Conservative Replication and Transcription of *Saccharomyces cerevisiae* Viral Double-Stranded RNA In Vitro

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All double-stranded RNA viruses have capsid-associated RNA polymerase activities. In the reoviruses, the transcriptase synthesizes the viral plus strand in a conservative mode and the replicase synthesizes the viral minus strand, again conservatively. In bacteriophage $\phi 6$ and in some fungal viruses, the transcriptase activity is semiconservative, acting by displacement synthesis. In this work we demonstrate *Saccharomyces cerevisiae* viral RNA replication in vitro for the first time and, using more sensitive techniques than those previously used, show that both the transcriptase and the replicase appear to act conservatively, like those of reovirus. There is therefore clearly no universal life cycle for the double-stranded RNA viruses.

Fungal viruses are double-stranded RNA (dsRNA) viruses without infectious cycles, which persist indefinitely in their host cells and are transmitted only by mating and cell division; their multiple dsRNA segments are separately encapsidated (14, 15). The *Saccharomyces cerevisiae* viruses (ScV) have been studied rather intensively, partly because one of their segments (M) encodes an extracellular toxin (killer toxin) that kills sensitive cells (2). There are a number of killer subtypes with different toxin and immunity specificities, of which killer type 1 has been studied most intensively. In this subtype a large viral dsRNA (L_1) of about 4.8 kilobases encodes the major viral capsid polypeptide (13). Like all dsRNA viruses, ScV have a capsid-associated RNA polymerase (4, 12, 26, 27) that synthesizes primarily the viral plus strand and is thus a transcriptase (4).

Despite attempts by several groups (4, 18, 21), it has not been possible to determine unambiguously whether ScV transcription and replication are conservative, like that of reovirus (22), or semiconservative, like that of bacteriophage $\phi 6$ (25) and some fungal viruses (8, 19). Conservative transcription and replication in reovirus result in the uncoupled synthesis of plus and minus strands. Semiconservative transcription in $\phi 6$ and *Aspergillus foetidus* virus S (AfV-S) result from the synthesis of a new plus strand that displaces the parental plus strand in the template viral dsRNA. In ScV, all results are consistent with either conservative transcription by all active particles or multiple rounds of semiconservative transcription by a minority of the particles. It has been estimated that a maximum of 2% of virus particles could be involved in semiconservative transcription in vitro (4) or in semiconservative replication in vivo (21). Our present experiments demonstrate for the first time that replication (synthesis of minus strand) occurs in virus particles in vitro and that ScV transcription and replication are conservative.

MATERIALS AND METHODS

Virus particle preparation. Virus particles from strain LO14 of *S. cerevisiae* were prepared as previously described (4, 26). After purification by sucrose density gradient centrifugation, 1-ml fractions were collected from the top of the gradient and assayed for A_{260} and transcriptase activity (4).

In vitro synthesis of RNA. Transcriptionally active sucrose

gradient fractions were pooled and pelleted at 40,000 rpm in a 50 Ti rotor for 4 h. Pelleted virus particles were suspended in low-salt buffer IV (4). In a 0.5-ml reaction volume, 8 A_{260} units of suspended virus particles were incubated with in a solution containing ATP, CTP, GTP (all at 0.5 mM), 1 mCi of [α - 32 P]UTP (specific activity, 619 Ci/mmol; ICN Pharmaceuticals Inc.), and 5 mg of bentonite per ml at 37°C for 3 h with constant agitation.

CF11 column chromatography. Total RNA from incubated virus particles was phenol extracted and run on a CF11 column (9) to separate single-stranded RNA (ssRNA) from dsRNA. Some 10 μ g of S14 (19 pmol) was recovered for the experiments shown in Fig. 4 and 5.

RNA ligase labeling of dsRNA. dsRNAs were labeled with [32 P]pCp by T4 RNA ligase as previously described (5).

Gel electrophoresis. S14 RNA labeled by the polymerase activity was isolated from total labeled dsRNA by agarose gel electrophoresis followed by electroelution. Denaturing polyacrylamide gel electrophoresis and strand separation polyacrylamide gel electrophoresis were performed as described previously (17). The same conditions used for DNA were used with dsRNA.

Southern hybridizations. Southern blots with nitrocellulose were performed as described previously (16). Southern blots with GeneScreen Plus (New England Nuclear Corp.) were performed as specified by the manufacturer.

Total digests of RNA. P1 nuclease digests of RNA were performed with 10 μ g of enzyme per ml in 50 mM sodium acetate (pH 5.0)–0.01 mM $ZnCl_2$ –1 mM serine at 65°C for 2 h. Alkaline hydrolysis of RNA was performed by incubation in 0.2 N NaOH overnight at 37°C. Analysis was by electrophoresis on Whatman 540 paper at pH 3.5 for 1 h at 5 kV (5). Spots localized by autoradiography were cut out and Cerenkov counted.

RESULTS

There are two radioisotope methods for distinguishing conservative from semiconservative viral RNA synthesis in vitro. One method involves the use of in vivo-labeled dsRNA. If semiconservative (displacement) synthesis occurs, then labeled plus-strand RNA should appear in the transcription products in the absence of labeled precursors. This method gave our previous estimate of a maximum of 2% of particles involved in semiconservative synthesis (4). More recent experiments gave an estimate of a maximum of 0.7%

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of the particles involved in semiconservative transcription (J. D. Reilly and J. Bruenn, unpublished observations). These results are not shown, because they are less definitive than those obtained by a second method, which involved the determination of the nature of the labeled dsRNA that results from RNA synthesis in vitro by unlabeled viral particles by using labeled precursors. Semiconservative transcription results in labeling of the viral plus strand in template dsRNA. Conservative replication, by synthesis of minus strand on plus-strand templates in "subviral" particles containing only the viral plus strand, would result in the labeling of the viral minus strand. The ScV RNA polymerase activities, from their very discovery (12), have been known to result in labeling of dsRNA to the extent of 1 to 3% of the total RNA synthesized. We have characterized this labeled dsRNA.

To make synthesis, isolation, and characterization of full-sized products as easy as possible, we chose to use virus particles containing defective-interfering viral dsRNAs (S) that are derived from M by internal deletion (5, 6, 10, 24). These are the smallest *S. cerevisiae* viral dsRNAs. The yeast strain that we used (LO14) has dsRNAs of two sizes: L, of 4.8 kilobases, and S14, of 793 bp. Transcription of the S dsRNAs (24) appears to be similar to that of L and M: only the viral plus strand appears in the single-stranded products. The plus strand of L₁ has been identified by in vitro translation combined with sequencing of the 5'-terminal pause products of the viral transcriptase (3, 4) and by reverse transcription on the L plus strand primed by synthetic oligonucleotides (D. Pietras and J. Bruenn, unpublished data). The plus strand of M₁ has been established by sequence analysis of the cDNA clones of the toxin gene (1, 23) combined with N-terminal sequence analysis of the mature toxin polypeptides (1) as well as by direct RNA sequencing of transcript (11). The plus strand of S3 is the same as the plus strand of M₁ (24).

Our analysis of ScV in vitro RNA synthesis products has involved the use of cDNA clones derived from S14. S14 has 540 bp from the 3' terminal portion of the M₁ plus strand and 253 bp from the 5' terminal portion of the M₁ plus strand. We have constructed cDNA clones from all regions of S14 (M. Lee, D. Pietras, M. Nemeroff, B. Corstjanje, L. Field, and J. Bruenn, submitted for publication). Our sequence of S14 agrees with that previously determined by direct RNA sequencing of the 3' terminal 321 bp from another S dsRNA (S3), with the exception of several base differences (24).

To determine the strandedness of in vitro viral RNA synthesis products, we hybridized them to the viral DNA of M13mp9 subclones of cDNA derived from the cloned region. These two clones have a single *TaqI* fragment of 288 bp from the region 376 bp to 664 bp from the 5' end of the plus strand inserted into the *AccI* site in M13mp9. One clone has the S14 plus strand, and the other has the S14 minus strand from this region.

Since the ScV dsRNAs are separately encapsidated, it is possible to partially separate ScV L and ScV S particles. ScV particles enriched in S14 particles were obtained from the more slowly sedimenting side of the ScV peak in a sucrose gradient, but ScV S14 was not well separated from ScV L (see, e.g., Fig. 1).

To label dsRNA at as high a specific activity as possible, we used 1 mCi of high-specific-activity [α -³²P]UTP (619 Ci/mmol) as the sole source of UTP in our in vitro synthesis. This resulted in a rather low concentration of UTP (3.2×10^{-6} M), and so most of the S14 transcript synthesized was not full sized. More than 90% of in vitro transcript is full

FIG. 1. Agarose gel electrophoresis of dsRNA labeled by the ScV polymerase. Agarose gel (1%) of dsRNA from ScV particles incubated with [α -³²P]UTP and three nonradioactive nucleoside triphosphates. Lanes: A, Autoradiograph; E, ethidium bromide stain of the same gel.

sized, with optimal concentrations of nucleoside triphosphates. Total incorporation into ssRNA, isolated by CF11 chromatography, was about 25% of input label, corresponding to a minimum of 1.6 pmol of S14 ssRNA product from 19 pmol of S14 particles. A nondenaturing agarose gel of the resultant labeled dsRNA is shown in Fig. 1. From gels of this kind we estimate that the crude dsRNA has no more than 10% contaminating labeled ssRNA (as a percentage of total radioactivity). The labeled material that did not enter the gel was not further characterized; this material does not occur in every experiment. Clearly, there is incorporation into dsRNA by both ScV L and ScV S particles. Incorporation into dsRNA was 1.3% of incorporation into transcript, of which incorporation 9.3×10^{-3} pmol was in S14 (assuming synthesis of one complete strand in each labeled duplex). Thus approximately $100 \times (9.3 \times 10^{-3})/19$, or 0.05%, of the particles have labeled dsRNA after in vitro synthesis.

The labeled S14 was excised from the gel and electroeluted. The purified dsRNA was analyzed on an 8% polyacrylamide-7 M urea sequencing gel with labeled denatured dsRNA markers (Fig. 2). The labeled dsRNA markers were [³²P]pCp-labeled *Ustilago maydis* virus (UmV) subtype P1 M₁ (1,500 bp), UmV P1 L (355 bp), and ScV S14 (793 bp). The in vitro-labeled S14 is primarily full sized, since the denatured RNA comigrates with denatured [³²P]pCp-labeled S14. Some smaller products are visible. These vary in quantity from preparation to preparation. Some of these may

FIG. 2. Denaturing gel electrophoresis of in vitro-labeled S14. The figure shows an 8% polyacrylamide-7 M urea sequencing gel of in vitro-labeled denatured S14. Lanes: 1, [32 P]pCp-labeled denatured S14; 2, denatured S14 labeled by in vitro synthesis. Markers were denatured UmV M1 (1,500 bp), UmV L (355 bp), and S14 (793 bp), all labeled by T4 RNA ligase with [32 P]pCp.

be degradation products generated in the RNA preparation rather than incomplete strands, since some appear to be present in the [32 P]pCp-labeled S14 as well (see Fig. 2 and 5).

Both the S14 dsRNA (excised from the agarose gel) and the crude single-stranded fraction from CF11 were used as probes in separate Southern blots with the M13 clones carrying the *TaqI* fragment in opposite orientations. As

FIG. 3. Southern blot of S14 cDNA probed with in vitro transcript. The *TaqI* 288-bp fragment is cloned in M13mp9 with the plus strand in the phage (+) or with the minus strand in the phage (-). Phage DNA (1 μ g) in each lane was probed with 10^6 cpm of ScV in vitro-labeled ssRNA. (A) Autoradiograph of Southern blot; (E) ethidium bromide stain of gel used for the Southern blot.

expected, the ssRNA is primarily transcript, since it hybridizes to the *TaqI* fragment with the sequence of the minus strand (Fig. 3). A small amount of the ssRNA is minus strand, since there is some detectable hybridization to the plus strand as well. There is no detectable hybridization to M13 without an insert (7; data not shown). In contrast, the S14 dsRNA hybridizes mainly to the plus strand and is consequently minus strand (Fig. 4). Note that this hybridization was performed with a maximum amount of M13 cDNA clone DNA on the filter, since the specific activity of the dsRNA is only about 2.5×10^5 dpm/ μ g, while the specific activity of the ssRNA is 4.9×10^8 dpm/ μ g. In each case, the DNA on the filter is in molar excess to labeled RNA. The Southern blots in Fig. 3 and 4 were cut up and Cerenkov counted. For S14 dsRNA, 785 and 72 cpm above background hybridized to the plus and the minus strand, respectively; for the S14 transcript, 117 and 2,577 cpm above background hybridized to the plus and the minus strand, respectively. These results show that 96% of the ssRNA is transcript and 92% of the label in dsRNA is minus strand.

We confirmed, by a strand separation experiment, the synthesis of minus strand alone in the dsRNA labeled by in vitro synthesis. Both strands of S14 are equally labeled by RNA ligase in vitro. In 5% polyacrylamide gel electrophoresis of denatured S14, the faster-migrating strand is the plus strand and the slower-migrating strand is the minus strand, as identified by hybridization to the M13 subclones used in the previous experiment and by direct RNA sequencing of [32 P]pCp-labeled separated strands (Lee et al., submitted). Only the minus strand of S14 is labeled in dsRNA by the ScV polymerase activity in vitro (Fig. 5). No labeled plus strand is apparent, although there are some degradation products present in both dsRNA preparations.

We performed two sets of control experiments to show that the label incorporated into double-stranded S14 was not artifactual and occurred throughout the RNA. First, since the 32 P incorporated should, in every case, be 5' to a UMP residue in the RNA, hydrolysis of the RNA with a nuclease creating nucleoside 5'-monophosphates should release only labeled 5' UMP. Such an enzyme is P1 nuclease, and it does release radioactivity in nothing but UMP (Table 1). Alkaline hydrolysis of RNA should release all nucleotides as the nucleoside 2',3'-monophosphates. In this case, the 32 P should be distributed among the four nucleoside monophosphates according to the frequency with which each occurs as

FIG. 4. Southern blot of S14 cDNA probed with S14 dsRNA labeled by in vitro synthesis. Conditions are as for the experiment shown in Fig. 3, but 10 μ g of phage DNA in each lane was probed with denatured S14 dsRNA labeled by ScV particles in vitro (2×10^5 cpm).

a 5' nearest neighbor of U. The results are consistent with a nearly random incorporation of UMP throughout the S14 minus strand (Table 1). We conclude that incorporation of label into dsRNA by ScV particles is not artifactual. In a second set of experiments, we hybridized labeled S14 dsRNA to the phage DNA of M13 hybrid phages with cDNA inserts complementary to bases 224 through 347. After hybridization, filters were treated with pancreatic RNase in $6\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) to remove any unhybridized regions. The results were like those of Fig. 4: radioactivity was in the S14 minus strand (results not shown). Similar results were obtained when the filter of Fig. 4 was treated with pancreatic RNase in $6\times$ SSC. We conclude that label is incorporated into all regions of the S14 minus strand tested (residues 224 through 664).

DISCUSSION

We observed that the label appearing in ScV dsRNAs during incubation of particles with labeled precursors in vitro was almost entirely in the minus strand. Both transcription and replication in ScV thus appear to be conservative, like those processes in reovirus.

The small proportion of labeled plus strand present in the isolated S14 dsRNA could be due to contamination with transcript, since 98.7% of the incorporation is transcript. There are no extensive inverted repeats in S14 (Lee et al., submitted), so we are not observing hybridization of minus

FIG. 5. Strand separation of S14 dsRNA labeled by in vitro synthesis. The figure shows a 5% polyacrylamide gel of denatured dsRNA. Lane 1 has denatured S14 dsRNA labeled by in vitro synthesis. Lane 2 has denatured S14 dsRNA labeled with [32 P]pCp by T4 RNA ligase. Plus strand (+) and minus strand (-) are indicated. Direction of migration is top to bottom.

TABLE 1. Total hydrolysis products of S14 dsRNA^a

Hydrolysis product	Fraction of total radioactivity ^b after:	
	P1 nuclease digestion	NaOH digestion
CMP	0.0008 (0)	0.25 (0.27)
AMP	0.000 (0)	0.29 (0.25)
GMP	0.002 (0)	0.25 (0.26)
UMP	0.997 (1)	0.21 (0.22)

^a RNAs were digested, and the hydrolysis products were separated and analyzed as described in Materials and Methods.

^b Numbers in parentheses are predicted fractions.

strand to itself. Strand separation gel electrophoresis confirmed that the vast majority of the labeled dsRNA is minus strand.

If transcription in ScV were semiconservative, then only those particles with a labeled plus strand could have been engaged in transcription. We can estimate that this would be a maximum of $0.084 \times 9.3 \times 10^{-3}$ pmol/19 pmol, or 4.1×10^{-5} , of the particles. Here 0.084 is the maximum fraction of label in dsRNA present in plus strand, 9.3×10^{-3} pmol is the quantity of labeled S14 dsRNA molecules, and 19 pmol is the total amount of S14 dsRNA present in particles in the experiment. Previous estimates were that this fraction had to be smaller than 2% (4).

We do not believe that it is reasonable to ascribe all in vitro transcription, which can amount to as much as one transcript per particle, to 0.004% of the particles. At least 0.05% of the particles are active in replication (synthesis of minus strand), yet only 1.3% of the RNA synthesized is minus strand. It seems very unlikely that less than 1/10 as many particles (0.004%) synthesize 80 times as much RNA. We therefore conclude that transcription is conservative in ScV.

The synthesis of S14 minus strands that appear in S14 dsRNA is not accompanied by synthesis of plus strands (at least not more than 8.4% of the time). If the preexisting plus strands that serve as templates for this minus strand synthesis were synthesized in the same manner as the observed plus strand synthesis in vitro, then replication must be conservative. The presence of a small amount of minus strand in the transcript fraction may be due to the release of aborted minus strands from particles or from partial degradation of dsRNA during isolation. There is no evidence for the presence of free minus strand in vivo (21). The nature of the ScV particles engaged in replication remains to be determined, but since only minus strand is synthesized, they may be subviral particles containing only the plus strand, as in reovirus. At present, the resolution of our sucrose gradients is inadequate to determine the nature of the replicating particles, owing to the heterogeneity of the viral particles (20).

ACKNOWLEDGMENTS

We thank T.-H. Chang for advice and D. Kowalski for providing nuclease P1.

This work was supported by Public Health Service grant GM22200 from the National Institutes of Health and by grant H104N from the Research Foundation of the State University of New York.

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