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Yeast viral RNA polymerase is a transcriptase

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ABSTRACT

ScV-L is a simple double-stranded RNA virus of yeast, consisting of a 4.8 kilobase pair double-stranded RNA (L) encapsidated in isometric particles composed mainly of one polypeptide (ScV-P1) of 88,000 daltons. L encodes ScV-P1. There is a capsid-associated RNA polymerase that synthesizes in vitro predominantly single-stranded RNA. We show that this polymerase activity is a transcriptase, at least one product of which is the mRNA for ScV-P1. The transcript, like its template, is uncapped.

INTRODUCTION

The fungal viruses are double stranded RNA (dsRNA) viruses with segmented genomes separately encapsidated in isometric particles, and generally lacking an infectious cycle (1,2). The simplest of these is the virus of Saccharomyces cerevisiae (ScV), which has two separately encapsidated dsRNAs (3,4). The larger of the two dsRNAs (L) is about 4.5-4.9 kilobase pairs (kbp) in length (5,6,7,8) and encodes the major capsid polypeptide (9). The smaller dsRNA (M) is about 1.9 kbp in length (6,8,10) and encodes a secreted polypeptide toxin ("killer factor") lethal to strains without M (11,12,13). The major capsid protein of L-containing (ScV-L) particles and M-containing (ScV-M) particles is identical (14,15) while L and M have very little sequence homology (6). Defective interfering particles (ScV-S) that replace ScV-M in "suppressive sensitive" strains have genomes that are the result of internal deletion of M (6,10,16,17). ScV-M and ScV-S particles, but not ScV-L particles, are dependent on the expression of a number of chromosomal genes (18,19). The 5' termini of L and M are pppGp (20), and the denatrued viral RNAs are tranlated in the wheat germ (9,12) and the Schreier-Staehelin (this work) systems. There are two recent reviews of the ScV system (8,21).

By virtue of the exotic nature of their genomes, double-stranded RNA

viruses do not have the option open to single-stranded RNA viruses: that is, to code for an RNA-dependent RNA polymerase on the infecting strand. Rather, the double-stranded RNA viruses must have capsid-associated RNA polymerases. In those cases most thoroughly studied, the polymerase activity is, indeed, inseparable from an intact capsid (or inner capsid) with enclosed dsRNA (22,23). The fungal viruses, even though they are permanently imprisoned in their host cells, are not exceptions to this rule. Of the 14 or so dsRNA fungal viruses known (2), capsid-associated RNA polymerases have already been described for several: Aspergillus foetidus virus (AfV-S), Penicillium chrysogenum virus, Penicillium stoloniferum virus (PsV-S), and Saccharomyces cerevisiae virus (ScV) (24,25,26,27,28,29). There are two distinct RNA polymerase activities characteristic of the best studied dsRNA viruses, the reoviruses. These are a transcriptase, associated with mature virions treated with SDS or chymotrypsin, and a replicase, associated with sub-viral particles present as intermediates in virus replication in vivo (22). The nature of the fungal virus RNA polymerases is much less clear. The product of the PsV-F and PsV-S RNA polymerases seems to be dsRNA that remains within the viral capsids (26,27), while the products of the AfV-S polymerase are ssRNA copies of the viral genome that are released from the particles (28). This latter synthesis, however, appears to represent displacement synthesis on the dsRNA template, so that the ssRNA released from the particle represents a displaced strand of the parental dsRNA (28). The RNA polymerase associated with stationary phase ScV capsids synthesizes predominantly genome length ssRNA (29,30) that is released from the virions (see review, ref. 21). This activity requires all four rNTPs and Mg^{++}, is inhibited by high salt concentrations, pyrophosphate, and ethidium bromide, but is unaffected by DNAse or actinomycin D (see review, ref. 21). There is also an RNA polymerase activity associated with logarithmic phase ScV particles that apparently synthesizes predominantly dsRNA (31).

In the present work, we show that the stationary phase ScV-L RNA polymerase is a transcriptase: the product of the reaction is complementary to only one strand of the template and is translated in vitro to produce all the polypeptide products synthesized on its denatured dsRNA template, including the major viral capsid polypeptide.

MATERIALS AND METHODS

Isolation of ScV particles

Particles were isolated from yeast strain S7 (7), which has only ScV-L particles, as previously described (30). The last step in the purification is a 10-40% or 5-20% sucrose gradient centrifugation in Buffer IV: 50 mM Tris-Cl pH 7.5, 10 mM $MgSO_4$, 0.15 M NaCl, 0.1 mM ethylene diamine tetraacetic acid (EDTA), 0.4 M KCl, 1 mM dithiothreitol (DTT), 20% (v/v) glycerol.

Isolation of ^{32}P ScV particles

^{32}P labeled ScV particles were isolated as described above from cells growing in 500 ml of low-phosphate YPD with 10 mCi of $^{32}PO_4^{-3}$ (20).

Transcriptase assay

Sucrose gradient fractions were assayed for transcriptase activity by the addition of 50 µl of each fraction to 50 µl of reaction mix, consisting of 1 mM ATP, CTP and GTP, 11.1 µM ^{3}H-UTP (ICN, final specific activity 2.5 Ci/mMole), 20 mM mercaptoethanol, 50 mM Tris-Cl pH 7.5 and 0.10 mM EDTA. Reactions were incubated for 2 hrs at 37°C and assayed by precipitation with 20% trichloroacetic acid (TCA) in the presence of 200 µg of carrier RNA. Precipitates were collected on glass fiber filters, extensively washed with 5% TCA and ethanol, dried, and counted in PPO-toluene.

Synthesis of large quantities of the viral transcript

Pooled fractions from the sucrose gradient were dialized against a modified Buffer IV, which has 40 mM NaCl, rather than 150 mM NaCl, and no KCl. 5 ml of particles in modified Buffer IV were added to 5 ml of the reaction mix (see above, and with the ^{3}H-UTP replaced with 1 mM UTP) and incubated for 3 hrs at 37°C in the presence of 1 mg/ml bentonite. Total RNA was isolated by phenol extraction and ethanol precipitation, followed by purification of the transcript by 1.4% agarose gel electrophoresis and/or CF11 cellulose chromatography (6).

Hybridizations

Hybridization mixes in 100 µl of 20% formamide were heated to 105°C in an ethylene glycol bath for about 5 min and cooled on ice. 400 µl of distilled water was added and 50 µl was taken as a 0 min time point. The remaining 450 µl of each reaction mixture was placed in a 70°C water bath and equilibrated for 10 min. 50 µl of 20 x SSC (SSC is 0.15 M sodium chloride, 0.015 M, citrate, pH 7) was added, to a final concentration of 2x SSC. Samples (50 µl) were diluted with 2 ml of 2 x SSC and split into two

portions, each of one ml: one was treated with 20 µg of pancreatic RNAse for 30 min at 37°C and the other placed on ice. 200 µg of tRNA carrier (in 100 µl) was added to each, followed by 100 µl of 100% (wt/vol) TCA. After precipitation on ice for 30 min, samples were filtered onto glass fiber filters and washed extensively with 5% TCA and ethanol. Dried filters were counted Cerenkov or counted in toluene-PPO scintillation fluid.

5' End Labelling

5' end labelling was carried out as described by Efstradiadis (32), using calf-intestine phosphatase (Boehringer Mannheim) purified as described and polynucleotide kinase (PL Biochemicals) with γ-^{32}P-ATP (New England Nuclear or Amersham, 3000 Ci/mMole). The labelled transcript was purified by phenol extraction, ethanol precipitation, and Sephadex G100 chromatography or 1.4% agarose gel electrophoresis.

^{32}P Labelled L

In vivo labelled, purified dsL was made as previously described (20) at a specific activity of about 10^6 dpm/µg.

Protein synthesis in vitro

Protein was synthesized in vitro using a fractionated cell-free system similar to that originally developed by Schreier and Staehelin (33) with some modifications (34). The reaction mixtures (50 µl) contained: 20 mM Hepes·KOH (pH 7.3), 2 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 6 mM creatine phosphate, a few crystals of creatine phosphokinase, 45 µM unlabelled amino acids except for methionine, 30 µCi of ^{35}S-methionine (approximately 500 Ci/mMole; New England Nuclear), 1.8 mM $MgCl_2$, 0.5 mM spermidine, 80 mM KCL, 0.1 A_{260} unit of 40S ribosomal subunits, 0.25 A_{260} units of 60S ribosomal subunits, 2 µg rabbit liver tRNA (Gibco), mRNA as indicated and saturating concentrations of pH 5 enzyme (50-100 µg protein), partially purified EF-1 (50-75 µg protein), and initiation factors (20-30 µg protein). Ribosomal subunits and pH 5 enzyme were prepared from mouse Erlich ascites cells (34). Partially purified EF-1 (Step 2; ref. 35) and initiation factors (34) were prepared from rabbit reticulocytes. Rabbit liver tRNA from Gibco was purified on DEAE cellulose prior to use (35).

Polyacrylamide gel electrophoresis

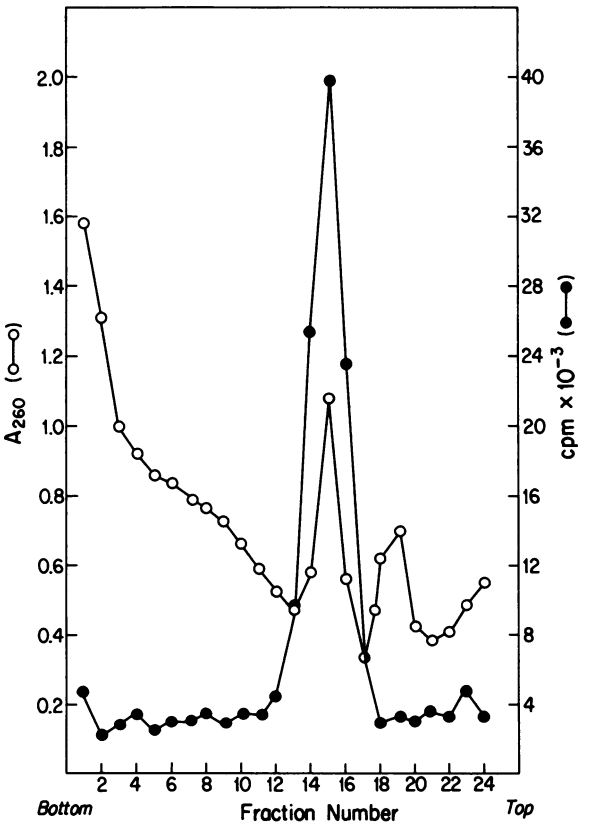
The SDS-polyacrylamide slab gels were prepared as described by Laemmli (36) except that the separation gel contained 6 M urea. Aliquots of in vitro reactions were diluted with electrophoresis sample buffer (0.125 M Tris/HCl, pH 6.8, 4% SDS, 10% glycerol, 10% ß-mercaptoethanol, 0.25%

bromophenol blue), and boiled for 3 min before being applied to the gel.

RESULTS

Particle isolation

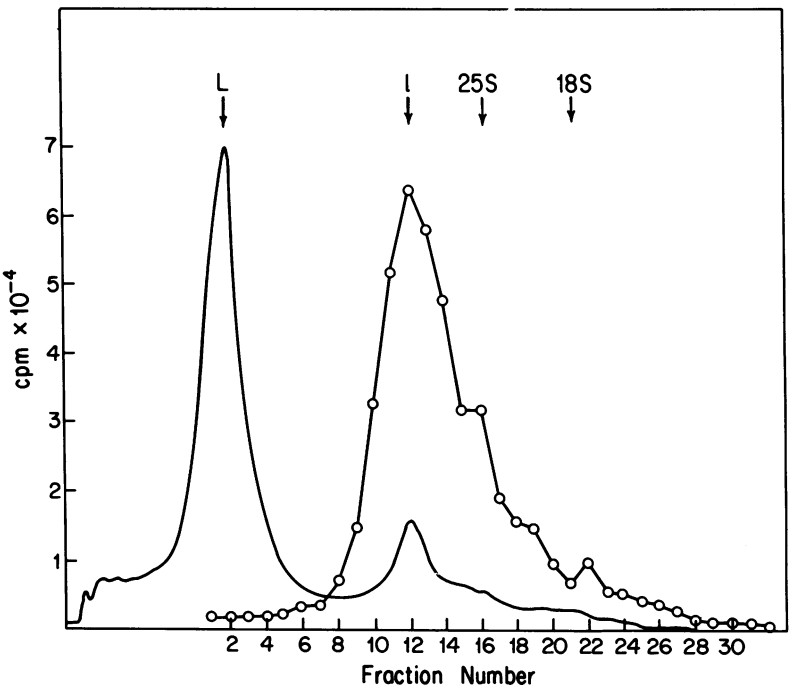
The procedure described for the isolation of ScV-L particles results in a reasonably pure preparation: a distinct peak in absorbance at 260 nm in the sucrose gradient corresponds to the peak in transcriptase activity (Fig. 1). This is a highly reproducible procedure, and the peak fractions usually contain no RNA other than L (not shown) and mainly the viral capsid protein, ScV-P1 (see Fig. 5 for example). Occasionally, there are small amounts of contaminating ribosomal RNAs present in the peak fractions. Incorporation into transcript is linear for 2-3 hrs.



1. Sucrose gradient of ScV-L particles. o——o A_{260} of fractions. ●——● Transcriptase activity.

Purification and size of the viral transcript

Total RNA isolated from transcriptase reaction mixes containing about 5 A_{260} of ScV particles from the peak fractions of several sucrose gradients was applied to a series of wells in 1.4% agarose slab gels. The results of such an experiment are shown in Fig. 2. The unincubated ScV-L particles contain only L. The transcriptase reaction mixes contain L and a new RNA migrating just slower than 25s rRNA. The transcript labelled in vitro with ^{3}H-UTP co-migrates with this new peak stained with ethidium bromide. After 3 hrs of incubation, the yield of transcript from a large reaction mix averages about 10-20 µg after



2. Agarose gel of transcript. In vitro reaction mixes were extracted for RNA and run on a 1.4% agarose slab gel. After being stained with ethidium bromide, the gel was photographed under ultraviolet light, and a densitometer tracing of the negative made (——). One reaction mix was run with 20 µM ^{3}H-UTP. The RNA from this reaction was run in a separate lane and the lane cut into 3 mm slices, which were scintillation counted in Aquasol (0——0). rRNA markers were run in an adjacent well.

elution from the gel, which represents about 10-20% of the weight of the template present. If only one strand were transcribed (as we intend to demonstrate) this would represent an average of one full sized transcript from each of 20-40% of the particles.

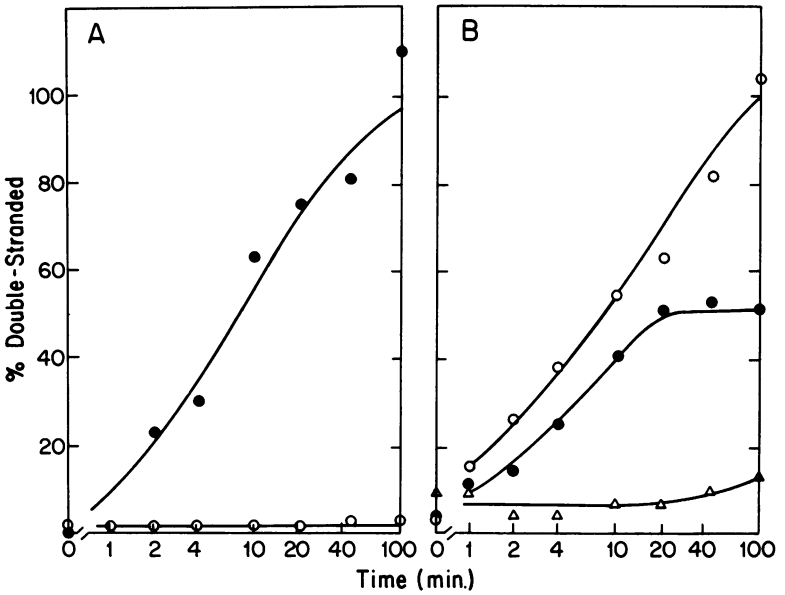
The size of the transcript estimated from non-denaturing gels such as that of Fig. 2 is about 4.9 kb, which is the expected size of a full-size transcript of L, which contains 4.5-4.9 kbp. This is the same size as estimated for the transcript by other investigators (see review, ref. 21). Methyl-mercury denaturing gel electrophoresis and electron microscopy demonstrates that the transcript 1 has the same size as the denatured template L strands (30, and data not shown).

Strandedness of the ScV-L transcript

We have previously shown that the RNA synthesized in vitro by the capsid-associated RNA polymerase of ScV-L particles is entirely complementary to the template L (30). This experiment is repeated with the full-size transcript isolated as described in Fig. 2. Excess non-radioactive dsL (2 µg) is hybridized to ^{3}H-UTP labelled transcript (about 0.036 µg, at 2.8 x 10^{5} cpm/µg). All of the transcript hybridizes to L, with a $Rot_{\frac{1}{2}}$ = 1.5 x 10^{-2} m-sec/l corrected to 0.12 M phosphate buffer (37) (Fig. 3A). This is the same $Rot_{\frac{1}{2}}$ as that of dsL to dsL probe (see below). The transcript does not self-hybridize to any detectable extent when incubated to a Rot = 0.13, and must therefore represent one strand of L. This is again demonstrated in Fig. 3B, which shows that excess non-radioactive transcript (2 µg) will protect from digestion only 50% of the ^{32}P-labelled dsL probe (0.013 µg) with a $Rot_{\frac{1}{2}}$ = 7.4 x 10^{-3}, while excess dsL, with the same probe, protects 100% of the probe with a $Rot_{\frac{1}{2}}$ = 1.5 x 10^{-2}. The transcript hybridizes to the probe twice as fast as does the same weight of dsL (with half the $Rot_{\frac{1}{2}}$), indicating that the transcript has half the complexity of its template dsRNA. This is precisely the expected result if the transcript is of one strand of L. The transcript protects from RNAse digestion only half the input dsL, even when incubated to a Rot = 0.18, again indicating that the transcript is of only one strand of L.

Mode of Synthesis of transcript

There are two methods of using radiolabelled RNA to determine if transcription of a dsRNA template is conservative or semiconservative. If newly synthesized RNA is labelled, the appearance of label in dsRNA template is suggestive of semiconservative synthesis. If the template is labelled, the appearance of label in the transcript is conclusive evidence of semi-



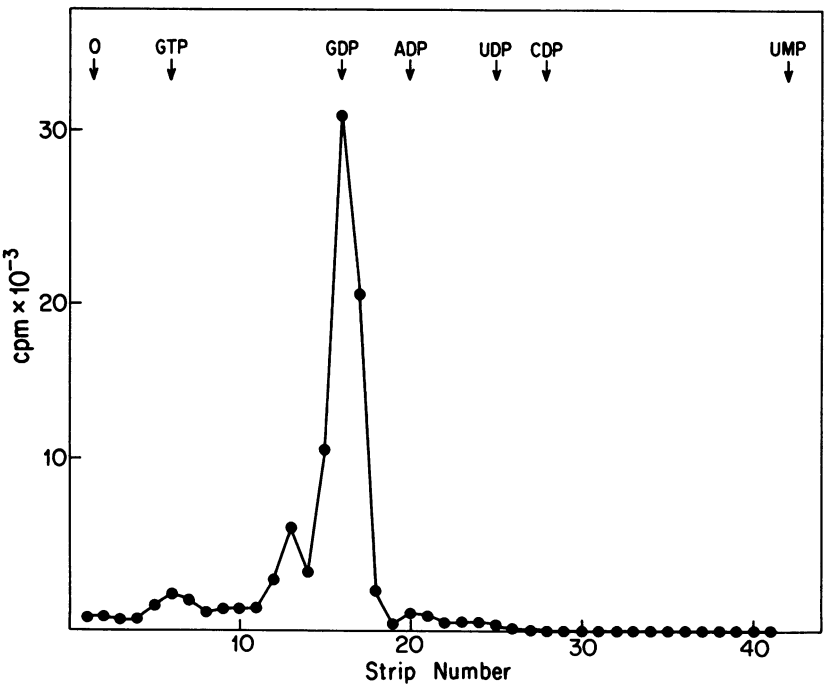
3. Hybridizations of transcript to template. A) Probe was ^{3}H-UTP labelled transcript of specific activity 2.8 x 10^{5} cpm/µg. Experiments were with 0.036 µg of transcript and 2 µg of template L (●——●) or with 1.4 µg of probe alone (0——0). B) Probe was in each case 0.013 µg of in vivo ^{32}P labelled ds template L of specific activity 10^{6} cpm/µg. Experiments were with 2 µg of non-radioactive L (0——0), 2 µg of non-radioactive transcript 1 (●——●), or probe alone (Δ——Δ). Complete conditions of denaturation and renaturation are in Materials and Methods. The 100 minute points in two of the experiments of A and B are fortuitously greater that 100% ds. This is experimental error. Later points did not deviate from the control (100%) by greater than 5%.

conservative synthesis. Neither experiment can give positive evidence of conservative synthesis. Usually, about 2% of the total radioactivity present in TCA-precipitable material after transcription appears in the L dsRNA fraction on non-denaturing agarose gels (Fig. 2). Our experiments with labelled template L have given equivocal results, but they are also consistent with there being a maximum of 2% of ScV particles engaged in semi-conservative synthesis of transcript (not shown). Since we do not know the fraction of particles active in synthesis of transcript, we are

unable to determine if synthesis is conservative as in reovirus (22) or semi-conservative as in Ø6 (38) and AfV-S (28).

The transcript is not capped

The in vitro transcription reaction does not require S-adenosylmethionine (SAM) (see Materials and Methods) and is not stimulated by its presence (not shown). Nor is the ^{3}H-label in the methyl group of ^{3}H-methyl-S-adenosylmethionine incorporated into TCA precipitable material in the in vitro reaction (not shown). It would therefore appear that, like the Penicillium stoloniferum virus S RNA polymerase (26), the ScV transcriptase is SAM independent (and the transcript uncapped). This conclusion is verified by 5' end labelling of the purified full-size transcript. After treatment with alkaline phosphatase, polynucleotide kinase readily labels the in vitro transcript: essentially all the label appears in pGp after alkaline hydrolysis (Fig. 4). This implies that the transcript is not



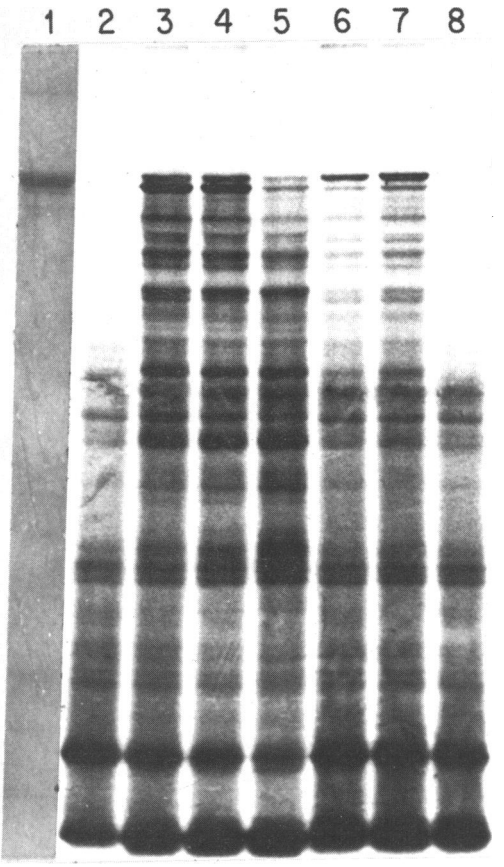
4. Alkaline digestion products of 5' end labelled transcript. DEAE paper electrophoresis was at 5,000 volts for 1 hr at pH 3.5. 1 cm strips were cut and Cerenkov counted. The positions of marker nucleotides are shown.

capped, and has the 5' end pppGp, as do the mature viral RNAs (20). In fact, some counts are present in GTP, which is probably the result of polynucleotide kinase catalyzed exchange between the β phosphate of a partially dephosphorylated pppGp 5' end and the γ phosphate of the γ-^{32}P ATP.

The 5' ends observed in the transcript have to be those of newly synthesized strands, even if transcription is semiconservative. If transcription were semiconservative and only 2% of the particles were active (see above) the synthesis of (an average of) 17% the mass of L (5 experiments) would correspond to 17 transcripts per active particle. Hence only 6% of 1 would be displaced viral strands. Since polynucleotide kinase labelled 14% of the transcript after alkaline phosphatase treatment, and essentially all of the label was in pGp (the authentic 5' terminus), most of the label must have been in newly synthesized strands.

Translation of the in vitro transcript

Denatured, dsL can be translated in the wheat germ system to produce predominantly one polypeptide, the viral capsid polypeptide, ScV-P1 of 88,000 daltons (9). In the Schreier-Staehlin system, danatured dsL produces up to 12 polypeptides, some of which may be premature termination products (Fig. 5). Denatured dsL produces, in the Schreier-Staehlin system, two polypeptides of very closely similar molecular weights, both of which may be present in purified virions (Lane 1, Fig. 5). Undenatured L produces no translation products at all (compare control lane 8 with lanes 6 and 7 in Fig. 5). The in vitro transcript, purified from transcription mixtures by phenol extraction, ethanol precipitation, and CF11 chromatography, is translated to produce all the products made by denatured L (lanes 3,4, and 5, Fig. 5). The in vitro transcript purified by 1.4% agarose gel electrophoresis produces the same translation products, although less efficiently (not shown). No dsL is present in the CF11 preparations. This is confirmed by the successful translation of the transcript without denaturation, a prerequisite for translation of L, as shown above. In fact, denaturation of the transcript prior to translation results in a decrease in its translation (lane 5, Fig. 5), presumably due to fragmentation of the RNA. No messenger RNAs other than the ScV-L transcript appear to be present, since all the translation products of the transcript are also present in in vitro translation of the denatured template L. Control translations of CF11 RNA purified from mock transcription mixtures not incubated prior to extraction show no translation products not present in the -mRNA



5. In vitro translation of transcript and template. Lanes 2-8 are an autoradiograph of an 8% polyacrylamide - 6 M urea gel. Lane 1: 1 µg of protein from ScV-L purified as in Fig. 1 (Coomassie Brilliant Blue stain). The major band is ScV-P1 (88 K). Lane 2: no mRNA. Lane 3: 0.5 µg of transcript. Lane 4: 1.5 µg transcript. Lane 5: 1.5 µg transcript denatured by boiling. Lane 6: 0.75 µg denatured template L. Lane 7: 1.5 µg denatured L. Lane 8: 1.75 µg undenatured L.

controls (not shown). We conclude that the ScV-L in vitro transcription products include the mRNA for the major viral capsid polypeptide.

DISCUSSION

Hybridization experiments with excess dsL and with radioactive transcript probe (l) show that l hybridizes to its template with the $Rot_{\frac{1}{2}}$ characteristic of L. Excess l hybridizes to dsL probe with half the $Rot_{\frac{1}{2}}$ and protects from digestion only half the template probe. The transcript l does not self-hybridize. These hybridization experiments demonstrate that l is the transcript of only one strand of its template.

By the criterion of size, the transcript appears to be a complete transcript of one strand of the template. The 5' end of the transcript is not capped and appears to be pppGp, as are the 5' ends of the template strands. At least one of the 3' ends of L, with the post-transcriptionally added terminal A_{OH} (17), is present in the in vitro transcript l (V. Brennan, L. Bobek and J. Bruenn, unpub. results). This also indicates that the transcript is an exact complement of its template strand.

The ScV-L in vitro transcript serves as a mRNA for the synthesis of the major viral capsid polypeptide (ScV-Pl) and for a number of other products also synthesized on denatured dsL. Translation of the transcript does (in some experiments) favor the synthesis of a polypeptide that may be present as a minor component in virions, slightly smaller than ScV-Pl, although the latter is also made. We currently do not know whether the 12 or so polypeptides other than ScV-Pl made in vitro in the Schreier and Staehelin system programmed with either denatured L or with transcript are artifactual.

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