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Complete replication of an animal virus and maintenance of expression vectors derived from it in *Saccharomyces cerevisiae*

(flock house virus/host factors/Nodavirus)

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ABSTRACT **Here we describe the first instances to our knowledge of animal virus genome replication, and of *de novo* synthesis of infectious virions by a nonendogenous virus, in the yeast *Saccharomyces cerevisiae*, whose versatile genetics offers significant advantages for studying viral replication and virus-host interactions. Flock house virus (FHV) is the most extensively studied member of the Nodaviridae family of (+) strand RNA animal viruses. Transfection of yeast with FHV genomic RNA induced viral RNA replication, transcription, and assembly of infectious virions. Genome replication and virus synthesis were robust: all replicating FHV RNA species were readily detected in yeast by Northern blot analysis and yields of virions per cell were similar to those from *Drosophila* cells. We also describe *in vivo* expression and maintenance of a selectable yeast marker gene from an engineered FHV RNA derivative dependent on FHV-directed RNA replication. Use of these approaches with FHV and their possible extension to other viruses should facilitate identification and characterization of host factors required for genomic replication, gene expression, and virion assembly.**

The understanding of virus–host interactions, including the identity of the host factors with which viruses interact to mediate their replication and cytopathic effects, remains a challenging frontier in virology. For a number of viruses, specific host factors have been implicated in some important infection processes such as cell attachment, DNA transcription and replication, and others. However, for many important virus functions, such as genome replication by (+) strand RNA viruses, the nature of the host factors involved remains almost completely unknown. For most viruses, identification of relevant host factors would be greatly assisted by ready genetic dissection of the host. Particularly effective eukaryotic genetics exists for the yeast *Saccharomyces cerevisiae*, as illustrated by prior identification of host genes required for the maintenance of its endogenous L-A double-stranded RNA virus (1).

To utilize this genetic potential for (+) strand RNA viruses, systems were recently developed that demonstrate the RNA-dependent replication, transcription, and persistence in yeast of derivatives of brome mosaic virus (BMV), a plant-infecting member of the alphavirus-like superfamily (2, 3). Yeast expressing the viral components of the BMV RNA replicase support replication and gene expression by BMV RNA derivatives. Such RNAs are transmitted through mitosis, so that Ura^+ strains can be obtained by transfecting Ura^- yeast with a BMV RNA replicon expressing the *URA3* gene. This system provides a basis for selecting mutations in host genes required for BMV RNA replication.

Given the feasibility of this approach, it was attractive to extend it to other viruses. Flock house virus (FHV) belongs to the Nodaviridae family of (+) strand RNA viruses, whose members infect invertebrates and vertebrates (for review, see ref. 4). FHV has many key features as a model for analyzing (+) strand RNA viruses. Its 4.5-kb genome is one of the smallest among animal viruses. Although FHV virions are infectious to insect cells, naked FHV genomic RNA is infectious to insect, plant (5), and mammalian (6) cells, suggesting that host factors necessary for its replication are highly conserved. An FHV plaque assay (7) and neutralizing antibodies (8) allow sensitive detection of FHV virions. Infectious transcripts from full-length cDNAs can be produced *in vitro* (9) and *in vivo* (10). The cis elements needed for genome replication have been characterized (11, 12) and FHV RNAs have been used to carry and express heterologous sequences (13). In addition, a replicase capable of full *in vitro* replication of added FHV RNA has been isolated from infected cells (14, 15).

The FHV genome is bipartite (Fig. 1*A*) (16), with both RNAs packaged in the same particle (7, 8). RNA1 (3.1 kb) (17) encodes all viral contributions to the FHV RNA replicase and can replicate autonomously (18). RNA1 serves as mRNA for protein A (112 kDa) (19), which contains the GDD amino acid sequence motif characteristic of RNA polymerases and is essential for FHV RNA replication (20). An RNA1-encoded subgenomic RNA, RNA3 (0.4 kb) (21), serves as mRNA for protein B (10 kDa) (22). Protein B is not required for production of (+) or (−) strand RNA1 or RNA3 (20). RNA2 (1.4 kb) encodes the virion coat protein precursor, α (43 kDa) (19, 23). Here we demonstrate that *S. cerevisiae* spheroplasts transfected with FHV RNA support full replication of FHV, including assembly of infectious virions, and we describe a system for *in vivo* expression and maintenance of heterologous marker genes dependent on FHV RNA replication.

MATERIALS AND METHODS

Plasmids. To construct p1R and p1(fs)R (see *Results*), the 5.3-kb *Sca*I–*Nar*I fragment of pFHV1[1,0] (10), containing an FHV RNA1 cDNA flanked 1 nt from its 5′ end by a T7 promoter and 0 nt from its 3′ end by a hepatitis delta virus ribozyme and a T7 terminator, was transferred to the 3.0-kb *Nar*I–*Sca*I fragment of the yeast shuttle plasmid YEplac112 (24). For p1(fs)R, the *Eag*I site at position 373 of the FHV RNA1 cDNA was digested, filled in, and religated. The 0.17-kb *Sac*I fragment containing the T7 terminator was deleted from p1R and p1(fs)R.

To construct the plasmid pDU (see *Results*), a 0.9-kb *Sac*I fragment containing a modified *URA3* gene from YEp24 (25) was transferred to the *Sac*I site of pBS-DI634 (11, 26). The 5′ end of the modified gene was a 238-bp *Sac*I–*Nco*I fragment of a product amplified by polymerase chain reaction (PCR) with the primers 5′-GCGGAGCTCTGTCGAAAGCTACATAT-AAGGAA-3′ and 5′-AGCGGCTTAACTGTGCCCTCC-3′, and the 3′ end was a 649-bp *Nco*I–*Sac*I fragment with a *Sac*I

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Abbreviations: BMV, brome mosaic virus; DI, defective interfering; FHV, flock house virus; hpt, hours posttransfection; pfu, plaque forming units.

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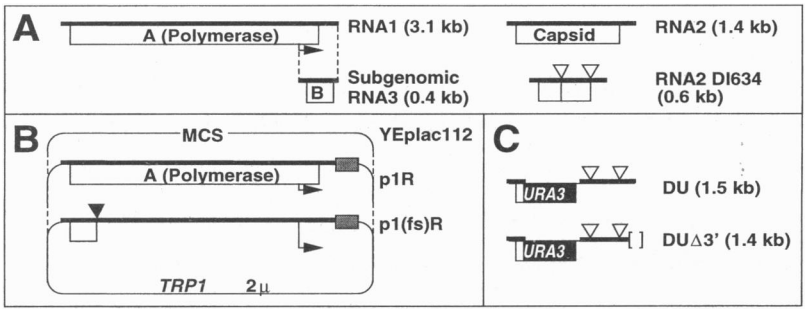

FIG. 1. Structure of the FHV genome and derivatives employed in this study. (*A*) Schematic drawing of FHV genomic RNA1 and RNA2. Subgenomic RNA3 is shown below RNA1. A derivative of RNA2, defective interfering RNA 634 (DI634), is shown below RNA2. Open boxes represent ORFs and lines represent nontranslated regions. The bent arrow below RNA1 represents the 5′-most nucleotide of RNA3. ORF A (polymerase) and ORF B overlap in different frames. The open triangles above DI634 represent two deleted RNA2 sequences. (*B*) Schematic drawing (not to scale) of plasmids YEplac112, p1R, and p1(fs)R. MCS indicates the multiple cloning site of YEplac112. The full-length FHV RNA1 cDNA is depicted as a heavy line. A hepatitis delta virus ribozyme cDNA is denoted with a shaded box. The filled triangle in p1(fs)R represents a 4-nt insertion following nt 377. The relative position of the *TRP1* gene and yeast 2μ origin of replication are indicated. (*C*) Schematic drawing of DU and DUΔ3′ RNAs. Sequences derived from FHV DI634 are represented as heavy lines. The *URA3* ORF, indicated by the filled box, is fused in-frame to the first 12 codons of the FHV capsid protein, indicated by the open box. The last 100 nt of DU are deleted in DUΔ3′, as indicated by the brackets.

linker inserted at the *Sma*I site in the 3′ noncoding region. To construct pDUΔ3′, the 106-bp *Msc*I–*Bam*HI fragment of pDU containing the 3′ 100 bp of the DI634 cDNA was deleted.

***In Vitro* Transcripts.** Unlabeled *in vitro* transcripts were generated using an Ampliscribe kit (Epicentre Technologies, Madison, WI), treated with DNase I, and purified by phenol–chloroform extraction and sephadex G50 (Pharmacia) spin column chromatography. (+) DU and (+) DUΔ3′ contained 4 nonviral nucleotides at their 3′ ends.

Strand-specific ^{32}P-labeled *in vitro* transcripts were generated as described (27). The probes for (−) and (+) RNAs 1 and 3 corresponded to or were complementary to nt 2718–3064 of FHV RNA1. The probe for (−) RNA2 corresponded to nt 425-1080 of FHV RNA2. The probe for (+) RNA2 was complementary to nt 62–1400 of FHV RNA2. The probe for (−) *URA3* corresponded to the entire 804-nt *URA3* open reading frame (ORF) plus 140 nt downstream. The probe for (+) *URA3* was complementary to the 3′ 598 nt of the *URA3* ORF plus 140 nt downstream.

Transformation and Transfection of Yeast. Plasmid DNAs were introduced into YPH500 (α *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*) cells as described (28). FHV virion RNA and *in vitro* transcribed RNAs were transfected into YPH500 spheroplasts using PEG and $CaCl_2$ (2, 29). Yeast were grown at 26°C in media selective for plasmid and RNA replicon maintenance.

Virion, RNA, and DNA Analysis. Yeast spheroplasts were pelleted at 5000 rpm for 5 min, resuspended in FHV buffer (50 mM NaHEPES, pH 7.0/0.1% 2-mercaptoethanol/0.1% bovine serum albumin) and stored at −80°C. The lysate was thawed and cleared of cell debris by centrifuging at 9000 rpm at 4°C for 30 min. Total virion content was determined by assaying the cleared culture and spheroplast lysate for plaque formation on *Drosophila* cell monolayers.

Total yeast RNA was extracted with phenol/chloroform as described (30) except that glass beads were omitted for spheroplasts. Total *Drosophila* RNA was isolated with phenol/chloroform (26) 20 hr after transfection. *URA3* sequences were amplified from 1.25 μg total yeast DNA or 0.25 ng YEp24 as described (2) except that *Taq* Extender (Stratagene) was used.

For Northern blot analysis, RNAs were denatured with glyoxal (27) and electrophoresed on a 0.8% agarose/10 mM phosphate gel. For Southern blot analysis, PCR products were electrophoresed on a 1% agarose gel. Nucleic acids were transferred to Zetaprobe (Bio-Rad) membranes that were prehybridized for 4 hr, hybridized overnight in 50% formamide at 60°C with 5 × 10^6 cpm/ml per probe, and washed at 60°C following the manufacturer's protocols. Quantitative results were obtained with a Molecular Dynamics PhosphorImager digital radioactivity imaging system.

***Drosophila* Cells.** The WR strain (19) of *Drosophila* cells was propagated (26), infectious virions were assayed on *Drosophila* cell monolayers (31), and FHV virions were treated with neutralizing FHV antibody (8) as described. *Drosophila* (2 × 10^6) cells were lipofectin-transfected (26) with 40 ng gel-purified viral RNA1 or 40 ng gel-purified viral RNA1 plus 100 ng *in vitro*-transcribed DU RNA.

RESULTS

Infectious FHV Virions from FHV RNA-Transfected Yeast. As a sensitive assay for biological activity of FHV RNA in yeast, yeast spheroplasts were transfected with phenol-extracted FHV genomic RNA1 and RNA2 and, at intervals, tested for infectious FHV virion production by plaque assay on *Drosophila* cell monolayers. As seen in Fig. 2, plaque forming units (pfu) were recoverable by 12 hr posttransfection (hpt), increased beyond 60 hpt, and plateaued by 72 hpt. These pfu corresponded to FHV virions, as their infectivity was abolished

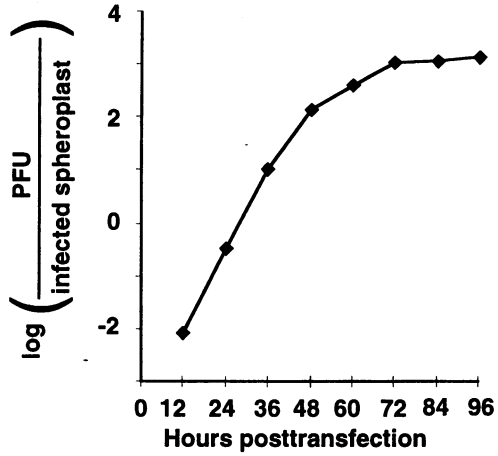

FIG. 2. Single step FHV growth curve in FHV virion RNA-transfected yeast spheroplasts. Yeast spheroplasts (5 × 10^8) were transfected with 5 μg FHV virion RNA, resuspended in 10 ml media, and incubated 96 hr. At 12-hr intervals, aliquots were assayed for pfu on *Drosophila* cell monolayers. The total number of pfu per ml divided by the number of infected spheroplasts per ml (as determined by endpoint dilution assay; see *Results*) is plotted on a log scale versus hpt.

by incubation (1 hr at 37°C) with a 1:10,000 dilution of FHV-neutralizing antiserum, while 0.1 mg/ml ribonuclease A had no effect. Consistent with the expected intracellular origin, ≈90% of these pfu were recoverable only after deliberate lysis of the yeast by freeze thawing. The exponential increase in FHV pfu between 0 and 48 hpt (see log scale in Fig. 2) suggested that these virions resulted from encapsidating the products of significant FHV RNA replication in yeast, rather than simply translating and encapsidating the input inoculum RNA. Conclusive evidence of this, including temporal and quantitative correlation between newly synthesized RNA and pfu accumulation, is given below.

In parallel controls, spheroplasts were mock transfected or transfected with intact FHV virions. Pfu were never detected in mock-transfected spheroplasts. Under the conditions used, there was no evidence of significant viral replication in spheroplasts transfected with FHV virions, rather than FHV RNA. Pfu were recovered in the media and the number of pfu remained constant from 0 to 4 days posttransfection. Thus, under the conditions of the experiment, extracellular virions were stable but unable to infect new spheroplasts.

Transfection Efficiency and Yield of Pfu. The pfu yields suggested that both transfection efficiency and pfu yield per infected spheroplast were high. To determine both parameters, an endpoint dilution experiment was conducted. Transfected spheroplasts were serially diluted in growth medium and multiple aliquots of each dilution placed in wells of microtiter plates at 0 hpt. Plates were affixed to a cell culture roller and incubated at 26°C. At 72 hpt, spheroplasts were lysed and lysates from each well were assayed for pfu. In two independent transfections, at a dilution corresponding to 125 spheroplasts per well, pfu were detected in only 17 of 50 and in 14 of 50 wells. Assuming Poisson's distribution, 0.3% of the spheroplasts were infected and the 31 wells with pfu contained 37 infected spheroplasts. Based on the sum of pfu from all wells (7.1×10^4 pfu), the average yield was 2000 pfu per infected spheroplast. This yield per transfected spheroplast correlates well with that in the transfection timecourse experiment at and beyond 72 hpt (Fig. 2), which was calculated assuming the same transfection efficiency.

Replication of FHV RNA in Yeast. Proof that progeny virions resulted from true replication, rather than repackaging of the inoculum, was obtained by hybridizing Northern blotted total RNA with strand-specific probes. No hybridization of FHV probes to total RNA extracted from mock-transfected or virion-transfected spheroplasts was observed.

The first product of genomic replication is (−) RNA, which is not present in the inoculum. As seen in Fig. 3*A*, (−) strand-specific probes first revealed genomic-sized (−) RNAs above background levels at 24 hpt (lane 3). (−) RNA levels increased until 72 hpt (lanes 4–7) and decreased somewhat by 84 and 96 hpt (lanes 8 and 9). A band comigrating with (−) subgenomic RNA3 appeared by 12 hpt (lane 2) in longer exposures, increased until 60 hpt (lanes 3–6) and decreased somewhat by 96 hpt (lanes 7–9). In addition to (−) RNA1-, (−) RNA2-, and (−) RNA3-sized RNAs, two other RNA species (marked by open arrows) were visible by 60 hpt (lanes 6–9). Both were also detected in longer exposures of total RNA extracted from transfected *Drosophila* cells (lane 12) and both hybridized to (−) RNA2 strand-specific probes. The larger band may represent incompletely denatured RNA2 replicative intermediate. The smaller band may represent a degradation product or (−) RNA of newly formed defective interfering (DI) RNA. DI RNAs readily arise from plaque-purified Nodaviruses (8, 26).

As seen in Fig. 3*B*, (+) strand-specific probes revealed low levels of input genomic (+) RNA1 and (+) RNA2, but not subgenomic (+) RNA3, a product of replication, at zero hpt (lane 1). The levels of genomic-sized RNAs decreased between 0 and 24 hpt (lanes 2 and 3), began to increase at 36 hpt (lane 4), then plateaued at 72 hpt (lanes 7–9). At 12 hpt (lane 2), in longer exposures, a band comigrating with subgenomic RNA3 (lane 12) could be detected. The level of this RNA increased from 12 to 72 hpt, then plateaued (lanes 3–9).

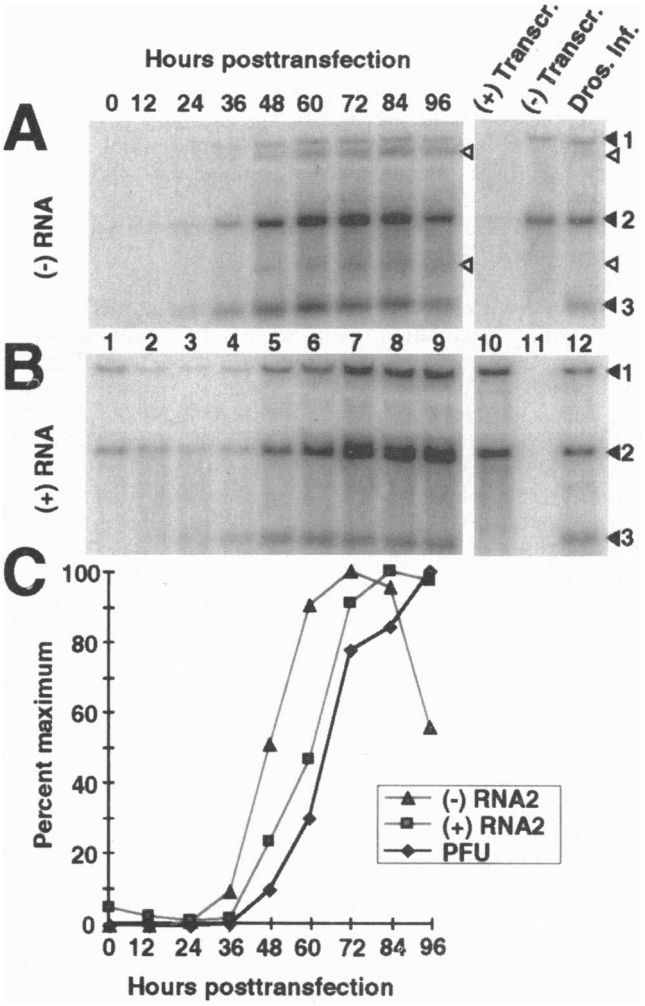

FIG. 3. FHV genome replication and transcription in FHV virion RNA-transfected yeast spheroplasts. Total RNA was extracted from spheroplasts in the culture described in Fig. 2. RNAs were denatured with glyoxal, electrophoresed on an agarose-phosphate gel, transferred to a nylon membrane, hybridized to the indicated strand-specific ^{32}P-labeled *in vitro* transcripts, and exposed to a PhosphorImager imaging plate. (*A*) Northern analysis of (−) strand FHV RNA. Lanes: 1–9, one-fifth of the total RNA isolated from spheroplasts in 1 ml of culture at the indicated time point; 10, 4 ng each of (+) strand FHV RNA1 and RNA2 *in vitro* transcripts; 11, 0.08 ng each of (−) strand FHV RNA1 and RNA2 *in vitro* transcripts; 12, total RNA extracted from *Drosophila* cells 20 hr after transfection with FHV virion RNA. Four micrograms of total yeast RNA was used as a carrier in lanes 10–12. The blot was hybridized simultaneously to two probes complementary to (−) strand FHV RNA3 and RNA2. The positions of FHV RNA1, RNA2, and RNA3 are indicated with numbered, filled arrows. The positions of two additional RNAs are indicated with open arrows (see *Results*). (*B*) The same as *A* except that four times less RNA was loaded per lane, the blot was hybridized simultaneously to two probes complementary to (+) strand FHV RNA3 and RNA2, and the image was printed at one-third the intensity level. (*C*) Accumulation of (−) RNA2, (+) RNA2 (*A* and *B*), and pfu (Fig. 2), as percent maximum of each, versus hpt.

As seen in Fig. 3*C*, FHV (−) and then (+) RNA accumulation foreshadowed pfu accumulation in yeast. Measurements of pfu and RNA show that the vast majority of yeast-derived FHV virions must contain FHV RNAs synthesized in yeast. The infectious virion yield at 72 hpt and beyond was 2×10^8

pfu per ml of culture. Unless yeast-derived FHV virions had higher specific infectivity than those from *Drosophila* cells, which have a particle to pfu ratio of 320 (7, 31), this corresponded to 160 ng encapsidated FHV RNA per ml. In comparison, the total level of cell-associated genomic RNA per ml of culture was ≈40-fold less than this at 0 hpt, but only ≈3-fold less at 72 hpt (as determined by PhosphorImager quantification of the bands in Fig. 3*B*, lanes 1 and 7, and similar experiments). The final 3-fold difference may reflect, in part, losses during RNA extraction.

Selection of Uracil Prototrophs from Yeast Transfected with an RNA Replicon. Since yeast supported FHV RNA replication, a system for *in vivo* expression and maintenance of heterologous markers dependent on FHV genomic replication was developed. To provide FHV trans-acting replication functions, plasmid p1R (Fig. 1*B*) was designed with an intact FHV RNA1 cDNA 1 nt downstream of the bacteriophage T7 RNA polymerase promoter, and with a hepatitis delta virus ribozyme cDNA positioned to cleave primary transcripts at the exact 3′ end of FHV RNA1 (10). A control plasmid, p1(fs)R (Fig. 1*B*), differs from p1R by a frameshift 10% into the polymerase gene. Control experiments showed that inclusion of a plasmid expressing T7 RNA polymerase in *S. cerevisiae* (32) was inconsequential for all experiments described below. Its function was presumably replaced by cellular polymerases acting at cryptic promoters in p1R and p1(fs)R. Similar results have been reported for FHV RNA2 cDNA constructs in mammalian cells (10).

To express and maintain a heterologous marker *in vivo*, RNA DU (Fig. 1*C*) contains the yeast *URA3* ORF fused to the first 12 codons of the FHV capsid protein precursor in the context of DI634, a naturally occurring, efficiently replicating DI RNA derived from RNA2 (11). A control variant of DU, DUΔ3′ (Fig. 1*C*), lacked the 3′ 100 bp of FHV RNA2, which are necessary for its replication.

To determine if DU could be replicated, express *URA3*, and persist in yeast, YPH500 (*ura3-52*) spheroplasts containing p1R or p1(fs)R were transfected with DNase I-treated, capped *in vitro* transcripts of DU, DUΔ3′ or B3URA3. B3URA3 is a BMV RNA3 derivative able to replicate and express URA3 in yeast expressing BMV replicase genes (2), but which lacks cis elements required for FHV-directed replication. As seen in Table 1, consistent with a requirement for both the trans and cis components of FHV genomic replication, no colonies were obtained when DU RNA was transfected into spheroplasts containing p1(fs)R or when spheroplasts containing p1R were transfected with DUΔ3′ or B3URA3. Consistent with replication and inheritance of the DU RNA replicon, Ura$^+$ colonies were obtained following DU RNA transfection into spheroplasts containing p1R.

Absence of Wild-Type *URA3* DNA in Prototrophs. To determine if the above Ura$^+$ phenotype was due to unexpected alteration at the level of DNA, a PCR assay was used. The inactivating *ura3-52* lesion in the starting yeast is a Ty transposon insertion into the *URA3* coding region (33). Primers that anneal 28 bp 3′ of the *URA3* initiation codon and 20 bp 5′ of the termination codon allowed simultaneous amplification of a 758-bp *URA3* amplimer and a 6.5-kb *ura3-52* amplimer. PCR products amplified from total DNA extracted from relevant yeast strains were analyzed by Southern blot analysis with a *URA3* probe.

Table 1. Representative results for selection of uracil prototrophs following transfection of *ura3-52* yeast with the indicated RNA

Yeast strain	Transfected with	Ura$^+$ colonies
p1R	100 ng DU	4.5
	1000 ng DU	36
	1000 ng DUΔ3′	0
	2000 ng B3URA3	0
p1(fs)R	1000 ng DU	0

YPH500 (*ura3-52*) spheroplasts (5×10^7) containing p1R or p1(fs)R (see Fig. 1) were transfected with DU, DUΔ3′ (see Fig. 1), or B3URA3 (see ref. 2) DNAse I-treated *in vitro* transcripts. The spheroplasts were then plated in regeneration agar lacking uracil to select for Ura$^+$ prototrophs. The numbers of colonies are the averages of two independent experiments.

As seen in Fig. 4, a 6.5-kb band corresponding to the expected *ura3-52* amplimer was detected in PCR products from all strains derived from YPH500 (*ura3-52*) (lanes 3–6). This band was not detected in PCR products from a control yeast strain with a wild-type chromosomal *URA3* locus (lane 7). A 0.8-kb band corresponding to the expected *URA3* amplimer was only detected in PCR products from YPH500 (*ura3-52*) cells containing YEp24, a control plasmid with a wild-type *URA3* gene, or from a control yeast strain with a wild-type chromosomal *URA3* locus (lanes 6 and 7). This band was not detected in PCR products from DU-transfected Ura$^+$ cells (lane 5). Two additional high molecular weight bands of unknown origin were also detected in PCR products from cells containing the empty p1R cloning vector (YEplac112) or YEp24 (lanes 3 and 6). As these bands were not present in samples from DU-transfected cells (lane 5), they do not confuse the results.

Presence of Replicating FHV RNAs in Prototrophs. To test for possible maintenance of DU RNA by FHV-directed RNA replication, total RNA was extracted from DU RNA-transfected Ura$^+$ yeast strains for Northern blotting. As seen in Fig. 5 *A* and *B*, (+) and (−) strand-specific FHV RNA3 probes revealed no detectable bands in total RNA from yeast cells containing p1R progenitor YEplac112 (lane 6) or in total RNA from mock-transfected *Drosophila* cells (lane 3). RNA species corresponding in size to (+) and (−) strand RNA1 and RNA3 were detected in total RNA extracted from yeast containing p1R (lanes 7 and 8) but not p1(fs)R (lane 9). An RNA species migrating just above (+) RNA1 was present in total RNA extracted from cells containing p1R or p1(fs)R (see

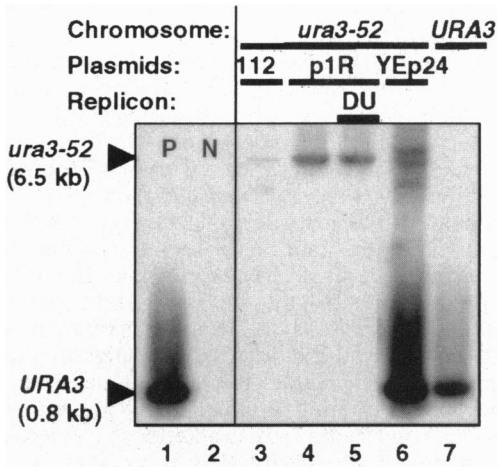

FIG. 4. Absence of *URA3* DNA in DU-transfected Ura$^+$ yeast cells. Total DNA was extracted from selected yeast strains grown overnight in liquid media. *URA3* sequences were amplified by PCR, electrophoresed on an agarose gel, transferred to a nylon membrane, and hybridized to a strand-specific ^{32}P-labeled *in vitro* transcript complementary to (+) strand *URA3*. Lanes: 1, P contains PCR products amplified from YEp24 DNA, a control plasmid containing the wild-type *URA3* gene; 2, N contains the products of a PCR reaction without added DNA template; 3–7, PCR products amplified from total DNA extracted from yeast cells having the characteristics shown at the top. Row 1 indicates whether the yeast strain was YPH500 (*ura3-52*) or S288C (*URA3*). Row 2 indicates whether the cells contained the plasmid YEplac112 (112), p1R or YEp24. In row 3, the heading DU indicates cells containing the DU RNA replicon. The positions of the PCR products of the *ura3-52* and *URA3* alleles are indicated.

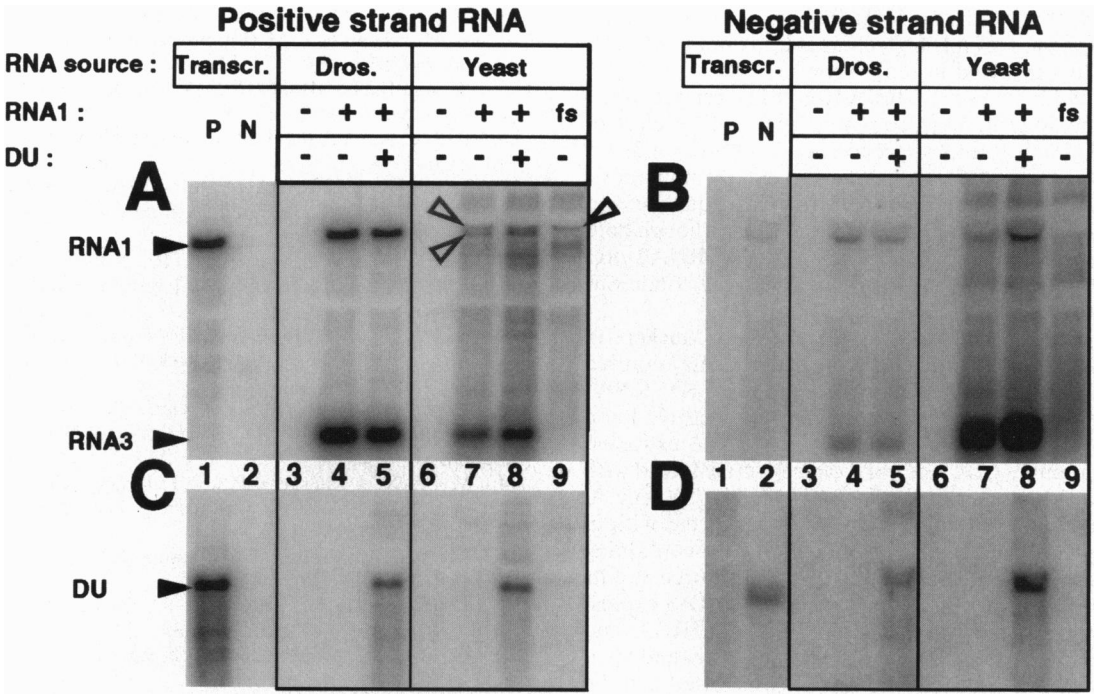

FIG. 5. Replication of *in vivo* transcribed FHV RNA1 and transfected DU RNA in yeast cells. Yeast cells containing the indicated RNAs were grown in liquid media to late log phase. Total RNA was extracted and processed as in Fig. 3, using the probes described below. (*A*) Northern analysis of (+) strand FHV RNA. Lanes: 1, P contains 1 ng each of *in vitro* transcribed (+) strand FHV RNA1 and DU (see Fig. 1); 2, N contains 0.01 ng each of *in vitro* transcripts of (−) strand RNA1 and a truncated DU (−) strand lacking the 3′ 264 nt; 3–5, total RNA extracted from *Drosophila* cells 20 hr after transfection with ddH_2O, with FHV RNA1 alone, or with RNA1 and DU, respectively. One microgram of total yeast RNA was used as a carrier in each of lanes 1–5. Lanes 6–8 contain 2.5 μg total RNA extracted from yeast cells containing YEplac112 alone, p1R alone, p1R and DU, or p1(fs)R alone (see Fig. 1), respectively. The blot was hybridized to a probe complementary to (+) strand RNA3. The positions of RNA1 and subgenomic RNA3 are indicated at left. The positions of two RNAs, one comigrating with RNA1 in lanes 7 and 8 and one migrating just above RNA1 in lanes 7–9, are indicated with open arrows (see *Results*). (*B*) The same as *A* except that 4-times more RNA was loaded per lane, the blot was hybridized to a probe complementary to (−) strand FHV RNA3, and the image was printed at 10-times the intensity level. (*C*) The same as *A* except that 70-times more *Drosophila* RNA was loaded in lanes 3–5 and the blot was hybridized to a probe complementary to (+) strand *URA3*. (*D*) The same as *C* except that 4-times more RNA was loaded per lane, the blot was hybridized to a probe complementary to (−) strand *URA3*, and the image was printed at 10-times the intensity level.

upper open arrow in Fig. 5*A*, lanes 7–9). This species may represent a transcript derived from p1R and p1(fs)R by upstream initiation by a cellular DNA-dependent RNA polymerase.

As seen in Fig. 5 *C* and *D*, (+) and (−) strand-specific *URA3* probes revealed no bands in total RNA from any YPH500 (*ura3-52*) yeast cells not transfected with DU RNA (lanes 6, 7, and 9) or in total RNA from mock- or RNA1-transfected *Drosophila* cells (lanes 3 and 4). However, total RNA extracted from *Drosophila* cells transfected with RNA1 and DU RNA or from DU-transfected Ura^+ yeast contained (+) and (−) DU-sized RNA bands (lanes 5 and 8). Therefore, DU RNA-transfected Ura^+ yeast prototrophs, at least 30 generations after transfection, contain RNAs that hybridize to (+) and (−) *URA3* probes and have mobilities indistinguishable from DU RNA replicated in *Drosophila* cells.

DISCUSSION

The results reported here establish that *S. cerevisiae* can support robust FHV genomic replication and infectious virion synthesis. They also show that an FHV-derived RNA replicon expressing a selected marker gene can be maintained persistently in yeast, conferring heritable phenotypic changes that should be useful in further genetic analysis of viral replication and gene expression.

***De Novo* Synthesis of FHV RNA and Infectious Virions in Yeast.** Following transfection of yeast spheroplasts with (+) strand FHV RNA1 and RNA2, all expected RNA products of FHV RNA replication and transcription appeared (Fig. 3 *A* and *B*). The dramatic increase in (+) RNA1 and RNA2 between 0 and 84 hpt, as well as the appearance of (−) strand RNA1 and RNA2 replication intermediates and subgenomic RNA3, which were not present in the inoculum, all demonstrated *de novo* RNA synthesis. As expected for RNA-dependent viral replication, accumulation of (−) strand RNA peaked first, followed in turn by (+) strand RNA, and then FHV pfu (Fig. 3*C*). The sensitivity of these pfu to FHV-neutralizing antibodies, but not to ribonuclease, showed that they represented virions. Moreover, these virions resulted from packaging RNA synthesized in yeast, rather than from translation and repackaging of the inoculum: pfu accumulation increased exponentially between 12 and 48 hpt, (+) genomic RNA accumulation foreshadowed, temporally and quantitatively, virion accumulation, and cell-associated inoculum at 0 hpt was insufficient to account for the number of FHV virions.

Robustness of FHV RNA Replication and Virion Synthesis in Yeast. FHV replication in yeast mirrored that in *Drosophila* cells in multiple ways. (*i*) The molar ratios of all FHV RNAs in yeast were similar to those in *Drosophila* cells. (*ii*) The calculated yield of 2000 pfu per infected spheroplast was comparable to the yield of 2400 pfu per infected *Drosophila* cell (31). This was initially surprising because yeast cells are normally much smaller than *Drosophila* cells. However, yeast spheroplasts are unable to regenerate their cell wall in liquid media (34), and our microscopy showed that spheroplasts increased in size from 70 μm^3 (35) at 0 hpt to about the size of a *Drosophila* cell, 360 μm^3, by 24 hpt, independently of infection. (*iii*) The ratio of (+) strand RNA to pfu showed that the specific infectivity of yeast-derived FHV virions was at

least as high as that of virions from *Drosophila* cells. Overall, the results imply surprisingly high compatibility between FHV and yeast factors involved in replication.

Previously, when (−) strand RNA from FHV replication in BHK (baby hamster kidney) cells was analyzed by electrophoresis, (−) strand RNA2 sequences were found to migrate in at least two bands: a less prevalent band of monomeric RNA2, and a predominant band with mobility equivalent to dimeric RNA2 or double-stranded RNA2 (10). Although both bands were observed (Fig. 3*A*), monomeric (−) RNA2 predominated in both insect and yeast cells under the conditions described here.

Expression and Maintenance of Heterologous Markers in Yeast by FHV RNA Replication. Viral components required for FHV RNA replication were provided from FHV RNA1 cDNA plasmid p1R. RNA species comigrating with (+) and (−) RNA1 and RNA3 were present in total RNA extracted from cells containing p1R, but not from cells transfected with a derivative with a frameshift in the FHV replication gene A.

When Ura^- cells containing p1R were transfected with *in vitro* transcripts of DU, an FHV RNA2-derivative containing the *URA3* gene, Ura^+ colonies were obtained. Three results indicated that the Ura^+ phenotype was due to URA3 expression from DU, which was replicated as a free RNA and inherited by daughter cells. (*i*) Colony formation depended on FHV components in trans and in cis. (*ii*) PCR showed that the Ura^+ phenotype was not a result of reverse transcription of the RNA to DNA. (*iii*) DU RNA and its (−) strand replication intermediate were found in Ura^+ prototrophs derived from DU-transfected spheroplasts many generations after transfection.

The ability of *S. cerevisiae* to support full FHV RNA replication and encapsidation, plus the ability of FHV to direct yeast phenotypes, means that the extensive resources of yeast can be used to analyze FHV. Among other opportunities, this provides powerful host genetics for identification and characterization of host factors required for FHV genomic replication, gene expression, and assembly.

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