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Yeast dsRNA viral transcriptase pause products: identification of the transcript strand

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ABSTRACT

ScV-L is a double-stranded RNA virus of the yeast Saccharomyces cerevisiae. The virus possesses a capsid-associated transcriptase activity the product of which is a single-stranded RNA complementary to only one strand of the double-stranded RNA template (L). We show that the U-rich 3' terminus of L is the initiation site of transcription and that a number of pause products are made. One prominant product has the sequence pppGAAAAAUUUUU-AAAUUCAUAUAACU$_{OH}$.

INTRODUCTION

Most strains of Saccharomyces cerevisiae possess a simple non-infectious, double-stranded (ds) RNA virus (ScV) with two separately encapsidated dsRNAs (1,2). The smaller of the two dsRNAs (M of 1.9 kbp) encodes a protein toxin (12,000 daltons) which is lethal to yeast strains not harboring ScV-M particles (3,4,5). The larger dsRNA (L of 4.8 kbp) encodes the major capsid protein (88,000 daltons) of both ScV-L and ScV-M particles (6,7,8). Internal deletion mutations of M result in defective - interfering particles (ScV-S particles; 9,10,11). Replication of ScV-M and ScV-S particles is dependent on the products of a number of nuclear genes not needed for ScV-L replication (12,13).

Both 5' ends of M and L are pppGp and both 3'-ends are CA$_{OH}$ suggesting the end structure Gppp$^{CA_{OH}}$ (14,15). L and M have a U-rich and a C-rich 3' terminus (15). Both 3'-ends of L and M are heterogeneous, the U-rich end being less heterogeneous than the C-rich end in each case (15). Aside from their ends, L and M have little sequence homology (9).

ScV-L and ScV-M particles isolated from stationary phase cells possess a transcriptase activity (16,17,18,19,20). In vitro transcription using intact ScV-L or ScV-M particles produces single-stranded RNAs (ssRNAs) equal in length to denatured L or M respectively (16,18,19,20). These newly syn-

thesized ssRNAs are extruded from the viral particles. The *in vitro* transcript of L is complementary to one strand of its double-stranded template (18,20) and can serve as a mRNA for the *in vitro* translation of the major capsid protein of ScV-L and ScV-M particles (20). The 5'-end of the L transcript is pppGp (20); it is uncapped, as are both 5'-ends of the dsRNA template (14,15). Since ScV-L and ScV-M contain the same major viral polypeptide, which is at least 98% of the protein in the virus (D. Reilly, unpub., K. Bostian, personal comm.) the transcriptase activity of ScV-L and ScV-M may be the same, and may therefore recognize the same sequences in L and M. There is only one such sequence at a 3'-end (where transcription originates) and that is at the U-rich end (15,21). Consequently, we have postulated that the viral transcriptase initiates at this end (15).

There are a number of variants of ScV with different toxin specificities, two of which, K1 and K2, have been extensively characterized (22). The dsRNAs of these are distinguishable in sequence and have been designated L_1 and M_1, L_2 and M_2 respectively (21). The current experiments concern only ScV-L_1 particles.

In this work we show that the U-rich 3'-end of L, as expected, is the initiation site of transcription and that the transcript initiates at the penultimate nucleotide C of the 3'-end. The 5'-end of the L_1 transcript has a sequence similar to that of Alfalfa mosaic virus RNA4 (23).

MATERIALS AND METHODS

Isolation of ScV particles

Viral particles were obtained from yeast strain S7, which contains only ScV-L_1 particles (21,24). Yeast cells were grown to stationary phase in 1% yeast extract, 1% peptone and 3% ethanol. ScV particles were isolated essentially as described (17), except that a second 5-20% (w/v) sucrose gradient was used for further purification.

Synthesis and 3'-end labeling of viral transcript

The fractions with the highest transcriptase activities from the second sucrose gradient of ScV-1 particles usually contained only L dsRNA although occasionally small amounts of ribosomal RNA contaminants were present. After the transcription reaction, the *in vitro* transcript for 3'-end labeling was purified by CF-11 cellulose chromatography to separate double-stranded and single-stranded RNA. The single-stranded fraction from the CF-11 column contained full length transcript (95%), no L dsRNA, and occasionally some rRNA contaminants, as judged by electrophoresis of a sample of this fraction

on 1.4% agarose slab gels. The transcript fraction from the CF-11 column was further purified by SP-Sephadex C-50 or Sephadex G-25 chromatography before 3'-end labeling.

The CF-11 column transcript fraction RNA was labeled at its 3'-ends with ^{32}pCp using T4RNA ligase (15). The labeled RNA was isolated by phenol extraction and ethanol precipitation. This was followed by Sephadex G-25 chromatography to separate the labeled RNA from any remaining free ^{32}pCp.

Analysis of the end-labeled transcript

The 3'-end labeled oligonucleotides isolated by the 2D gel system of De Wachter and Fiers (25) were sequenced by the chemical method of Peattie (26). We refer to the 3' terminal sequences without their added ^{32}pCp.

For complete alkaline hydrolysis, RNA samples were incubated at 37°C for 20 hours in 5 microliters of 0.2N NaOH. The products were separated on Whatman 540 paper (pH 3.5) at 5000 volts for 1 hour (27).

Hybridization of pCp labeled *in vitro* transcript to L dsRNA and *in vitro* transcript

Denatured L dsRNA (5 µg) and denatured *in vitro* transcript (5 µg) were baked onto separate nitrocellulose filters for hybridization with ^{32}pCp labeled *in vitro* transcript (28,29). The unlabeled RNAs were denatured in 8 microliters of 10 mM CH_3HgOH for 1 hour at 50°C then blotted onto nitrocellulose filters which had been equilibrated with 20 x SSC (3 M NaCl., 0.3 M sodium citrate). The filters were dried, then baked at 80°C for 2 hours under vacuum.

The filters were prehybridized for 3 hours at room temperature in 0.5 ml of hybridization mix (4XSSC, 0.1% sodium pyrophosphate, 5X Denhardt-0.1% BSA, 0.1% ficoll, 0.1% polyvinyl pyrrolidone, 0.1% SDS, 150 µg/ml denatured and sheared calf thymus DNA and 10% dextran sulfate). The mix was changed twice.

Hybridization to the filters was performed in glass scintillation vials containing 0.5 ml hybridization mix and 0.2 ml of the denatured labeled probe (40,000 cpm Cerenkov) at 50°C for 40 hours with shaking. Filters were washed with 1 M NaCl, 0.5 X Denhardt, 0.045 M Tris-Cl pH 7.9, 0.1% SDS, 0.1% sodium pyrophosphate until there were no detectable counts above background in the wash. Filters were dried and counted Cerenkov.

Isolation of L dsRNA used for hybridization

L dsRNA was purified as previously described (15). L dsRNA eluted from the agarose gels was further purified by SP-Sephadex C-50 chromatography.

RESULTS

Isolation of 3'-end labeled RNA

Samples of the 3'-end labeled CF-11 transcript were run on 1.4% agarose slab gels. The ^{32}P label did not run with the full length transcript but in a broad band at about the position of tRNA marker. This labeled single-stranded RNA was not visible in the gel by ethidium bromide staining. Consequently, we fractionated the 3'-end labeled RNA by 2D polyacrylamide gel electrophoresis (25). Figure 1 shows an autoradiograph of a 2D gel of 3'-end labeled CF-11 column transcript fraction RNA which was not digested with T1 RNase. Several large oligonucleotides (1,3,4,5 and 6) are present. Digestion of this RNA with T1 RNase results in the loss of the slowly migrating oligonucleotides 3,4,5 and 6 in Figure 1 and the appearance of the smaller

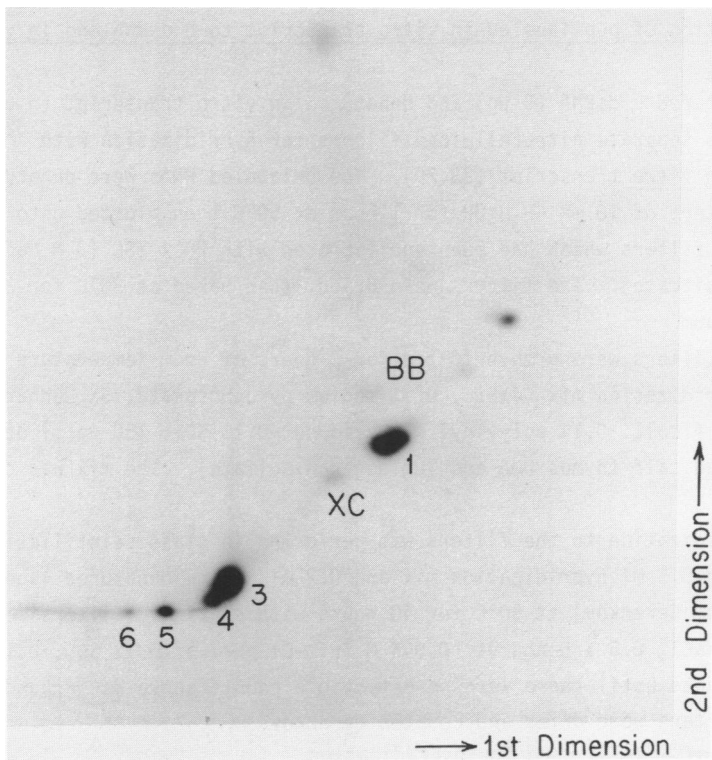

Figure 1. 2D polyacrylamide gel (25) of 3'-end labeled, undigested, CF-11 column transcript fraction RNA that was purified by SP-Sephadex C-50 chromatography. The bromophenol blue marker (BB) was run 13 cm. The xylene cyanol marker (XC) was run 7 cm.

3'-end labeled oligonucleotides 7 through 15 in Figure 2. This indicates that the smaller oligonucleotides (Figure 2) are T1 products of the larger oligonucleotides (Figure 1).

Oligonucleotide 2 in Figure 2 ran at about the same position as oligonucleotide 1 in Figure 1. The complete alkaline hydrolysis products of both of these 3'-end labeled oligonucleotides is Up. Oligonucleotide 2 differs from oligonucleotide 1 only in the removal of a 5'-terminal pppGp (see below).

The CF-11 column transcript fraction RNA used in Figures 1 and 2 was purified on a SP-Sephadex C-50 column before labeling with ^{32}pCp. Figure 3 shows a 2D gel of undigested 3'-end labeled CF-11 column transcript fraction RNA which was purified instead by Sephadex G-25 chromatography before the 3'-end labeling reaction. This RNA fraction contains not only the 3'-end label-

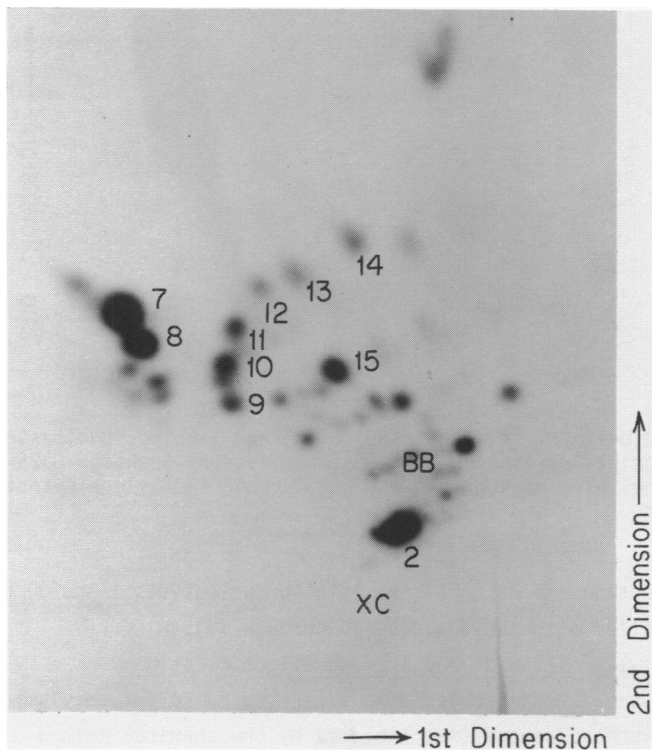

Figure 2. 2D polyacrylamide gel (25) of 3'-end labeled, T1 RNase digested, CF-11 column transcript fraction RNA that was purified by SP-Sephadex C-50 chromatography. The bromophenol blue marker (BB) was run 13 cm. The xylene cyanol marker (XC) was run 7 cm.

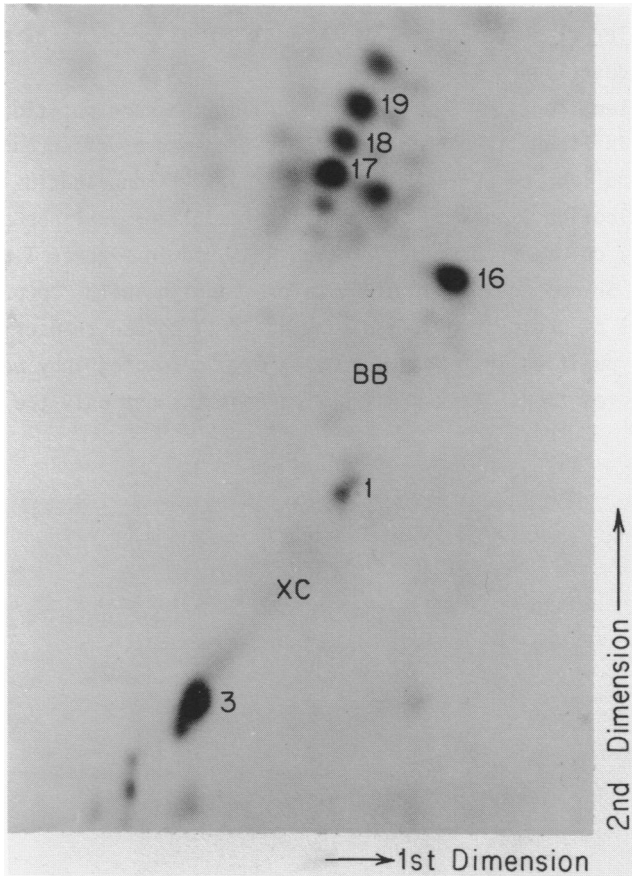

Figure 3. 2D polyacrylamide gel (25) of 3'-end labeled, undigested, CF-11 column transcript fraction RNA that was purified by Sephadex G-25 chromatography. The bromophenol blue marker (BB) was run 19 cm. The xylene cyanol marker (XC) was run 10 cm.

ed oligonucleotides found in Figure 1 (oligonucleotides 1 and 3) but also a number of smaller oligonucleotides (16 through 19).

Sequence analysis of the 3'-end labeled oligonucleotides

Some of the 3'-end labeled RNAs separated by 2D polyacrylamide gel electrophoresis were sequenced (see Table 1) by the chemical method of Peattie (24).

Oligonucleotide 1 from Figures 1 and 3 has the sequence pppGAAAAAUUUUU-AAAUUCAUAUAACU$_{OH}$ (data not shown). This sequence is complementary to the U-rich 3'-end of L dsRNA (see Table 2)

TABLE 1. Sequences of L 5'-end transcript fragments

3'-end labeled oligonucleotides	
1 (see figures 1 and 3)	pppGAAAAAUUUUUAAAUUCAUAUAACU$_{OH}$
2 (see figure 2)	AAAAAUUUUUAAAUUCAUAUAACU$_{OH}$
16 (see figure 3)	pppGAAAAAUUUU$_{OH}$
17 (see figure 3)	pppGAAAAA$_{OH}$
18 (see figure 3)	pppGAAAA$_{OH}$
19 (see figure 3)	pppGAA$_{OH}$

The 5' pppGp is deduced from previous 5'-end labeling experiments of L transcript (19).
The 3' OH is deduced from the specificity of T4 RNA ligase.

Figure 4 shows a sequencing gel of oligonucleotide 2 (Figure 2), that migrates to about the same position in the 2D gel system as oligonucleotide 1 (Figure 1). It has the sequence AAAAAUUUUUAAAUUCAUAUAACU$_{OH}$. The 5' terminal pppGp of oligonucleotide 1 has been removed by T1 RNase digestion. Oligonucleotides 3 and 4 have the same 5' sequence as oligonucleotide 1 but have an additional 3' sequence of about 40 nucleotides the first few of which correspond to the remaining sequenced region of L1 (not shown). Oligonucleotides 5 and 6 are about 300 and 600 nucleotides long, respectively, and originate from the same 5'-end as oligonucleotide 1 (not shown). We have sequenced some of the fast migrating 3'-end labeled oligonucleotides from the transcript fraction RNA purified by Sephadex G-25 chromatography (Figure 3). Their sequences (Table 1) are also complementary to the U-rich 3'-end of L dsRNA (Table 2). The 5' terminal G's were assigned by chemical cleavage, the 5' triphosphate from previous 5' labeling experiments (20) and from electrophoretic mobilities.

The oligonucleotides present in the CF-column transcript fraction that are labeled with ^{32}pCp could have originated by abortive synthesis of either

TABLE 2. Comparison of the U-rich 3'-end of L dsRNA (15,21) with the 5'-end of the L transcript

5'-------GUAUGGGGAGUUAUAUGAAUUUAAAAAUUUUUCA$_{OH}$ 3' (U-rich 3'-end of L)
3'--------------UCAAUAUACUUAAAUUUUUAAAAAGppp 5' (5'-end of L transcript)

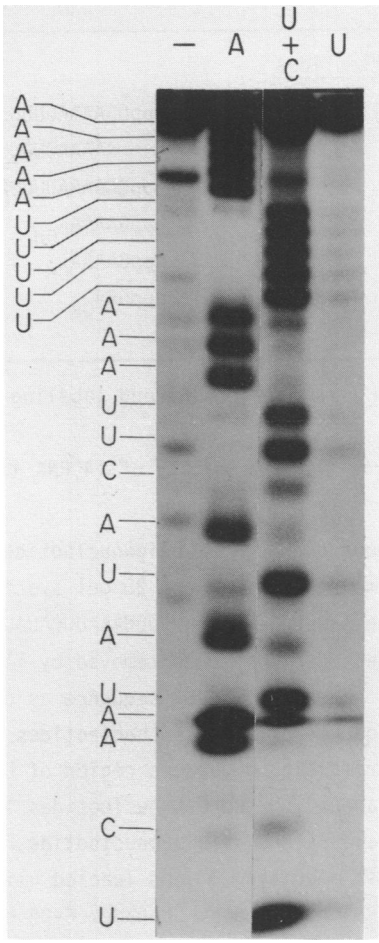

Figure 4. Sequencing gel of 3'-end labeled L transcript oligonucleotide 2 (see Figure 2). The chemical cleavage products of this 3'-end labeled, T1 oligonucleotide were run on a 20% polyacrylamide, 7M urea gel (26). The control and A lanes and the U & C and C lanes were run on separate regions of the same gel. They have been abutted for this figure.

strand of the viral dsRNA. If they arose by abortive transcription, (that is, synthesis of the message strand), they should be of the same strandedness as the full sized transcript ℓ. Abortive transcription products should hybridize to the dsRNA template L, but not to the full sized transcript ℓ.

Hybridization

The 3'-end labeled oligonucleotides contained in the in vitro transcript

fraction RNA purified by SP-Sephadex C-50 chromatography were hybridized to either denatured L dsRNA or to *in vitro* transcript that was isolated by the same procedure as the 3'-end labeled probe and therefore includes both full-length transcript (~95%) and the oligonucleotides resulting from abortive transcription. Denatured 3'-labeled transcript probe (4.0×10^4 cpm Cerenkov) was hybridized to denatured L dsRNA (5 µg) and denatured transcript (5 µg) on separate nitrocellulose filters. Under these conditions L or ℓ is in molar excess to the ^{32}pCp labeled oligonucleotides. After hybridization, the filters were washed until no more ^{32}p was removed from the filters. The results are shown in Table 3.

The 3'-end labeled transcript fraction RNA (oligonucleotides 1-6) did not hybridize to the *in vitro* transcript but did hybridize to denatured L dsRNA. Therefore the small products of *in vitro* transcription are of the same strandedness as the full sized transcript ℓ. A control experiment verifies that the full-sized transcript and the small *in vitro* transcription products are homologous. Both full-length transcript ℓ and the ^{32}pCp labeled oligonucleotides hybridize efficiently to the 300 bp insert of the same cDNA clone of L (Bobek, unpub.). The labeled oligonucleotides do not hybridize to the full-length transcript, even though the longer oligonucleotides (of 300 and 600 bp) are as long or longer than the cDNA insert.

DISCUSSION

The labeling of the 3'-ends of the *in vitro* products of the ScV-L transcriptase reveals a number of short 5' products of 3 to 600 nucleotides in length, but not the full-sized transcript ℓ. There are two reasons for the absence of ℓ among the labeled products. First, ℓ is present in low molarity. We estimate that the short products are about 5% of the RNA synthesized *in vitro*. They would then account for nearly 90% of the 3'-ends. Second, authentic ℓ isolated from agarose gels is very inefficiently labeled by T4 RNA ligase, presumably because its 3'-end is buried in secondary structure.

TABLE 3. Hybridization with 3'-end labeled transcript RNA

Nitrocellulose filters	cpm (Cerenkov) after hybridization
control filter (blank)	46
in vitro transcript RNA filter	105
L dsRNA filter	4,404

We have had similar difficulties with the large yeast ribosomal RNAs, but the ScVdsRNAs or 5S RNA eluted from agarose gels are efficiently labeled by T4 RNA ligase. The observed products are not nuclease artifacts: such a nuclease would have to be a 3' exonuclease since all the products terminate with a 3'-hyroxyl. No such activity is detectable in the T4 RNA ligase we use (15) nor in the transcriptase reaction mix, since the rRNAs and dsRNA present are not degraded: the only labeled products are from the ℓ transcript strand. In addition, in each of four preparations of transcript from four different ScV-L preparations, the same 5' truncated transcription products appeared. It is therefore very unlikely that a degradation process is responsible for them: they are probably the results of specific termination events. They are pause products of the capsid-associated transcriptase. Our previous determination of the 5' pppGp of the viral transcript (20) was also of the transcript pause products, since these are in vast molar excess.

ScV-L and ScV-M contain the same major viral capsid polypeptide, which constitutes at least 98% of the capsid protein (D. Reilly, unpub., K. Bostian, personal comm.). Consequently, the capsid-associated transcriptase of ScV-L and ScV-M is probably the same, and probably recognizes similar sequences in both dsRNAs. At least part of this sequence must be close to the region in which initiation occurs. Since the 3'-end with the most striking homology is the U-rich end, we have speculated that transcription initiates at this end (15). The present results confirm this expectation, and predict that the ScV-M and ScV-S transcripts should begin pppGAAAAAUAAAGAAAU.....

We have noticed structural similarities between the plus (transcript) strands of ScV and the plus strands of several segmented ssRNA plant viruses (the Ilarviruses) at their 3'-ends (21). Both have hairpins with A and U in the loop immediately followed by the sequence AUGC at the 3' terminus (21, 30,31). This similarity extends to the 5' non-coding region as well. Alfalfa mosiac virus (closely related to the Ilarviruses) initiates with a G and has no other G within the first 39 nucleotides at the 5'-end of its RNA4 (23). The ℓ transcript of ScV killer type 1 (ℓ1) described here initiates with a G and has no other G within at least its first 33 nucleotides. RNA4 is 85% A or U in its 5' non-coding region; ℓ1 is 76% A or U in its first 33 nucleotides (a region without AUG codons). Both RNAs have the sequence AUU UUUAA within 5 or 6 nucleotides from the 5'-end. These similarities are striking enough to warrant speculation that the segmented dsRNA viruses of yeast and this class of segmented ssRNA plant viruses share a common ancestor.

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