Native Replication Intermediates of the Yeast 20 S RNA Virus Have a Single-stranded RNA Backbone*

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20 S RNA virus is a positive strand RNA virus found in Saccharomyces cerevisiae. The viral genome (2.5 kb) only encodes its RNA polymerase (p91) and forms a ribonucleoprotein complex with p91 in vivo. A lysate prepared from 20 S RNA-induced cells showed an RNA polymerase activity that synthesized the positive strands of viral genome. When in vitro products, after phenol extraction, were analyzed in a time course, radioactive nucleotides were first incorporated into double-stranded RNA (dsRNA) intermediates and then chased out to the final single-stranded RNA products. The positive and negative strands in these dsRNA intermediates were non-covalently associated, and the release of the positive strand products from the intermediates required a net RNA synthesis. We found, however, that these dsRNA intermediates were an artifact caused by phenol extraction. Native replication intermediates had a single-stranded RNA backbone as judged by RNase sensitivity experiments, and they migrated distinctly from a dsRNA form in non-denaturing gels. Upon completion of RNA synthesis, positive strand RNA products as well as negative strand templates were released from replication intermediates. These results indicate that the native replication intermediates consist of a positive strand of less than unit length and a negative strand template loosely associated, probably through the RNA polymerase p91. Therefore, W, a dsRNA form of 20 S RNA that accumulates in yeast cells grown at 37 °C, is not an intermediate in the 20 S RNA replication cycle, but a by-product.

Positive strand RNA viruses encode their RNA-dependent RNA polymerases (RdRps)1 and utilize them to replicate their RNA genomes in conjunction with other viral or host proteins. A well known example is the RNA coliphage Qβ replicase, consisting of a viral-encoded RdRp and host proteins, which can synthesize the positive and negative viral RNAs in vitro (1). Unlike the holoenzyme of coliphage, most of the replicases of eukaryotic viruses are associated with intracellular membranous structures (2. 3). This makes their identification and characterization difficult. Although biochemical and genetic approaches have been attempted, replication mechanisms of eukaryotic viruses remain to be elucidated. If a membrane-free replication system is available, it may provide direct information on the replication mechanism and also on the components involved in this process.

Most laboratory strains of Saccharomyces cerevisiae harbor 20 S RNA virus. The virus belongs to the genus Narnavirus of the family Narnaviridae (4). It is a persistent virus and has no extracellular transmission pathway. Typical of fungal viruses, 20 S RNA virus does not kill the host nor render phenotypic changes to the host. This virus was originally described as an RNA species induced under nitrogen starvation conditions (5, 6). The copy number of 20 S RNA genome in some strains becomes almost equivalent to those of rRNAs under the induction conditions. The complimentary (negative) strands count less than 1–2% compared with the amount of positive strands, a ratio similar to those found among positive strand RNA viruses (7). The viral genome is small (2514 nt) and has no 3′-poly(A) tail and perhaps no 5′-cap structure (8). It encodes only a single protein of 91 kDa (p91) (9, 10). p91 contains four amino acid motifs well conserved among RdRps. Fewer strains of yeast harbor another narnavirus called 23 S RNA virus. Its genome is slightly larger than 20 S RNA genome (2891 nt) and encodes a single protein of 104 kDa (p104) (8, 11). The RdRp motifs of p91 and p104 are most closely related to those of the RdRps of RNA coliphages (12). These viruses are compatible in the same host and do not exclude each other. When yeast cells harboring these viruses are grown at high temperature (37 °C) the cells accumulate double-stranded RNAs (dsRNAs) called T and W (13). T and W are the dsRNA forms of 23 S RNA and 20 S RNA, respectively (8). Because the viral genomes do not encode coat proteins, the RNA genomes are not encapsidated into viral particles (14–16). Instead, they form ribonucleoprotein complexes with their cognate RNA polymerases in a 1:1 stoichiometry and reside in the host cytoplasm (17).

Previously we detected a 20 S RNA positive strand-synthesizing activity in lysates prepared from 20 S RNA-induced cells (18). The activity was not associated with host cell membranes and migrated in sucrose gradients almost the same as 20 S RNA/p91 complexes. In this study, we analyzed replication intermediates of 20 S RNA synthesis in vitro and found that the native replication intermediates have a single-stranded RNA (ssRNA) backbone, consisting of a positive strand less than unit length and a template negative strand loosely associated, perhaps held by p91. Deproteination with phenol converted the RNA in the intermediate to a double-stranded form. This indicates that W, the dsRNA form of 20 S RNA, is not a replication intermediate but a by-product of 20 S RNA replication. The completion of RNA synthesis in vitro resulted in the
release of the synthesized positive strand as well as the negative strand template from the replication complex. Our results suggest that the positive and negative strand ssRNAs released from replication complexes are associated with protein.

**EXPERIMENTAL PROCEDURES**

**Preparation of Cell Lysates**—Throughout this work, the yeast strain 37-4C (a kar1-1 leu1) 20 S RNA, 23 S RNA, L-A-g, L-B-g (13) was used to prepare cell lysates. Yeast cells were grown in YPAD broth (1% yeast extract, 2% peptone, 0.04% adenine sulfate, and 2% glucose) for 2 days at 28 °C and then transferred to 1% potassium acetate (pH 7.0) at 95 °C for 16 h to induce 20 S RNA. The cells were harvested and washed once with water. The cells were suspended in lysis buffer (0.1 M Tris-HCl, pH 8.0, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) and broken with glass beads by vortex mixing 10 times at the maximum speed for 15 s. Cell debris was removed by centrifugation in an Eppendorf centrifuge at the maximum speed at 4 °C for 5 min. The supernatant was centrifuged again at 80,000 rpm (228,000 g) in a Beckman TL 100.2 rotor at 4 °C for 1 h. The precipitate was suspended in the incubating buffer with 10% glycerol and kept at −70 °C before use.

The strain also harbors 23 S RNA virus, but the lysates prepared (Promega). The reaction was stopped at 5 min by the addition of 15 mM EDTA, 0.5 mM each of ATP, GTP, and CTP, and 20 mM dithiothreitol. After centrifugation, the filtrate was divided into aliquots. The aliquots were incubated again at 30 °C for 60 min with or without 15 mM MgCl2. Some aliquots were further supplemented with one of non-labeled NTPs (0.5 mM) or all of them together. After incubation, the complexes were electrophoresed onto a positively charged nylon membrane in TAE buffer at 270 mA for 1 h at 4 °C using an electro-transfer unit from the Mini Protean II series (Bio-Rad). The membrane was either directly exposed to an x-ray film for autoradiography (Fig. 5) or hybridized with a 32P-labeled probe specific to the negative strand of 20 S RNA (Fig. 6). The probe was made by T7 RNA polymerase from Smal-digested pALI-22 (17), which is complementary to the negative strand sequence from nt 1 to 227 numbered from the 5'-end.

**RESULTS**

**dsRNA Replication Intermediates**—20 S RNA virus-harbouring cells, when grown under nitrogen-starvation conditions, accumulate a large amount of positive strands of 20 S RNA. Lysates prepared from these cells, enriched in 20 S RNA, have an RNA polymerase activity in vitro. The RNA products, extracted with phenol and heat-denatured in the presence of 7 M urea, migrated in an agarose gel as mostly a single-stranded form of 20 S RNA (Fig. 1A). We showed previously that most of the in vitro products were full-length positive strands of 20 S RNA and that the incorporation of radioactive nucleotides required RNA synthesis and was not the result of a simple end-addition reaction to preexisting 20 S RNA molecules (18). When the in vitro products were hybridized with positive or negative strands of 20 S RNA or 23 S RNA transcribed from cDNAs, the labeled products mostly hybridized with 20 S RNA negative strands as observed previously (Fig. 2A). This demonstrates that the in vitro products were mostly positive strands of 20 S RNA. Scanning of the autoradiogram shown in Fig. 2A indicates that synthesis of 20 S RNA negative strands counts ~2% of that of positive strands, roughly the same as the ratio of negative to positive strands present in the lysate. When parts of the sample shown in Fig. 1A were analyzed without heat denaturation, we found another, slower moving mobility band whose mobility corresponded to W, a dsRNA form of 20 S RNA (Fig. 1B). During incubation, radioactive nucleotides were first incorporated into the dsRNA form with a peak at 20 min, and then the label in the dsRNA form decreased gradually (Fig. 1B). On the other hand, the appearance of the labeled ssRNA form was slightly delayed compared with that of the dsRNA form, but its amount increased steadily during the incubation (Fig. 1B). These results suggest a precursor-product relationship between them. This was confirmed by the pulse-chase experiments shown in Fig. 1D. During the first 5-min labeling period, most of labeled nucleotides were incorporated into the dsRNA form. After the addition of an excess amount of non-radioactive nucleotide, the label in the dsRNA form was converted into the final ssRNA products. When the same samples were heat-denatured in the presence of 7 M urea, the label in the dsRNA intermediates disappeared and was converted to ssRNA forms (Fig. 1C). This indicates that the labeled positive strands were non-covalently associated with negative strands. In the early labeling period we observed heterogeneous molecules moving faster than full-length 20 S RNA in the gel (Fig.
FIG. 1. Products of in vitro RNA synthesis. A and B, time course of 20 S RNA synthesis. A cell lysate prepared from 20 S RNA-harboring cells was incubated at 30 °C in the standard RNA polymerase reaction mixture containing \[^{32}P\]UTP. At the times indicated samples were withdrawn and extracted with phenol. Each sample was divided into two portions. One set of the samples was heat-denatured in the presence of 7 M urea and separated in an agarose gel (A). The other set was analyzed in the same gel but without heat denaturation (B). Autoradiograms of the gels are shown. The mobility of 20 S RNA positive (+) strands and W dsRNA, a double-stranded form of 20 S RNA, is indicated. C and D, pulse-chase of in vitro synthesis. A cell lysate was incubated in the standard RNA polymerase reaction mixture containing \[^{32}P\]UTP. At 5 min, non-labeled UTP (final concentration, 2 mM) was added to the mixture (indicated by the arrow), and the incubation was continued. Samples were withdrawn at the times indicated and processed as described above. The heat-denatured (C) or non-denatured (D) samples were analyzed in an agarose gel and exposed to an x-ray film. E, a cell lysate was incubated as described in A and B. The RNA polymerase products were precipitated with 10% trichloroacetic acid in the presence of salmon sperm DNA as carrier and counted.

FIG. 2. RNA products in vitro are mostly positive strands of 20 S RNA made by chain-elongation. A, non-labeled RNA containing the full sequence of the positive (+) or negative (−) strand of 20 S or 23 S RNA was made by T7 or T3 RNA polymerase and separated in an agarose gel. After electrophoresis the RNAs were denatured in the gel and blotted onto a nylon membrane. The membrane was hybridized with \[^{32}P\]labeled RNA polymerase products made in vitro by a cell lysate. The upper panels show the ethidium bromide staining of the gel (left) and an autoradiogram of the membrane (right). The mobility of \(\lambda\) HindIII markers is also shown. From the autoradiogram it is estimated that the amounts of 23 S RNA-positive strands and 20 S RNA-negative strands synthesized in vitro are 8% and 2%, respectively, compared with that of 20 S RNA positive strands. A diagram of the non-labeled 20 S RNA and 23 S RNA positive and negative strands used is shown under the panels. B, small RNAs containing the 5′- or 3′-end terminal sequence of the positive and negative strands of 20 S RNA were separated in an agarose gel and processed as in A. After hybridization the membrane was treated with RNase A to digest the single-stranded portions of the \[^{32}P\]labeled products bound to the small RNAs before the final stringent wash. The upper panels show the ethidium bromide-staining of the gel (left) and an autoradiogram of the membrane (right). A diagram of the positive (+) and negative (−) strands of 20 S RNA and the small terminal fragments used (indicated by the arrows) with their nucleotide sizes is shown under the panels.
Conversion of dsRNA intermediates to ssRNA products requires RNA synthesis. A cell lysate was incubated at 30 °C in the standard RNA polymerase reaction mixture containing \(^{32}P\)UTP. At 5 min EDTA was added to the mixture (lane 1). A small portion of the mixture was incubated for another 60 min at 30 °C (lane 2). The rest of the mixture was filtrated through a G-50 spin column (lane 5), ATP (lane 6), or the four NTPs, 0.5 mM each (lane 7) was added (the filtrate in lane 3 received none), and these samples were incubated at 30 °C for another 60 min. All the samples were phenol-extracted and separated in an agarose gel. An autoradiogram of the gel is shown.

Conversion of dsRNA intermediates to ssRNA products requires RNA Synthesis. The conversion of the dsRNA form to the ssRNA form requires RNA synthesis, ATP, Mg\(^{2+}\), CTP, and UTP. This suggests that there are no multiple rounds of RNA synthesis. During the chasing period these molecules became full-length. Therefore, they are 20S RNA positive strand molecules shorter than full-length. In the absence of heat denaturation, however, these molecules were not detected at the same position in the gel (Fig. 1D). Instead, we observed a broad smear band between the dsRNA and ssRNA forms. During the following chasing period, this broad band moved upwards until reaching the position of the dsRNA. Furthermore, there was no incorporation into materials larger than the dsRNA form (Fig. 1D). These observations suggest that (i) the dsRNA form consists of a full-length template negative strand and a single molecule of full-length or almost full-length positive strand and (ii) that the smear broad band between the dsRNA and ssRNA forms has the same RNA composition as the dsRNA form but with a positive strand much shorter than full length and heterogeneous in size.

Conversion of dsRNA intermediates to ssRNA products requires RNA synthesis. The conversion of the dsRNA form to the ssRNA form by incubating the reaction mixture on ice or just by adding an excess amount of EDTA can be stopped by incubating the reaction mixture on ice or just by adding an excess amount of EDTA.

In Vitro Synthesis Is Done by Chain Elongation at the 3'-Ends of Positive Strands. In a time course, the incorporation of radioactive nucleotides into trichloroacetic acid-insoluble materials was not uniform during the incubation time. We observed a vigorous RNA synthesis in the first 10 min of incubation, followed by a much slower incorporation (Fig. 1E). This suggests that there are no multiple rounds of RNA synthesis in vitro.

We confirmed this by the hybridization experiment shown in Fig. 2B. A set of four small RNA fragments containing either the 5'- or 3'-end sequence of the 20S RNA was hybridized with the RNA fragments in the membrane. After hybridization, the membrane was treated with RNase A under high salt conditions, both RNA bands disappeared (lanes 4 and 5). After RNase treatment, the RNA products were extracted with phenol and separated in an agarose gel. An autoradiogram of the gel is shown. Lane 1, non-RNase A treated sample run in parallel as control.

This indicates that the positive strands in the dsRNA intermediates are almost but not full-length and that the release of ssRNA products from dsRNA intermediates requires the completion of positive strand synthesis.

The RNA in Native Replication Complexes Is Mostly Single-stranded. As shown in Fig. 4, lane 1, phenol-extracted in vitro products resolved into two bands corresponding to the dsRNA intermediates and the final ssRNA products in an agarose gel. RNase A treatment under high salt conditions completely digested the ssRNA products, whereas the dsRNA intermediates remained undigested (lanes 4 and 5). When the same sample but without phenol extraction was treated with the enzyme under high salt conditions, both RNA bands disappeared (lanes 2 and 3). This indicates that RNA in native replication complexes is mostly single-stranded and that deproteinization with phenol converted the negative strand template and the positive strand product in the complex into the dsRNA intermediates. Hereafter we call these native intermediates “replication complexes” to distinguish them from phenol-extracted dsRNA in-
Fig. 5. Detection of native 20 S RNA replication intermediates. A–D, a cell lysate was incubated at 30 °C in the standard RNA polymerase reaction mixture containing [\(^{32}\)P]UTP. After 5-min incubation non-labeled UTP (2 mM) was added and incubation was continued. Samples were withdrawn at the times indicated and divided into three parts. Two sets (A and C) of the samples were directly applied to an agarose gel. The other set was phenol-extracted and then applied to the same gel (B). After electrophoresis at 4 °C, two sets of the samples were electrophoretically loaded onto a positively charged nylon membrane (A and B). The other set of the samples (C) was electrophoretically loaded onto a nitrocellulose membrane. Then the blotted membranes were exposed to x-ray films. Autoradiograms of the membranes (A–C) are shown. D shows ethidium bromide staining of the phenol-extracted samples in the gel, photographed before electrophoresis. In C a phenol-extracted sample collected at 60 min was also run as control (the far right lane). The brackets indicate the broad band of native replication intermediates. In D, the mobility of some \(\lambda\) HindIII markers is indicated by their sizes in base pairs on the left of the panel. Note that the amount of ssRNA products bound to the nitrocellulose membrane shown in C appears to be much less compared with the one bound to the nylon membrane shown in A. This is caused by the co-migration in the gel of the labeled products with a bulk of non-replicating 20 S RNA/p11 complexes and by the poorer binding capacity of the nitrocellulose membrane. E, the mobility of 18 S and 25 S rRNAs and 20 S and 23 S RNAs in the gel shown in D were plotted semilogarithmically against their sizes on kilobases (Kb). The mobility of the upper edge of the broad band corresponding to the native replication intermediates shown in A and C is indicated by the arrow.

Visualization of Replication Complexes in Non-denaturing Gels—Because the native complexes are directly involved in the 20 S RNA positive strand synthesis, we tried to detect and characterize them in their native state. For this purpose, we pulse-labeled RNA molecules by incubating lysates in the standard RNA polymerase reaction mixture and loaded the samples directly onto non-denaturing agarose gels (Fig. 5). After electrophoresis the labeled RNA products were electrophoretically loaded to a positively charged nylon membrane (Fig. 5A) or to a nitrocellulose membrane (Fig. 5C). This blotting procedure eliminated high background caused by unincorporated radioactive nucleotides. As control, parts of the samples were phenol-extracted, separated on the same gel, and electrophoretically loaded to the positively charged nylon membrane (Fig. 5B). In the control, radioactivity was incorporated mostly into the double-stranded forms during the first 5-min labeling period (Fig. 5B). After addition of an excess amount of non-labeled UTP, the label in the dsRNA form was rapidly converted to the ssRNA products, a pattern similar to the one observed in Fig. 1D. When the same, but native samples were analyzed, we obtained quite different results (Fig. 5A). During the 5-min labeling, radioactivity was incorporated into a broad band that moved slightly slower than the phenol-extracted ssRNA products, but much faster than the dsRNA forms. The radioactivity in the band was rapidly transferred to the ssRNA products during the following chasing period. There was no incorporation into the dsRNA form in the native samples (Fig. 5A). This is not an artifact related to the blotting procedure, because we obtained the same results when the gel was dried and exposed directly to an x-ray film (not shown).

Although native replication complexes moved as a broad band, the lower edge of the band never ran faster than the ssRNA products, thus suggesting that the RNA content in the replication complex is more than a single molecule of 20 S RNA. After the addition of non-labeled UTP, the lower edge of the band shifted upwards, converging to the upper edge of the band in the first 10-min incubation (Fig. 5A). During the following incubation, all of the label in the broad band was converted to the ssRNA products. There is a good correlation between the mobility of four phenol-extracted ssRNAs (18 S rRNA, 20 S RNA, 23 S RNA, and 25 S rRNA) in the gel and their nucleotide sizes (Fig. 5E). Because 20 S RNA is 2514-nt long, this is equivalent to two molecules of 20 S RNA. This suggests that these intermediates at the upper edge contain a full-length negative strand and an almost full-length positive strand per complex. When the same native samples shown in Fig. 5A were electrophoretically loaded to a nitrocellulose membrane, we again observed a broad band corresponding to the replication intermediates (Fig. 5C). The upward movement of the lower edge of the band during incubation became more evident. Because nitrocellulose membranes are slightly negatively charged, phenol-extracted ssRNA products did not bind to the membrane under the blotting conditions used (17; also compare the far right lanes in Fig. 5, B and C). On the contrary, the same products but without phenol extraction bound to the membrane. This suggests that the native ssRNA products re-
We were interested in the fate of the negative strand templates released from replication complexes (Fig. 6, B). Because the lysate predominantly synthesized 20 S RNA positive strands over negative strands in *vitro* (the latter synthesis counted <2% compared with that of the former ones), and replication complexes engaging positive strand synthesis are expected to contain negative strand templates equimolecular to the products, most of the increase of negative strands in the single-stranded form was not caused by its synthesis *in vitro* but derived from negative strand templates released from replication complexes. During incubation we observed a 3- to 5-fold increase of negative strands in the single-stranded form. This suggests that most of negative strands in 20 S RNA-induced cells were present in replication complexes, thus engaging in positive strand synthesis. When negative strands were electroblotted onto a nitrocellulose membrane (Fig. 6B), we observed again an increase in the amount of negative strands in the single-stranded form and a concomitant decrease of negative strands present in the replication complexes. Interestingly, when a sample taken at 10 min was phenol-extracted and loaded onto the same gel, the single-stranded form of negative strands did not bind to the nitrocellulose membrane (compare the far right lane with the third lane from the left in Fig. 6B) but to the positively charged nylon membrane (Fig. 6A, far right lane). Therefore, these results suggest that the negative strand templates released from replication complexes are also associated with protein.

**DISCUSSION**

*Non-double-stranded Nature of Replication Intermediates*—In this work we have analyzed the synthesis of 20 S RNA *in vitro* in lysates prepared from 20 S RNA virus-induced cells and detected replication intermediates of 20 S RNA virus. The majority of the products were 20 S RNA positive strands, and the negative strands accounted for only a few percent of total synthesis. The *in vitro* products, when phenol-extracted and analyzed in an agarose gel, resolved in two forms, dsRNA and ssRNA. The dsRNA form, resistant to RNase A under high salt conditions, was converted to the ssRNA form by heat denaturation with 7 M urea, thus indicating a non-covalent association of the positive strand with the negative strand template. Time-course and pulse-chase experiments demonstrated their precursor-product relationship; radioactive nucleotides were first incorporated into dsRNA intermediates, and then they were converted to the ssRNA products (Fig. 7). This conversion requires the presence of all four NTPs and can be stopped by the addition of EDTA. Thus the conversion process requires a net RNA synthesis.

In contrast to the phenol-extracted intermediates, the native intermediates were fully sensitive to RNase A under high salt conditions. It suggests that the growing positive strand was loosely associated with the negative strand template in the replication complex and that denaturation with phenol converted them to an RNase A-resistant dsRNA form (Fig. 7). Similar effects of protein denaturants on the RNA component of replication intermediates have been well documented in some positive strand RNA viruses (see below). The non-double-stranded nature of RNA in replication complexes is consistent with their electrophoretic mobility in non-denaturing gels. The native complexes moved much faster than the phenol-extracted dsRNA.

**RNA Constituents of Replication Complexes**—The broad band of the native replication complexes seen in the early labeling period reflects the heterogeneity in length of the growing positive strands in the complexes (Fig. 5, A and C). In the
Native replication complexes have a ssRNA backbone

| dsRNA intermediates | ssRNAs released after in vitro synthesis |
|---------------------|-----------------------------------------|
| 5' (+) 3' | 5' (+) 3' |
| 3' (-) 5' | 3' (-) 5' |
| Phenol treatment | |

FIG. 7. Schematic diagrams of positive strand synthesis of 20 S RNA virus in vitro. Upper diagram, replication complexes synthesizing the positive strands of 20 S RNA virus consist of, as RNA components, a full-length negative strand template (→) and a positive strand less than full-length (+). They incorporate radioactive nucleotides (†) mostly into the 3'-end region of the positive strands in vitro. The two RNA strands in the complex are loosely associated, perhaps held together by the RdRp, p91 (filled circles), or the replicase machinery. Upon completion of RNA synthesis, the positive strand product and the negative strand template are released from the replication complex. The fate of p91 during this process is not known (see "Discussion"). Lower diagram, deproteination with phenol converts the two RNA strands in the replication complex into a dsRNA intermediate. Once RNA synthesis is completed, however, the positive strand product and the negative strand template released from the replication complex remain as ssRNAs, even after phenol-treatment. The 5'- and 3'-ends of the positive and negative strands are indicated.

following chasing period the lower edge of the band shifted quickly to the upper edge of the band, and all the label was eventually converted to the ssRNA form. This indicates that the upward shift of the label during this period was due to positive strand RNA synthesis. Consistently, the front edge of the broad band ran as fast as 20 S RNA (or 20 S RNA/p91 resting complexes, see below), and the upper edge of the band migrated as a ssRNA of ~5 kb in size, twice the size of 20 S RNA (2514 nt). These results, thus suggest that the replication complexes contain, as RNA components, one full-length negative strand template and one growing, but less than full-length, 20 S RNA positive strand. There are several lines of evidence that support this. First, as shown in Fig. 2B, most of the in vitro synthesis was done by chain elongation of positive strands pre-initiated in vivo. Thus there are no multiple rounds of positive strand synthesis in vitro from the same template. Consistently, vigorous RNA synthesis ceased after 10-min incubation (Fig. 1E), accompanied with the accumulation of the dsRNA form. Second, in pulse-chase experiments, we observed molecules smaller than the dsRNA form during the early incubation period (Fig. 1D). Heat denaturation with urea converted them into molecules smaller than full-length positive strands (Fig. 1C). There was no incorporation into materials larger than the dsRNA form. Therefore, these observations indicate that the total positive strand content in replication complexes never exceeds a single molecule of 20 S RNA per complex. This implies that a strand-replacement mechanism is not responsible for the conversion of dsRNA intermediates to the final ssRNA products. If a full-length positive strand is displaced by a newly synthesized positive strand, then the total amount of positive strand RNA in the complex should be more than a single molecule of 20 S RNA. Finally, we detected negative strand templates in replication complexes. When RNA in lysates was separated in non-denaturing agarose gels, a negative strand-specific probe detected two bands (Fig. 6). The faster moving band corresponds to the negative strand templates present in replication complexes. During incubation, the second band disappeared and a concomitant increase in the single-stranded form was observed. Because negative strand synthesis in vitro was almost negligible, the increase of the single-stranded form was mostly derived from the negative strand templates released from replication complexes. In good agreement, the increase of negative strands in the single-stranded form coincided with the appearance of positive strand products released from replication complexes during incubation.

Although the replication complexes contain p91, the contribution of protein components to their mobility in the gel appears to be minimal. The mobility of protein-stripped 20 S RNA (808 kDa) is hardly distinguishable from that of 20 S RNA/p91 resting complexes (Ref. 17; see also Fig. 5, A and B). Interestingly, both the positive and negative strands released from replication complexes bound to a nitrocellulose membrane by electroblotting but did not bind to the same membrane when extracted with phenol. This suggests that both strands were associated with protein.

Comparison with Replication Complexes from Other Positive Strand RNA Viruses—The single-stranded nature of the RNA components of 20 S RNA replication intermediates is quite similar to that of native replication intermediates of some positive strand RNA viruses. It has been well documented that the RNA in native replication intermediates of the RNA coliphage Qβ is fully single-stranded (20). Deproteinization with phenol converts it into a form that is largely double-stranded. Once extracted with phenol, the annealed RNA is unable to infect protoplasts of the host cells nor to serve as template for Qβ replicase. However, both activities can be restored by heating the RNA to melt it (21, 22). Although multistranded intermediates have been observed, a single Qβ replicase is capable of synthesizing RNA multiple rounds. After completion of product-strand elongation, both the product and template strands are released from the replication complex as single-stranded RNA. Then the replicase is free to bind to a new template to proceed the next cycle of RNA synthesis (23).

In polio virus-infected cells, a short exposure to [3H]uridine predominantly labels a minor virus-related RNA species called replicative intermediate (RI). The RI consists of a genome-
length negative strand template RNA and complementary RNA with an average of five to seven nascent single-stranded tails (24, 25). Although deproteinated RI has a backbone mostly double-stranded, a careful electron microscope analysis using a membrane-permeable cross-linking reagent demonstrated that the native RI in vivo has a predominantly single-stranded backbone attached to several nascent RNA chains with few or no regions of extensive base-pairing (26). Despite similarity in their single-stranded nature, replication intermediates of 20 S RNA virus do not have multiple nascent RNA chains. Because 20 S RNA virus is not infectious, this may reflect its slower replication rate compared with the infectious counterparts. Alternatively, 20 S RNA virus may have some mechanism to prevent a second round of initiation from templates still engaging in the synthesis of complementary strands.

W dsRNA Is a By-product of 20 S RNA Replication—20 S RNA virus-harboring cells, when grown at 37°C, accumulate W dsRNA. As shown in this work, however, native replication complexes have a single-stranded RNA backbone and deprotection with phenol converted them to dsRNA intermediates. Therefore, W dsRNA is not an intermediate of 20 S RNA replication but a by-product. Interestingly, W dsRNA isolated from cells grown at 37°C had the intact 3'-end sequences in both the positive and negative strands (8). Growth at the higher temperature may simply accelerate duplex formation between single-stranded positive and negative strands. Alternatively, the higher temperature might affect replication complexes in such a way that the nascent strand is allowed more easily to re-hybridize with the template strand behind the site of RNA synthesis without affecting the catalytic activity of the polymerase machinery. In contrast to W dsRNA, the dsRNA intermediates detected in vitro after phenol treatment were formed only when the labeled positive strands were still associated with replication complexes. Furthermore, their release from replication complexes required net RNA synthesis. Therefore, these observations suggest that the positive strands in dsRNA intermediates were not full-length. It appears that RNA synthesis slows down considerably near the 3'-end of the positive strand. It is interesting to know if there is a specific pausing site(s) for positive strand synthesis. Such a pause(s) could be caused by the structure of the template RNA or by binding of protein to the template RNA. Considering the importance of the formation of ribonucleoprotein complexes in narnavirus replication (see below), the latter case may be of particular interest. 20 S and 23 S RNA genomes do not encode capsid proteins. Thus their RNA genomes are not encapsidated into intracellular viral particles. Instead these positive strand RNAs form resting ribonucleoprotein complexes with their cognate RdRps in a 1:1 stoichiometry and reside in the cytoplasm. These RNAs have no poly(A) tails at the 3'-ends and perhaps no 5'-cap structures, thus resembling intermediates of mRNA degradation. Therefore, we expect these RNAs, if alone without forming complexes with their RdRps, to be susceptible to the exonucleases involved in mRNA catabolism. 20 S and 23 S RNA genomes share the same 5' nt inverted repeats at both ends (5'-GGGGC...GCCCC-OH) (8). Recently we have succeeded in generating 23 S RNA virus in vivo from a vector containing the entire viral cDNA (27). Using this system we found that the cluster of 4 Cs at the 3'-end of the positive strands is a part of a bipartite 3' cis signal for replication (28). We also found that this bipartite signal is essential for the formation of a ribonucleoprotein complex in vivo between the 23 S RNA positive strand and its RdRp, p104 (29). It points out the importance of the ribonucleoprotein complex formation in the life of 23 S RNA virus. In the case of 20 S RNA virus we have developed a similar virus launching system and found that the cluster of four Cs at the 3'-end of the 20 S RNA positive strand is also a part of a 3' cis signal essential for replication. 2 On the analogy of 23 S RNA virus, we expect that p91 interacts with this 3' signal to form a ribonucleoprotein complex. In fact, when p91/20 S RNA resting complexes were extensively digested with RNase A, anti-p91 antiserum immunoprecipitated 3'-end fragments as well as 5'-end fragments of the 20 S RNA positive strands. We located the 5' p91 binding site on the second stem structure from the 5'-end (nt 72–104, numbered from the 5'-end). These data strongly suggest that a single p91 molecule circularizes a 20 S RNA positive strand in a resting complex by binding at both ends of the RNA. This may give a good explanation of why 20 S RNA virus can survive in the yeast cytoplasm without its RNA genome being digested by exonucleases, provided that the negative strand genome is also protected by a similar mechanism. Our available data indicate that negative strands can be immunoprecipitated with anti-p91 antiserum from a sucrose gradient fraction containing non-replicating 20 S RNA genomes (30). Therefore, 20 S RNA negative strands also form ribonucleoprotein complexes with p91. Recently we found that 20 S RNA negative strands transcribed from a vector can also generate the virus in vivo if an active p91 is provided in trans from a second vector. Using this two-vector system we observed that the cluster of 4 Cs at the 3'-end of the 20 S RNA negative strand again is a part of a 3' cis signal for replication. It is likely that this signal is also involved in the formation of complexes with p91. So far our knowledge on the negative strand is limited, because the low amount of 20 S RNA negative strands in the cells makes it difficult to study them in detail. In this context, this two-vector system can be exploited, especially to analyze cis signals on the negative strands to form complexes with p91 by expressing a higher amount of these RNAs from a vector in vivo. It will be more difficult to understand the structural features of replication complexes, given their heterogeneity and complexity compared with resting complexes. Some of the features, however, may not be fundamentally far from those seen in resting complexes. For example, if negative strands in resting complexes are circularized by p91 for protection, as suggested for positive strands, then such protection might also occur in negative strand templates in replication complexes. We have observed that positive strand synthesis in vitro apparently decelerated when the replication machinery approached the 5'-end of the template. This slowdown might be caused by p91 bound to the 5'-end region of the template strand, because the polymerase machinery has to displace this p91 from the template to complete the positive strand synthesis. This possibility could be verified experimentally by comparing p91 binding sites on the negative strand in resting complexes, with pausing sites of positive strand synthesis located on the template strand. If it is the case, then this scenario leads to another interesting question of whether the “replicating” p91 in the polymerase machinery is the same molecule “bound” to the negative strand template. If the same single molecule has these two functions, then either the positive strand product or the negative strand template released from a replication complex will need an additional p91 molecule to form resting complexes. Alternatively, if there are two p91 molecules with distinct functions in a replication complex, then, we may expect their non-random distribution to the respective strands released from the complex, thus resulting in (or leading to) the formation of two resting complexes. As we have observed in this work, both the positive and negative strands released from replication complexes in vitro are asso-

2 R. Esteban, L. Vega, and T. Fujimura, manuscript in preparation.

3 T. Fujimura and R. Esteban, manuscript in preparation.
ciated with protein. Because each replication complex contains at least a single molecule of p91, either the positive or negative strand released should be associated with p91 (or p91 might be distributed between them randomly). To answer this question, therefore, we need to know which strand released from the replication complex is associated with p91 and what is the stoichiometry of their association. Although we expect that these experiments are technically challenging, the membrane-free nature of 20 S RNA virus replication complexes may allow us to answer these questions. These results will provide fundamental insights to understand the replication mechanism of positive strand RNA viruses in yeast.

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