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Expression of the RNA genome of an animal virus in *Saccharomyces cerevisiae*

(heterologous gene expression/yeast/intracellular traffic/vesicular stomatitis virus)

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ABSTRACT **The nucleocapsid of vesicular stomatitis virus (VSV) was introduced into the cytoplasm of *Saccharomyces cerevisiae* by low pH-dependent fusion of the viral envelope with the spheroplast plasma membrane. This led to *de novo* synthesis of the three major structural proteins of the virus—the G, N, and M proteins—as shown by immunoprecipitation of [^{35}S]methionine-labeled spheroplast lysates. In NaDodSO$_4$/polyacrylamide gel electrophoresis, M and N proteins comigrated with those of the virion, whereas the yeast-made G protein migrated as two bands with apparent molecular sizes of 60 and 70 kDa. Both polypeptides appeared to be N-glycosylated, since only one polypeptide with the apparent molecular mass of ≈55 kDa was produced in the presence of tunicamycin. Phase separation into Triton X-114 suggested that the unglycosylated G protein was membrane bound. According to immunofluorescent surface staining of live spheroplasts, at least part of the G protein was transported to the plasma membrane. Spheroplasts expressing the VSV genes could be fused together by low pH to form polykaryons, indicating that G protein synthetized by yeast was fusogenic—i.e., biologically active.**

Yeast is an attractive host for production of foreign proteins by recombinant DNA technology, since it is able to glycosylate and secrete them. Intracellular transport and posttranslational modifications of soluble enzymes of *Saccharomyces cerevisiae* have been well-characterized (1), whereas much less is known about membrane proteins. Extensive glycosylation, characteristic of secretory enzymes and mannoproteins of yeast (2, 3), can be suspected to impair biological functions of foreign glycoproteins expressed in this organism. Studies of intracellular transport and glycosylation of heterologous proteins in yeast require laborious recombinant DNA constructions. We have used a different approach to study the direct expression of the proteins of an animal virus in *S. cerevisiae*, taking advantage of the activation of the particle-associated transcriptase (L protein) of vesicular stomatitis virus (VSV) (4, 5). The nucleocapsid of VSV was introduced into the yeast cytoplasm by fusion of the virus envelope with the spheroplast plasma membrane. The genes encoding the major structural proteins (G, M, and N) were expressed within 30–120 min. The G protein was transported to the plasma membrane where its fusogenic activity could be elicited in acid medium, resulting in polykaryon formation.

MATERIALS AND METHODS

Cells and Virus Preparations. The strain OL1 (6) of *S. cerevisiae* was grown in YPD medium (growth medium) containing 1% yeast extract (Oxoid, Basingstoke, U.K.), 2% Bacto-peptone (Difco), and 2% glucose (BDH), at 30°C in a shaker to densities of 4–15 × 10^7 cells per ml. Spheroplasts were prepared with zymolyase 60,000 (Seikagaku, Tokyo, Japan) as described (7). All media used for spheroplasts contained 1.2 M sorbitol to prevent osmotic lysis. Unlabeled and [^{35}S]methionine-labeled VSV (Indiana serotype) were grown in baby hamster kidney cells and purified as described (8).

Fusion of Virus with Spheroplasts. Spheroplasts were washed once with citric acid phosphate buffer containing 1.2 M sorbitol (pH 4.0) (pH 4 medium) and resuspended into pH 4 medium at a concentration of 1.5 × 10^8 spheroplasts per 100 μl. After addition of 9 μg of VSV (viral protein), the spheroplasts were incubated for 5 min at 37°C. One milliliter of cold phosphate-buffered saline, containing 1.2 M sorbitol (pH 7.4) (pH 7.4 medium) was added to stop the fusion reaction. The spheroplasts were pelleted and washed twice with pH 7.4 medium. The fusion process will be characterized in detail elsewhere.

Phase Separation into Triton X-114. Spheroplasts were lysed with 0.03% NaDodSO$_4$ containing 2 mM phenylmethylsulfonyl fluoride (PhMeSO$_2$F) (Sigma), Trasylol (100 units/ml) (Bayer, Leverkusen, F.R.G.), and 1.5% Triton X-114 (9) (Sigma). The lysate was centrifuged for 3 min at 3000 rpm. The supernatant was diluted 1:5 with 20 mM Tris·HCl (pH 8.0) containing 0.15 M NaCl, 0.1 mM EDTA, 0.75% Triton X-114, 2 mM PhMeSO$_2$F, and Trasylol (100 units/ml), and it was incubated successively for 5 min at 0°C and 37°C. After centrifugation for 5 min at 10,000 rpm, the aqueous phase and the detergent phase were separated from each other and subjected to immunoprecipitation.

Immunoprecipitation. The spheroplasts were lysed by adding 100 μl of hot 0.05% NaDodSO$_4$, or subjected to phase separation into Triton X-114. Then 400 μl of 20 mM Tris·HCl containing 0.15 M NaCl, 0.1 mM EDTA, and 1% Nonidet P-40 (pH 8.0) (NET buffer) and 50 μl of normal rabbit serum were added (only to the lysates and the detergent phase), and the preparations were then incubated for 30 min at room temperature under rotation. Fifty microliters of 10% protein A-Sepharose (Pharmacia, Sweden) was added and the incubation was continued for 30 min. The protein A-Sepharose was pelleted (2 min at 10,000 rpm) and the supernatant was incubated with 5 μl of anti-VSV antiserum for 60 min, and another 60 min after addition of 50 μl of 10% protein A-Sepharose. The protein A-Sepharose was again pelleted and the pellet was washed with 0.5 M NaCl in NET buffer, then twice with NET buffer lacking detergent, and twice with 10 mM Tris·HCl (pH 7.5). The pellet was resuspended in Laemmli sample buffer (1% NaDodSO$_4$) and subjected to polyacrylamide gel electrophoresis according to ref. 10 as described (7).

Immunofluorescent Staining. Spheroplasts were fixed with 3% paraformaldehyde, prepared in pH 7.4 medium, for 30 min at room temperature. After three washes with pH 7.4

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Abbreviations: VSV, vesicular stomatitis virus; PhMeSO$_2$F, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate.

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medium, anti-VSV antiserum diluted 1:2 with pH 7.4 medium containing 0.2% bovine serum albumin and 0.5 M NaCl was added (pH 7.4 medium/BSA/NaCl). After a 1-hr incubation at room temperature, the spheroplasts were washed twice with pH 7.4 medium/BSA/NaCl and incubated for 1 hr at room temperature with fluorescein isothiocyanate (FITC)-conjugated anti-IgG antibody (DAKO-immunoglobulins A/S, Copenhagen, Denmark), diluted 1:20 with pH 7.4 medium/BSA/NaCl. The spheroplasts were then washed three times and mounted on concanavalin A-coated microscopic slides for observation with a Polyvar microscope (Reichert-Jung, Vienna, Austria) equipped with a ×100 oil immersion objective, fluorescence filters, and Normarski optics. Agfapan 400ASA film (Agfa-Gevaert, Leverkusen, F.R.G.) exposed for 45 sec was used for photography. Surface-staining of live nonfixed spheroplasts was done similarly, except that all treatments were carried out in the cold and incubations with the antibodies were for 30 min.

Other Materials and Methods. Cycloheximide and tunicamycin were from Sigma and fungal proteinase K was from Merck. Scintillation counting was carried out in xylene/Nonidet P-40/Permablend cocktail or in toluene/Permablend in an LKB-Wallac 1211 RackBeta counter.

RESULTS

Expression of the Structural Proteins of VSV in *S. cerevisiae*. VSV was fused with the plasma membrane of *S. cerevisiae* spheroplasts. Nonfused virus was washed off, and the spheroplasts were incubated for 2 hr at 37°C in YPD medium in the presence of [^{35}S]methionine. The spheroplasts were then lysed and subjected to immunoprecipitation using anti-VSV antiserum, which reacts with the three major structural proteins of the virus (11). Analysis by NaDodSO$_4$/polyacrylamide gel electrophoresis revealed four polypeptides that were absent from immunoprecipitates of control spheroplast lysates. One of the bands comigrated with the virion M protein and one comigrated with the virion N protein. No band was detected at the position of the virion G protein. Instead, two other bands with apparent molecular masses of 70 and 60 kDa were found, which probably represented anomalously glycosylated forms of G protein (Fig. 1*A*). The 70- and 60-kDa proteins were N-glycosylated, since they could not be detected when the spheroplasts were labeled after VSV fusion in the presence of tunicamycin. Instead, NaDodSO$_4$/PAGE of the immunoprecipitates revealed one band with the apparent molecular mass of 55 kDa (Fig. 1*B*). This polypeptide was amphipathic, since it could be recovered into the detergent phase in phase separation using Triton X-114 (Fig. 1*B*), whereas the N and M proteins could be immunoprecipitated from the aqueous phase (Fig. 1*C*).

Synthesis of VSV-specific proteins, after fusion of the virus with spheroplasts, could be visualized by indirect immunofluorescent staining. After 2 hr of incubation at 37°C, the spheroplasts were fixed and treated with anti-VSV antiserum, followed by FITC-conjugated anti-IgG antibody. The spheroplasts were brightly stained and formed aggregates (Fig. 2*a*). Only weak background staining could be seen when spheroplasts were stained right after fusion of the virus (Fig. 2*b*) or when spheroplasts were not treated with virus (Fig. 2*c*). Fixation permeabilizes the spheroplasts, and thus the fluorescence in Fig. 2 visualizes antigens on the surface as well as inside the cell.

Transport of G Protein to the Spheroplast Surface. Indirect immunofluorescent staining of live spheroplasts was carried out to monitor possible surface expression of the newly synthesized G protein. VSV was fused with the plasma membrane as before, and the implanted G protein was removed from the surface by proteinase K digestion in the cold. Spheroplasts were then treated immediately or after incubation in YPD medium at 37°C with anti-VSV antiserum, followed by FITC-anti-IgG antibody. The staining procedure was carried out in the cold to prevent endocytosis (7). Proteinase K digestion removed all detectable implanted G protein from the surface, as shown in Fig. 3*a*. Increasing fluorescent staining of the plasma membrane could be observed when spheroplasts were incubated at 37°C for different periods of time (Fig. 3 *a–c*). This indicates that newly synthesized G protein had been transported to the surface. Surface expression was largely suppressed when the incubation was performed in the presence of cycloheximide (Fig. 3 *e* and *f*). The slight staining in Fig. 3*f* is due to the incomplete shutoff of protein synthesis with cycloheximide, which under the present conditions was ≈75%, as demonstrated by incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material. In many yeast strains, inhibition of protein synthesis by cycloheximide is inefficient (12).

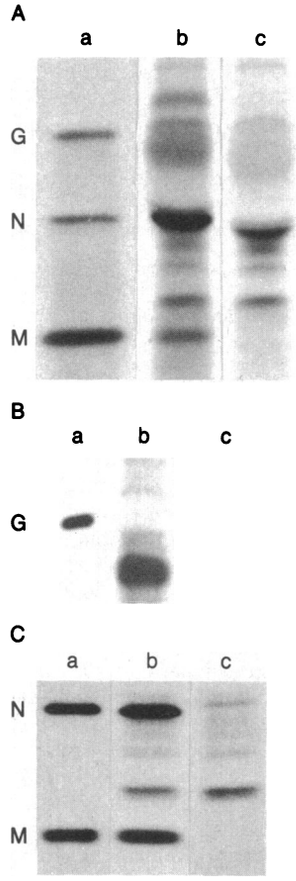

FIG. 1. Immunoprecipitation of VSV-specific proteins from spheroplast lysates. (*A*) *S. cerevisiae* spheroplasts (1.5×10^8) were incubated for 5 min at 37°C in 100 μl of pH 4 medium in the presence (lane b) or absence (lane c) of 9 μg of VSV. Nonfused virus was washed off, and the spheroplasts were incubated for 2 hr at 37°C in YPD medium in the presence of 140 μCi of [^{35}S]methionine (1 Ci = 37 GBq). They were then lysed with NaDodSO$_4$ and subjected to immunoprecipitation using anti-VSV antiserum. [^{35}S]Methionine VSV (lane a). (*B* and *C*) Spheroplasts were incubated after fusion with VSV with 125 μCi of [^{35}S]methionine as in *A*, but in the presence of tunicamycin at 20 μg/ml (lane b). The spheroplasts were then lysed and subjected to phase separation into Triton X-114. The detergent phase (*B*) and the aqueous phase (*C*) were immunoprecipitated using anti-VSV antiserum. Lanes: a, [^{35}S]methionine VSV; c, same as lane b, except that no virus was fused with the spheroplasts.

G Protein Synthesized in *S. cerevisiae* Is Fusogenic. Spontaneous fusion between *S. cerevisiae* spheroplasts is a very rare event (13). We could not find any polykaryons in

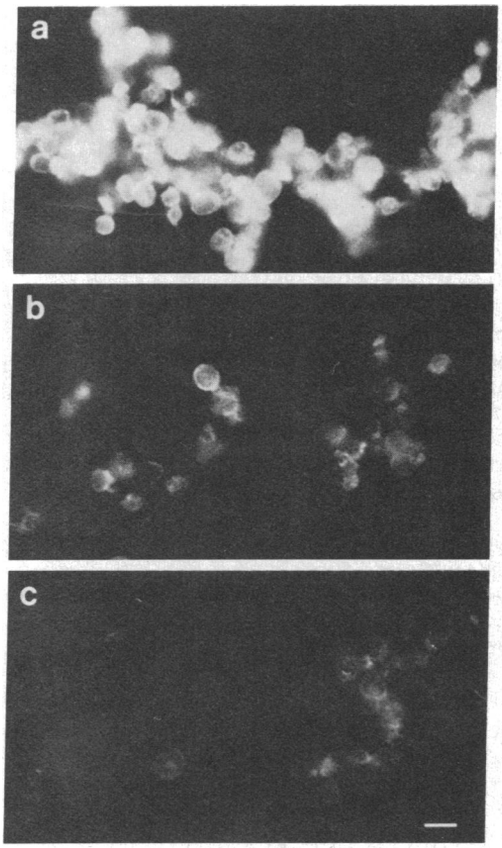

FIG. 2. Immunofluorescent staining of permeabilized spheroplasts. Spheroplasts were incubated in pH 4 medium in the presence (*a* and *b*) or absence (*c*) of VSV, as in Fig. 1. Nonfused virus was washed off, and the spheroplasts were fixed immediately (*b* and *c*) or after incubation for 2 hr at 37°C in YPD medium (*a*). All samples were subjected to indirect immunofluorescent staining using anti-VSV antiserum. (Bar = 5 μm.) (×870.)

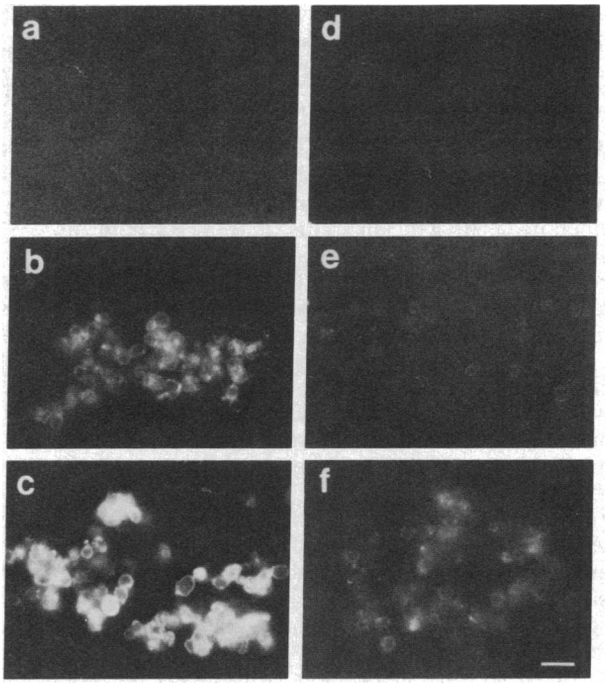

FIG. 3. Surface staining of spheroplasts. Spheroplasts were incubated in pH 4 medium in the presence (*a*, *b*, *c*, *e*, and *f*) or absence (*d*) of VSV, as in Fig. 1. After washes, all samples were digested for 15 min at 0°C with proteinase K (0.5 mg/ml) in pH 7.4 medium to remove the implanted G protein from the plasma membrane. After two washes, they were subjected to indirect immunofluorescent surface staining in the cold, using anti-VSV antiserum, either immediately (*a* and *d*), after 30 min (*b* and *e*), or after 60 min (*c* and *f*) of incubation at 37°C in YPD medium. In *e* and *f*, the YPD medium contained 100 μg of cycloheximide per ml. Nomarski optics showed that *a* and *d* contained at least similar amounts of spheroplasts as *e*. (Bar = 8 μm.) (×560.)

spheroplast preparations that were not exposed to VSV. To search for polykaryons, we took advantage of the fact that in most yeast cells there is only one vacuole (the digestive organelle). This large organelle is easily revealed by Nomarski optics. The finding of several vacuoles enclosed by one membrane of a giant cell was taken as evidence for polykaryon formation. The following experiment was carried out to study whether spheroplasts expressing newly synthesized G protein on their surface could be fused together. Spheroplasts with fused VSV were incubated for 1.5 hr at 37°C in YPD medium and then transferred to pH 4 medium and incubated further for 30 min at 37°C. Large polykaryons with tens of vacuoles could be seen by light microscopy using Nomarski optics (Fig. 4*a*). The formation of polykaryons was dependent on two factors—namely, preincubation in YPD medium, which allowed synthesis and surface expression of G protein, and the subsequent low pH treatment. No polykaryons could be detected right after the virus–spheroplast fusion, or when the low pH incubation was omitted (Fig. 4*b*). Polykaryon formation confirmed that G protein had reached the surface of the spheroplasts and indicated that it was biologically active—i.e., fusogenic.

DISCUSSION

Yeast has become an attractive model for both molecular and cell biologists because of its reasonably limited genetic capacity, as compared to animal cells, and because of the existence of a firm genetic background. One of the reasons for the increased interest in yeast is evidently also its potential suitability for production of valuable glycoproteins, which can be secreted into the growth medium. The secretory machinery of yeast has been recently approached by modern molecular biological techniques (14). In these studies, secretion-deficient temperature-sensitive mutants have turned out to be invaluable tools (15). Introduction of cDNA molecules encoding potentially useful proteins has been achieved by recombinant DNA techniques using yeast plasmids and

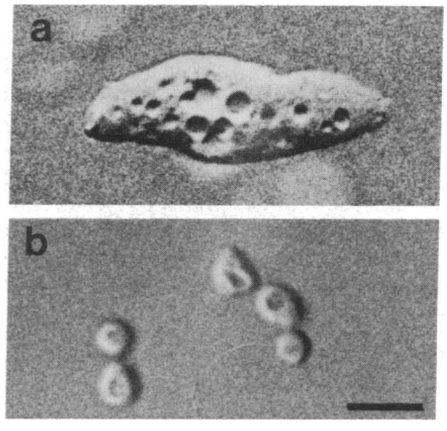

FIG. 4. Polykaryon formation. (*a*) VSV was fused with spheroplasts as described. Washed spheroplasts were incubated in YPD medium for 90 min and then in pH 4 medium for 30 min. Samples of nonfixed live spheroplasts were gently layered on concanavalin A-treated microscopic slides and viewed with the microscope using Nomarski optics, which clearly reveals the vacuoles. (*b*) Incubation in pH 4 medium was omitted. (Bar = 5 μm.)

promoters of genes isolated from the yeast genome (16). The yields of the foreign proteins have been relatively low as compared with the bacterial production systems (17).

Evidently our knowledge of the secretion process and membrane traffic in general is still too fragmentary to allow modification of yeast cells to increase their secretory capacity. This is why we started to study membrane traffic in yeast. We showed recently that the yeast *S. cerevisiae* has a functional endocytic apparatus. In these experiments, VSV, Semliki Forest virus, α-amylase, and FITC-conjugated dextran were used as markers (7, 18). Similar findings were reported using Lucifer yellow to demonstrate endocytosis (19).

We have again used VSV as a tool, now to study the biosynthesis of membrane proteins in yeast. This virus offers two advantages: First, the G protein is fusogenic in acid medium (20), which should allow the introduction of the nucleocapsid directly into the yeast cytoplasm by fusion of the virus envelope with the plasma membrane. Second, the nucleocapsid contains its own transcriptase, which is activated *in vitro* after the removal of the viral envelope (21). This enzyme transcribes mRNA molecules that direct the synthesis of the virion proteins (M, N, G, L, and NS proteins; see refs. 4 and 5). Thus, we reasoned that primary transcription and, hopefully, also primary translation would take place in yeast. If so, one should be able to detect the synthesis of structural proteins during a short transient observation period.

Indeed, we were able to demonstrate the synthesis of the major structural proteins N, M, and G of VSV in spheroplasts of *S. cerevisiae* during an observation period of 2 hr after the virus–spheroplast fusion, indicating that the virion transcriptase was activated in the yeast cytoplasm. The N and M proteins migrated in $NaDodSO_4$/PAGE like those from the virion. No clear band migrated at the position of the virion G protein. Instead, two other bands (70 and 60 kDa) could be detected after immunoprecipitation. When the labeling of the proteins was carried out in the presence of tunicamycin, which inhibits the synthesis of asparagine-linked glycans (22), a single band was detected after immunoprecipitation. It had an apparent molecular mass of 55 kDa, which is the molecular mass of nonglycosylated G protein (23). Thus the 70-kDa band probably represented a hypermannosylated form of G protein. Assuming that both glycosylation sites of the G protein (23) were occupied with similar glycans, these would consist of ≈45 mannose residues. The 60-kDa band could represent G protein with primary glycans. Thus, the signal sequence of G protein was functional in *S. cerevisiae*. G protein can be recovered into Triton X-114 in phase separation both from the virus as well as from membranes of the animal cell (24). The 55-kDa G protein was membrane-associated in the yeast spheroplast since it behaved in Triton X-114 extraction like an amphipathic polypeptide, while the N and M proteins behaved like soluble polypeptides.

Since the extension of the primary glycans takes place in the Golgi apparatus (25), at least the 70-kDa protein must have reached this organelle. Surface immunofluorescent staining of living spheroplasts, after removal of the implanted G protein by proteolytic digestion, revealed that the newly synthesized G protein accumulated on the cell surface. This implies that the membrane glycoprotein of an animal virus is properly recognized by the yeast cell machinery responsible for transport of plasma membrane glycoproteins. This would mean that the basic principles of addressing membrane proteins are of old evolutionary origin.

In mammalian cells, polykaryon formation has been used to show surface expression of viral fusogenic membrane proteins (26). Also yeast spheroplasts, which had accumulated G protein on their surface, could be fused with each other to form polykaryons when exposed to acid medium. Some of the polykaryons contained >20 vacuoles. This shows that the fusogenic activity of the yeast-made G protein was similar to that demonstrated in animal cells (20, 26), in spite of probable aberrant glycosylation. When VSV was added to spheroplasts in acidic medium, no polykaryon formation due to the implanted G protein could be detected. This may be due to the fact that maximally 90 particles could be fused per spheroplast (M.M., H. Sareneva, and C.-H. von Bonsdorff, unpublished data). The resulting density of the G protein in the plasma membrane may not have been high enough to induce spheroplast fusion.

Here the expression of the VSV proteins in yeast was evidently dependent on primary transcription and translation, which do not require replication. Quantitation of the immunoprecipitates showed that maximally 1–2% of the total protein synthesis was confined to the synthesis of virus-specific proteins. The levels of different soluble animal and plant proteins expressed in yeast from recombinant plasmids have been in the range of 0.01–5% of total protein (16, 17, 27–29). So far, one animal virus envelope glycoprotein, the hemagglutinin of influenza virus, has been expressed in *S. cerevisiae*. It was glycosylated, but its intracellular localization remained unclear (30).

The transient expression system described here might be used to study the synthesis and transport of glycoproteins of other negative-stranded fusogenic viruses in yeast, especially those that mature in specific organelles—e.g., Bunuyaviruses (Golgi-specific; see refs. 31 and 32) and Coronaviruses (endoplasmic reticulum-specific; 33, 34). To obtain valuable information about the evolution of the function of the secretory machinery in eukaryotic cells, temperature-sensitive transport mutants of viruses (35) could be combined with secretory mutants of *S. cerevisiae* (1, 36). The remarkably simple expression system we have described here circumvents the construction of recombinant DNA molecules and the search for yeast transformants that would survive the expression of alien, perhaps toxic, membrane glycoproteins.

Note Added in Proof. Recently, expression of the structural proteins of Sindbis virus and the G protein of VSV from recombinant plasmids in *S. cerevisiae* was published (37). The E_1 and G proteins were glycosylated, albeit not hypermannosylated, and transported partly to the cell surface.

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