Synthesis of Semliki Forest Virus RNA Polymerase Components nsP1 through nsP4 in *Saccharomyces cerevisiae* by Expression of cDNA Encoding the Nonstructural Polyprotein

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A Semliki Forest virus nonstructural polyprotein, P1234, expressed in the yeast *Saccharomyces cerevisiae* in the absence of a replicative RNA template appeared to be properly cleaved into nsP1 to nsP4. All nsPs were membrane associated, and nsP2 was also transported to the nucleus. The membrane fraction containing nsPs showed guanine-7-methyltransferase and guanylyltransferase-like activities, typical for Semliki Forest virus nsP1.

The Semliki Forest virus (SFV) 42S RNA genome codes for two polyproteins. One is translated from a subgenomic 26S mRNA and is processed into four structural proteins (C and E1 to E3); the other (P1234) is translated directly from the SFV genome and is autocatalytically cleaved into nonstructural proteins (nsPs) nsP1 to nsP4 (10, 22), which are the virus-specific components of the membrane-bound alphavirus RNA replication complex (3, 6, 10). We have characterized properties of the nsPs after expression in mammalian (13–15, 18) and insect cells (11) and in *Escherichia coli* (11, 19). Here we report the expression of SFV P1234 in the yeast *Saccharomyces cerevisiae*, which has been successfully used for the expression of brome mosaic virus nonstructural proteins, resulting in the assembly of active RNA polymerase (9).

Construction of expression vectors. *E. coli* XL-1 from Stratagene was used to propagate all plasmids. *S. cerevisiae* Y294 (MAT α *his3 Δ 1 leu2-3,112 ura3-52 trp1-289 GAL⁺*) and yeast shuttle vectors pYADE4 and pYPGE2 were kindly provided by Joseph Brunelli and Martin Pall (4). Plasmids pYAP1234 and pYKP1234 were constructed by inserting the entire coding region for SFV P1234 (without the 5' RNA replication sequences) between the plasmids' *ADH2* or phosphoglycerate kinase (*PGK*) promoters, respectively, and the *CYC1* transcription terminator of plasmid pYADE4 or pYPGE2, as follows. (i) A *Bam*HI-*Eco*RV restriction fragment, which encodes a *Bam*HI site directly 5' to the initiation codon and amino acids 1 to 64 of the polyprotein, was isolated from plasmid pBNS1 (11), which contains the nsP1 coding region. (ii) A second

restriction fragment, *Eco*RV-*Bam*HI, which encodes amino acids 65 to 2431, the termination codon of the polyprotein, and 45 untranslated nucleotides, including the *Bam*HI site, was isolated from plasmid pSFV1 (12), thereby encoding the entire P1234 polyprotein. Both restriction fragments were mixed together and ligated into pYADE4 or pYPGE2 plasmid by a triple-fragment ligation. Strain Y294 was transformed with the plasmids by the Li-acetate method (7).

Expression of SFV P1234 in yeast cells. *S. cerevisiae* Y294 was transformed with the shuttle plasmid pYAP1234, in which the expression of P1234 was under the control of the inducible *ADH2* promoter. Cells containing pYAP1234 were first grown to mid-log phase in synthetic complete medium (21) lacking tryptophan, to maintain the plasmid, but containing glucose, to suppress P1234 expression. The cells were then switched to a rich medium containing ethanol and glycerol to induce synthesis of SFV P1234. Northern (RNA) analysis (20, 24), using a probe encoding the nsP2 to nsP4 sequences, showed a band of the expected size of about 8 kb, which was not detectable from cells transformed with the parent plasmid (data not shown). Western analysis (immunoblotting) (11) of protein extracts from yeast cells (8), using monospecific antisera against nsP1 to nsP4, demonstrated that the individual nsPs were present in cells expressing P1234 mRNA (data not shown).

Because induction of P1234 showed no obvious deleterious effects on cell viability, another yeast shuttle plasmid, pYKP1234, was constructed, in which the transcription of the P1234 coding region was under the control of the constitutive

FIG. 1. Fractionation of yeast cells transformed by pYKP1234 plasmid analyzed by Western blotting. (A, B, C, and D) Treatment with antisera against nsP1, nsP2, nsP3, and nsP4, respectively. Lanes 1 in panels A to D show migration of recombinant marker proteins nsP1 to nsP4. Lanes 2 and 3 show the P15 and S15 fractions, respectively. Panel B, lane 4 shows a nuclear fraction treated with anti-nsP2 antiserum. Lanes 4 and 5 in panel D show the P15 and S15 fractions, respectively, from parent plasmid (pYPGE2)-transformed cells. (E) Lanes 1 and 2 show Coomassie blue-stained proteins in the P15 and S15 fractions, respectively.

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FIG. 2. Flotation separation of membranes from the P15 fraction from yeast cells transformed by pYKP1234 plasmid and analyzed by Western blotting. On the left, nsP1 to nsP4 denote the antisera used for immunostaining. TX denotes Triton X-100 treatment of the P15 fraction before centrifugation. Lanes: C, recombinant nsP1 to nsP4 from *E. coli*; T, top of the gradient; 2 to 3, fractions 2 to 3 (5% sucrose); 4 to 7, fractions 4 to 7 (50% sucrose); 8 and 9, fractions 8 to 9 (60% sucrose, the loading layer); 10 to 11, fractions 10 to 11 (67% sucrose cushion); B, bottom of the gradient.

PGK gene promoter. Since the transformed cells expressed the 8-kb mRNA and all of the individual nsPs, differential centrifugation was used to see whether the nsPs were associated with membranes, as in SFV-infected cells. For this purpose, yeast spheroplasts were made with lyticase (Sigma) (20,000 U in 50 ml of 1 M sorbitol in phosphate-buffered saline–1 mM phenylmethylsulfonyl fluoride for 60 min at 37°C). After being washed, the spheroplasts were broken with a Dounce homogenizer in hypotonic buffer (50 mM Tris [pH 8.0], 15 mM MgCl₂, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). Nuclei were separated (3,000 × *g*, 10 min), and the postnuclear supernatant was centrifuged for 20 min at 15,000 × *g* to obtain the pellet (P15) and supernatant (S15) fractions. Western blotting was carried out for the crude cellular fractions (Fig. 1). All of nsP1 (Fig. 1A, lane 2) and most of nsP3 (Fig. 1C, lane 2) and nsP4 (Fig. 1D, lane 2) were found in the P15 fraction, whereas nsP2 was mostly distributed between the membrane and nuclear fractions (Fig. 1B, lanes 2 and 4). None of the other nsPs were found in significant amounts in the nuclear fraction (data not shown). Lanes 4 and 5 in Fig. 1D show P15 and S15 fractions from yeast cells not expressing the nsPs to identify host proteins recognized by our anti-nsP4 antiserum. Coomassie blue staining of proteins from the P15 (Fig. 1E, lane 1) and S15 (lane 2) fractions demonstrates their relative amounts in equal volumes, emphasizing the preferential sorting of the nsPs to the membrane fraction. To ensure that the association

FIG. 3. Indirect immunofluorescence staining of yeast cells transformed with pYKP1234 (A, B, D, F, and H) or pYGE2 (C and E). Treatments with anti-nsP1 (A to C), anti-nsP2 (D and E), anti-nsP3 (F), and anti-nsP4 (H) antiserum are shown.

of the nsPs to P15 was not due to sedimentation of aggregated proteins, the P15 fraction was subjected to flotation separation in a discontinuous sucrose gradient (14). Thus, if membrane associated, the nsPs should float up through a 50% sucrose layer to the interface between 5 and 50% sucrose, but if no association exists, the nsPs should remain in the 60% sucrose layer. Western analysis performed on fractions taken from such gradients showed that nsP1 to nsP3 floated with the membranes to the interface (Fig. 2, lanes 3). Longer exposure of anti-nsP4-treated immunoblots also revealed nsP4 in the membrane fraction (the double band in Fig. 2, lane 3). In contrast, when the membranes in the P15 fraction were first solubilized with 2% Triton X-100, the two nsPs tested, nsP1 and nsP2, remained in the original 60% sucrose loading layer (Fig. 2, lanes 8).

Intracellular location of nsPs. Indirect immunofluorescence was performed with paraformaldehyde-fixed, Triton X-100-permeabilized yeast cells (17) transformed with pYKP1234. Anti-nsP1 antiserum revealed peripheral staining, suggesting that nsP1 was associated with the cytoplasmic aspect of the yeast plasma membrane (Fig. 3A). When the focal plane was changed, a less intense vesicle-like staining pattern could be seen in the cytoplasm (Fig. 3B). Anti-nsP2 antiserum produced intense staining of yeast nuclei and a punctate staining pattern in the cytoplasm (Fig. 3D). This result supports the subcellular fractionation data, which suggested that a portion of the nsP2 molecules is transported to the nucleus in yeast cells, as in mammalian cells (15), indicating that the nuclear localization signal of nsP2 (18) functions also in yeast cells. Immunofluorescence with anti-nsP3 (Fig. 3F) and anti-nsP4 antiserum (Fig. 3H) showed punctate staining patterns. No staining was seen in control cells treated with the antisera, as shown for anti-nsP1 (Fig. 3C) and anti-nsP2 (Fig. 3E) antisera.

Analysis of SFV nsP1-specific enzymatic activities. Guanine-7-methyltransferase activity was measured in the P15 and S15 fractions from cells transformed with pYKP1234 and pYGE2, with *S*-adenosyl[*methyl*-³H]methionine as the methyl donor and dGTP as the methyl acceptor (11) (Fig. 4A). It has been shown previously that nsP1-specific methyltransferase activity is enhanced by pretreatment with deoxycholate (DOC), whereas endogenous cellular activity is not (11). DOC caused at least a fivefold increase in the methyltransferase activity in the P15 fraction from pYKP1234-transformed cells (Fig. 4A, bars 2 and 3). Low levels of activity were detectable in the control cells and in the S15 fraction from pYKP1234-transformed cells (Fig. 4A, bars 1 and 4, respectively).

To assay for covalent complex formation between 7-methyl-GMP and nsP1, P15 fractions from yeast cells were incubated with [α -³²P]GTP and *S*-adenosylmethionine as described previously (1). The reaction mixture was immunoprecipitated (16) with anti-nsP1 antiserum, and the product was visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. α -³²P-labeled nsP1 was found in yeast cells expressing the nsPs (Fig. 4B, lane 3) and was similar in size to the complex formed by recombinant nsP1 synthesized in insect cells (lane 4). No such complex could be seen in the negative control (Fig. 4B, lane 2). These results demonstrate that nsP1 produced in yeast cells by cleavage from the polyprotein was enzymatically active.

The putative RNA polymerase activity of the P15 and S15 fractions of pYKP1234-transformed cells was assayed as described previously (6) with *in vitro*-transcribed RNA from a truncated derivative of pSFV 1 plasmid (12) with a central deletion between the *EcoRV* and *Bgl*III restriction sites (nucleotides 278 to 6715) as a template. Attempts were also made to detect this activity *in vivo* by transforming *in vitro*-made RNA

FIG. 4. nsP1-specific enzyme activities in yeast cells transformed with plasmid pYKP1234. (A) Guanine-7-methyltransferase activity. Bars: 1, the P15 fraction from yeast cells transformed with parent plasmid pYGE2 (negative control) and treated with DOC; 2, the P15 fraction from yeast transformed with pYKP1234; 3, same as for bar 2, except that the P15 fraction was first treated with DOC; 4, the S15 fraction from yeast transformed with pYKP1234 and treated with DOC. (B) Autoradiography of covalent 7-methyl-GMP-nsP1 complex. Lanes: 1, molecular weight markers (from the bottom, 30, 46, 69, 97, and 200 kDa); 2, the P15 fraction from yeast transformed with parent plasmid pYGE2; 3, the P15 fraction from yeast transformed with pYKP1234; and 4, recombinant nsP1 expressed in insect cells (1).

from the above-mentioned plasmid containing the yeast *URA3* gene cloned under the 26S subgenomic RNA promoter to yeast cells expressing the nsPs. However, neither direct nor indirect evidence of RNA polymerase activity could be demonstrated.

In this study, we produced SFV nsPs by transforming *S. cerevisiae* with a plasmid containing the SFV P1234 coding region. Western analysis with monospecific antisera revealed proteins comigrating with nsP1, nsP2, nsP3, and nsP4 markers by SDS-PAGE, suggesting that complete P1234 had been synthesized in the yeast cells. However, no P1234 or its intermediate cleavage products (P123, P12, or P34) (22) were detected by Western blotting, suggesting that the processing was fast, as in SFV-infected animal cells (16, 23). The apparently correct sizes of the nsPs suggest proper autocatalytic cleavage of P1234 by nsP2 (22). At least nsP1 and nsP2 had biological activities similar to those of SFV-infected cells, supporting their authenticity. In SFV-infected cells, the RNA replication complex is associated with membranes which sediment at 15,000 × *g*

(P15). This complex contains modified endosomes and lysosomes, viral replicative intermediate RNAs, RNA polymerase activity, and nsP1 to nsP4 (2, 3, 5, 6, 13). We have shown that nsP1 expressed alone in HeLa and BHK cells is associated with the plasma membrane, endosomes, and lysosomes, as in SFV-infected cells (13, 14), making it a possible candidate for the targeting of the replication complex to membranes. When P1234 was expressed in yeast cells, the cleavage products nsP1 to nsP4 were mostly associated with membranes (except nsP2), suggesting that they form a complex. Since only nsP1 has affinity for membranes in mammalian cells, it may also be responsible for membrane targeting of the complex in yeast cells. Our attempts to detect an active RNA polymerase complex in yeast cells were unsuccessful. However, the powerful arsenal of yeast genetics offers possibilities to address questions concerning individual nsPs (e.g., host factors involved in nsP1 targeting to membranes and nsP2 targeting to the nucleus).

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