m is made by M-containing particles and shows complete sequence homology to M but not to L. Product l is made by L-containing particles and shows complete homology only to L. The products show no self-homology, indicating asymmetric transcription. Therefore, the polymerase appears to function in vitro as a double-stranded RNA transcriptase. The lack of sequence homology between M and L is confirmed.

INTRODUCTION

Killer strains of Saccharomyces cerevisiae harbor two major species of double-stranded RNA (dsRNA), denoted L (or P1) and M (or P2), encapsulated in cytoplasmic virion particles.[1-6] The M RNA appears to be the cytoplasmically-inherited killer plasmid,[2] while L seems to encode the major capsid protein component of the virions.[7] Thus, the dsRNA species are genetically plasmids of yeast but physically reside in intracellular virus particles. Varying molecular weights have been reported for these dsRNA species based on different analytic methods. Recent values reported for L range from 2.5 to 3.5 x 10^6 daltons[1,2,8-12] and for M from 1.10 to 1.7 x 10^6 daltons.[1,2,8-10,12,13] Less variation has been noted in those estimates based on electrophoretic analysis of completely denatured dsRNA, with estimates of 3.0-3.3 x 10^6 daltons for L[10-12] and 1.10-1.18 x 10^6 daltons for M.[10-13] Ribonuclease T_1 oligonucleotide analysis has failed to reveal any sequence homology between L and M.[10]

As was described in the preceding paper, RNA polymerase activity catalyzing the synthesis of single-stranded RNA co-purified with dsRNA-containing virions from stationary phase ethanol-grown killer yeast cells.

An activity similar to this one has previously been described in virions of non-killer cells, although co-purification of the activity with the virions was not demonstrated.[12,14] The previous references indicated that the product formed showed some ability to hybridize with denatured L dsRNA; kinetic analysis of the hybridization reaction was not reported. In the preceding paper the properties of the polymerase activity in intact virions purified from killer cells (strain A364A X S7) were elucidated. The present report demonstrates the products of the reaction to be full-length transcripts of the virion dsRNA, with kinetic analysis of hybridization with denatured dsRNA demonstrating the fidelity and asymmetry of transcription.

MATERIALS AND METHODS

Materials. [α-^{32}P]-UTP (535 mCi/mmole) and carrier-free [^{32}P]-phosphoric acid were from I.C.N. Pharmaceuticals. [5,6-^{3}H]-UTP (1 Ci/mmole) and [8-^{3}H]-adenine (18.9 Ci/mmole) were from New England Nuclear. Pancreatic ribonuclease A was from Worthington Biochemical Corp. and ribonuclease T_1 was from Calibochem-Behring Corp. Bacteriophage ϕ6 dsRNA was generoulsy provided by J. Van Etten. Torula RNA was from Calbiochem-Behring. V. Stollar generously provided BHK cell RNA and [^{14}C]-Sindbis Virus RNA (26S and 42S). Ultrapure formamide was from Bethesda Research Laboratories, and Agarose (Type II) for electrophoresis was from Sigma Chemical Corp. Buffer 1 X SSC was 150 mM NaCl, 15 mM sodium citrate (pH 7.0). All other reagents were of analytical grade and were sterilized by autoclaving or ultrafiltration.

Yeast cells. The haploid nonkiller strain S7[15] and the diploid killer A364A X S7 were grown into stationary phase on ethanol as described in the preceding paper. Saccharomyces carlsbergensis Y379-5D, a haploid α strain lacking detectable 2-micron DNA and dsRNA, was provided by L. Hartwell.

Preparation of ribosomal RNA (rRNA). Strain Y379-5D was grown for 21 hours at 28°C in 1% yeast extract, 2% peptone, 2% dextrose containing [^{3}H]-adenine (2.5 μCi/ml). Cells were broken in a French Press in 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, 0.1 M LiCl, made 0.5% in SDS and extracted twice with phenol-chloroform-isoamyl alcohol (24:24:1). RNA was then precipitated from the aqueous phase with 2 volumes of ethanol at -20°C, redissolved in 2 X SSC and single-stranded RNA was precipitated with an equal volume of 4M LiCl at 4°C for 40 hours. The precipitated RNA was redissolved in 1 X SSC and fractionated on a 15-30% sucrose gradient in

10 mM Tris-HCl (pH 7.4), 60 mM NaCl, 1 mM EDTA, 0.2% SDS in a centrifuge rotor (Beckman SW27) run at 26,000 rpm for 27 hours at 20°C to separate 18S and 25S rRNA. Fractions containing each rRNA species were alcohol precipitated and redissolved in 1 X SSC. Purified 18S RNA contained 3.03 X 10^6 cpm/mg, 25S contained 3.16 X 10^6 cpm/mg. Purity was confirmed by electrophoresis under denaturing conditions (see below).

Virion Purification. Virions containing dsRNA and RNA polymerase activity were purified as described in the preceding paper. RNA polymerase was assayed as described in that paper. In all purifications, particles prepared from A364A X S7 were noted to have two partially separable peaks of virion polymerase activity on the final sucrose gradient. In the previous paper all active fractions were pooled. However, in the experiments reported here the two peaks were pooled separately prior to the final concentration step. The more rapidly sedimenting particles (peak A, sucrose gradient fractions 14-15) had a final specific enzyme activity of 0.361 U/mg protein and constituted 12.2% of the initial enzyme activity. The more slowly sedimenting particles (peak B, gradient fractions 16-17) had a specific activity of 0.264 U/mg protein and contained 14.1% of the initial activity. Purification of particles from strain S7 resulted in a single activity peak on the sucrose gradient sedimenting in the position of peak A with a specific activity of 0.032 U/mg protein and 18.5% recovery.

Preparation of Reaction Products. Virion preparations from peaks A (96 mU/ml) or B (100 mU/ml) of virions from A364A X S7 or preparations from S7 (5 mU/ml) were used to catalyze the synthesis of product RNA under the standard reaction conditions in the presence of either [^{3}H]-UTP or [α-^{32}P]-UTP, and with the addition of bentonite[16] (1.5 mg/ml). Bentonite addition prolonged the linear portion of the incorporation reaction to at least 5 hours thus increasing the yield of RNA in long reactions, although it reduced the initial rate of incorporation by 50%. Reactions were run for 3 hours, and then digested for 1 hour at 28°C in the presence of proteinase K (0.2 mg/ml), SDS (0.2%), and EDTA (5mM). Reaction mixtures were then extracted once with chloroform-isoamyl alcohol (24:1 v/v), precipitated overnight with 2 volumes of ethanol at -20°C and the precipitated RNA was dissolved in 1 X SSC. This RNA was then fractionated by preparative agarose (1.5%) gel electrophoresis on a vertical gel apparatus (Model SE520, Hoeffer Scientific Company) for 16 hours at 3.8 volts/cm in 90 mM Tris-acetate, 2.5 mM EDTA (pH 7.0). After bands

were identified by autoradiography, RNA was extracted from the gel by homogenization and phenol extraction as described,[17] with an additional extraction with two volumes of chloroform-isoamyl alcohol (24:1 v/v) to remove phenol prior to ethanol precipitation. Product concentration was estimated from the recovered radioactivity, assuming that approximately 25% of the nucleotides in the product are uridylate.[2,10,18]

Preparation of dsRNA. Cells of A364A X S7 were grown into stationary phase on ethanol-containing medium and the phenol extraction of dsRNA from unbroken cells was performed essentially as described,[13] except that cells were pre-incubated in 50 mM Tris-SO_4 (pH 9.0) and residual phenol was extracted with chloroform-isoamyl alcohol (24:1 v/v) prior to ethanol precipitation. Preparative agarose gel electrophoresis[13] resulted in preparations of pure L and M dsRNA. For the preparation of [^{32}P]-dsRNA, cells were grown in the usual medium after removal of inorganic phosphate[17] and the addition of 20-50 μCi/ml of [^{32}P]-phosphoric acid. Purified [^{32}P]-dsRNA had a specific activity of 75,000 cpm/μg(L) and 20,000 cpm/μg(M).

RNA Molecular Weight Determination. Radioactive RNA samples including unknowns and standards were denatured by heating at 60°C in 2.2 M formaldehyde in 50% formamide, 18 mM Na_2HPO_4, 2 mM NaH_2PO_4 for 5 minutes for single-stranded RNA and 10 minutes for dsRNA.[19] Agarose gel electrophoresis was then performed on 0.75% agarose gels in 2.2 M formaldehyde, 18 mM Na_2HPO_4, 2 mM NaH_2PO_4 for 4.5 hours at 75 volts at room temperature in a vertical slab gel electrophoresis apparatus. Subsequently each lane of the gel was cut out, frozen and sliced into 1 mm slices with a Hoeffer gel slicer, and the radioactivity in each fraction was determined by liquid scintillation in a toluene cocktail containing 2,5-diphenyloxazole (4 g/l), p-bis[2-(5-phenyloxazoyl)] benzene (50 mg/l), and 8% (v/v) NCS (Amersham).

Hybridization reactions. RNA-RNA hybridization was performed by a modification of Method A as described.[20] Reaction mixtures were prepared in siliconized polypropylene tubes and contained 50% (v/v) formamide, 6 X SSC, 0.1% SDS, and BHK cell RNA (100 μg/ml) as carrier. Double-stranded RNA was added to the reaction mixture and denatured and heat sheared at 115°C (2 min for M, 5 min for L and ϕ6 dsRNA). Where included, single-stranded RNA was added at the end of this incubation and annealing was immediately begun at 60°C. At indicated annealing times, aliquots were diluted 10-fold in cold 2 X SSC, and frozen in dry ice-ethanol. Upon completion of the experiment, samples were thawed simultaneously, and duplicate portions were diluted 25-fold in 2 X SSC containing ribonucleases

A (20 µg/ml) and T_1 (40 units/ml) or in 2 X SSC alone and digested for 15 min at 37°C. Precipitation was performed in the presence of 100 µg of Torula RNA as carrier with cold 5% TCA containing 0.1 M sodium pyrophosphate, followed by collection of GF/B filters and washing with cold 5% TCA-0.1 M sodium pyrophosphate (7 X 2 ml), 5% TCA-0.1 M Na_2HPO_4 (7 X 2 ml), and 95% ethanol (2 X 2 ml). Radioactivity was then measured by liquid scintillation counting. $C_rt1/2$ values for denatured [^{32}P]-dsRNA species were calculated as the values for the product of the RNA concentration X time at which one half of the maximal reannealing observed would occur.[20]

RESULTS AND DISCUSSION

Identification of 1 and m products. As is indicated in Figure 1, peak A virions isolated from strain A364A X S7 (killer) catalyzed the synthesis of two species of RNA migrating faster than the virion dsRNA on gel electrophoresis. These two species of product are denoted 1 (larger) and m (smaller). Peak B particles from A364A X S7 predominantly catalyzed the synthesis of the m species. On the other hand, virions isolated from strain S7 (non-killer), which only contained L dsRNA, catalyzed the synthesis of a single species of product, co-migrating with 1 synthesized using peak A particles from A364A X S7. In these experiments more than 90% of the TCA-precipitable radioactivity from each reaction was found to migrate on the preparative gel as the discrete species 1 and m.

Molecular weight of product. Figure 2 shows the molecular weight determination of the [^{32}P]-RNA 1 and m products of the reaction run in the presence of virions purified from A364A X S7. These products were isolated by preparative gel electrophoresis as described in Materials and Methods. These results contrast with the findings described in 37°C reactions using the virions from non-killer yeast, where a preponderance of low molecular weight heterogeneous product was formed,[14] although a large product predominates at 30°C.[12] As is shown in Figure 2, product 1 migrates on electrophoresis with a mobility indicating a molecular weight of 1.7 x 10^6 daltons, while the molecular weight of m is 0.56 x 10^6 daltons. On the same type of gel, denatured L migrated with a molecular weight of 1.6 x 10^6 daltons and denatured M was 0.55 x 10^6 daltons. RNA product made from virions isolated from the non-killer strain S7 co-migrated with product 1 RNA on electrophoresis with and without denaturation. Similarly, denatured dsRNA from S7 co-migrated with denatured L dsRNA from strain A364A X S7. Thus, 1 and m appear to very closely approximate the molecular weights of denatured L and

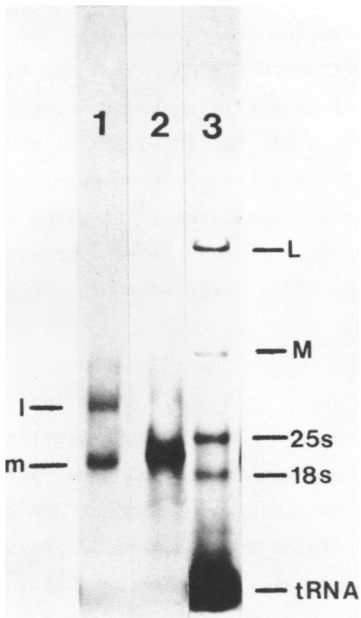

Figure 1. Agarose gel electrophoresis of the reaction products. [^{3}H]-RNA product was prepared from reactions run as described in Materials and Methods in the presence of either Peak A or Peak B virions from strain A364A X S7. Here a fluorograph of a gel identical to that used in the usual preparative agarose gel electrophoresis is shown. Fluorography was by a modification of the method of S. Gillies and V. Stollar (personal communication). The gel was soaked twice for 90 minutes in methanol, then for 16 hours in 2,5-diphenyloxazole (10% w/w) in acetone, dried onto filter paper and exposed at -70°C to pre-flashed Kodak XR-5 X-ray film. The sample in lane 1 is the product of a reaction containing peak A virions, that in lane 2 is the product of a reaction containing peak B$_{13}$ particles, and lane 3 shows total phenol-extracted RNA from whole cells[3] of strain A364A X S7 grown in the presence of [^{3}H]-adenine.

M, respectively.

Hybridization analysis of RNA product. Figures 3 and 4 summarize hybridization experiments demonstrating that the products of the polymerase reaction are asymmetric transcripts of the endogenous dsRNA molecules found in the polymerase-containing virions. In Figure 3, the self-annealing of denatured, heat-sheared L dsRNA is shown to follow a monophasic C_rt curve with the reaction proceeding until 85% of the input dsRNA has reannealed. The $C_rt_{1/2}$ for L under these conditions is 8.8×10^{-3} mole-sec/liter. The l product of the polymerase reaction is driven into hybrid by denatured

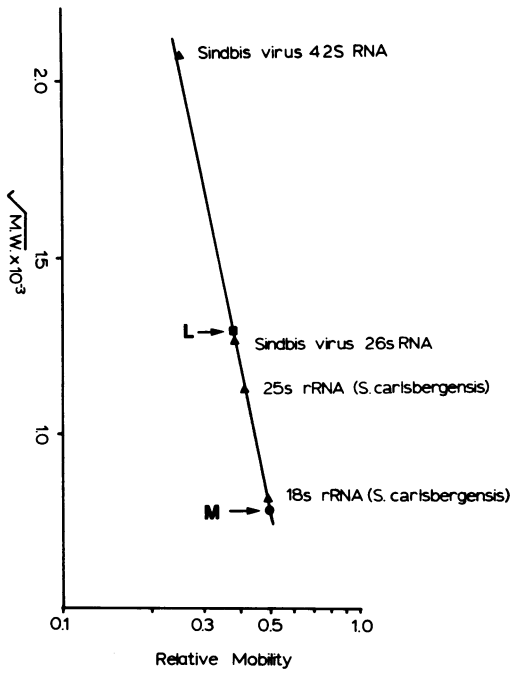

Figure 2. Molecular weight of RNA polymerase products. [^{32}P]-RNA products were synthesized in vitro and purified as described in Materials and Methods utilizing virions purified from strain A364A X S7. The molecular weight of formaldehyde-formamide-denatured RNA was determined by electrophoretic mobility as described above. [^{14}C]-Sindbis RNA[21] and S. carlsbergensis [^{3}H]-rRNA[22] were used as standards. The migrations of standards (▲), m (●), 1 (■), and denatured M and L dsRNA (arrows) are indicated.

L dsRNA driver, with the same kinetics of dsRNA formation shown by L dsRNA alone, indicating sequence identity between 1 and L within the limits of detectability. The L dsRNA fails to drive [^{32}P]-m product into hybrid. The failure of [^{32}P]-1 to form dsRNA on denaturing and reannealing indicates a lack of self-homology, as would be seen if 1 were a copy of a unique strand of L dsRNA. The [^{32}P]-1 product is not driven into dsRNA by ϕ6 dsRNA.

Figure 4 shows that M dsRNA also reanneals in a monophasic fashion, with a $C_r t_{1/2}$ of 2.3 x 10^{-3} mole-sec/liter under these conditions. The heating procedure for heat shearing and denaturation lowers the extent of "snap-back" otherwise seen for the M species.[23] At a $C_r t$ of 10^{-5} only 1.7% of input denatured M dsRNA was reannealed. In these experiments a maximum of 65% of the input denatured M dsRNA reannealed. The annealing of [^{32}P]-m

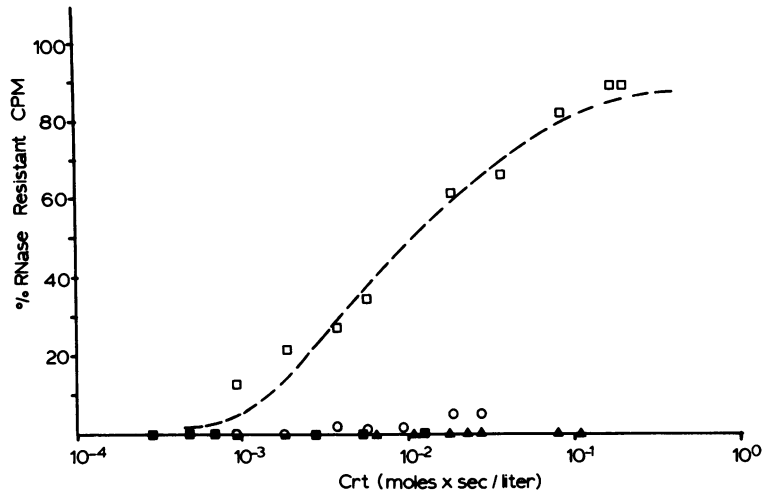

Figure 3. Hybridization kinetics of RNA products of virion RNA polymerase with L dsRNA. All RNA species were prepared as described in Materials and Methods. Virion polymerase products containing [^{32}P] were prepared using virions isolated from strain A364A X S7: [^{32}P]-l from reactions containing peak A virions and [^{32}P]-m from reactions containing peak B virions. Hybridization reactions were performed as described in Materials and Methods. In all experiments the non-radioactive driver species was added at 100-fold the nucleotide concentration of the [^{32}P]-RNA species. The data shown represent the reannealing of [^{32}P]-L dsRNA alone (---), [^{32}P]-l product with L dsRNA driver (□), [^{32}P]-l product without driver (■), [^{32}P]-m product with L dsRNA driver (○), and [^{32}P]-l product with ϕ6 dsRNA driver (▲).

in the presence of denatured M dsRNA driver shows kinetics identical with that of M dsRNA alone, proving the sequence identity of m and M within experimental limits. Annealing of [^{32}P]-l shows little if any ability to be driven into hybrid by denatured M. As was the case with product 1, [^{32}P]-m fails to reanneal without M dsRNA driver, indicating a lack of self-complementary sequences in both polymerase products. Neither product is driven into dsRNA by denatured ϕ6 dsRNA driver.

CONCLUSION

The m and l products of the dsRNA-virion-associated RNA polymerase from stationary phase yeast cells grown on ethanol are full-length single-stranded transcripts of unique strands of the M and L dsRNA species,

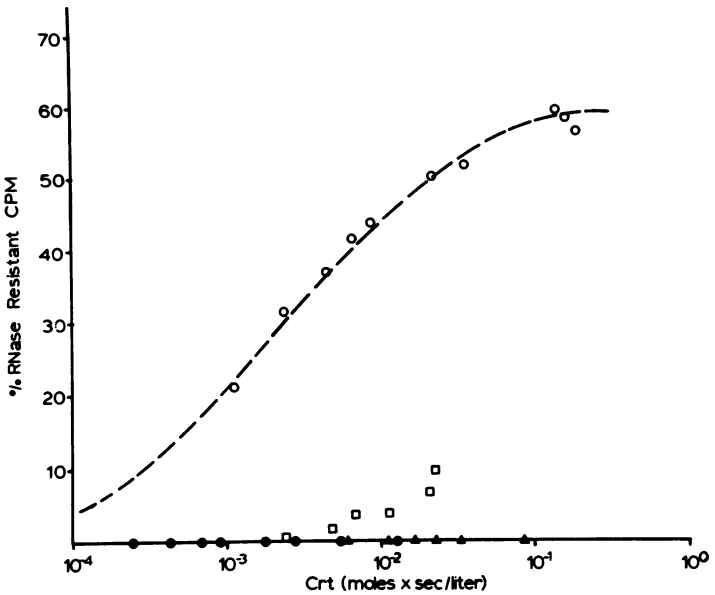

Figure 4. Hybridization of RNA products of virion RNA polymerase with M dsRNA. The experiments were performed as in Figure 3. The data represent reannealing of [^{32}P]-M dsRNA alone (---), [^{32}P]-m product with M dsRNA driver (○), [^{32}P]-l product with M dsRNA driver (□), [^{32}P]-m product without driver (●), and [^{32}P]-m product with ϕ6 driver (▲).

respectively. Although the biological function of this enzyme activity has not yet been proven, the properties of the products suggest that they may function as precursors to messenger RNA coding for the gene products of the viral genomes. Mutants defective in various aspects of killer function will be studied in an attempt to elucidate the physiological role of the viral polymerase, and its relationship to the maintenance and expression of the cytoplasmically inherited killer character of yeast. Post-transcriptional modification[24] of the RNA products of this *in vitro* reaction has not yet been demonstrated.

Hybridization analysis has shown that m can reanneal to M but not L, and l to L but not M, thus proving the absence of any significant homology between the M and L dsRNA species. This lack of homology was previously supported by T_1-ribonuclease "fingerprint" patterns.[10] The dependence of M replication on 27 chromosomal genes which are not absolutely required for L replication[25] may be a biological result of the differences in

sequence between these two molecules.

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