Fusion of Yeast Protoplasts Induced by Polyethylene Glycol

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Protoplasts, prepared from auxotrophic strains of *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Hansenula wingei*, were mixed to give intraspecific complementary combinations. Polyethylene glycol (PEG) was added to induce agglutination and fusion. Some of the fused products grew on the surface of solid minimal medium forming large vacuolated bodies. Others reverted to hybrid cells when embedded in solid minimal regeneration medium. The cytological and preliminary genetical analyses suggest a synkaryon formation and integration of genetic markers from parental strains. The frequency of intragenetic fusions assessed from the number of protoplasts growing on the surface of minimal agar was estimated to be 1 to 3 %, while the frequency of hybrid colony formation in regeneration medium was less than 1 %.

INTRODUCTION

Protoplasts of fungi are useful models for cytological studies. They can be readily prepared, subjected to various treatments and cultivated. Under defined conditions they regenerate their walls within several hours and revert to normal cells (Nečas, 1971). The differentiation of the whole organism involves only the reversion of the regenerated protoplast into a normal cell; species-specific morphogenesis is already renewed by the first, second or third generations (Svoboda & Nečas, 1974). For these reasons, protoplasts can serve as unique tools for fusion experiments. Successful fusions have already been reported between fungal protoplasts (Ferenczy *et al.*, 1975; Anné & Peberdy, 1975, 1976) and between yeast protoplasts (Sipiczki & Ferenczy, 1977; van Solingen & van der Plaats, 1977; Yamamoto & Fukui, 1977).

Recently (Svoboda, 1976) I studied the possibility of fusion of yeast protoplasts derived from opposite mating-type cells. Sex-specific fusion occurred only after the wall had regenerated, indicating that mechanisms similar to those operating in intact cells are involved. Here I report the formation of artificial hybrids, between yeast auxotrophs, induced by PEG (Kao & Michayluk, 1974) and a simple method for the detection of developing hybrid protoplasts.

METHODS

Organisms and growth conditions. Haploid strains with detectable biochemical markers were used. *Saccharomyces cerevisiae* strains 9 *his* α and 5 *trp* *a* were obtained from Dr M. Opekarová, Institute of Microbiology, Prague, strain *rec291 leu trp1-1 ura4 met4 tyr7* α from Dr R. K. Mortimer, University of California, Berkeley, and strains 390 1 *C his* α and 8282 *ade* α from Dr V. Kováčová, Komenský University, Bratislava. *Schizosaccharomyces pombe* strains *leu1-32 h⁻* and *ade6-250 h⁻* were obtained from Dr U. Leupold, University of Bern, and *Hansenula wingei* strains 5 *cyh lys* and 21 *ade his* were from Dr M. Crandall, University of Kentucky, Lexington.

All strains were maintained on wort agar slants. The cultures were grown in liquid wort medium at 28 °C and before conversion to protoplasts they were cultivated in minimal medium (Leupold, 1955) for

2 h. The protoplasts were cultivated in similar medium supplemented with 0.7 M-mannitol and 2 % (w/v) Difco agar (solid minimal medium).

Protoplast preparation and fusion. Protoplasts were prepared by the routine method using snail enzyme (Eddy & Williamson, 1959). Cells were harvested in the exponential phase of growth, washed twice with distilled water and incubated in 0.01 M-mercaptoethanol for 30 min at 28 °C. *Saccharomyces cerevisiae* cells were then suspended in a solution containing 0.7 M-mannitol, 1 % (w/v) lyophilized snail enzyme and 0.15 M-citrate/phosphate buffer (pH 5.4). *Schizosaccharomyces pombe* cells were suspended in a similar solution but with a lower (0.6 M) mannitol concentration. For *H. wingei* cells, the buffered mannitol was replaced by 0.8 M-MgSO₄. Fresh protoplasts were washed with non-buffered 0.7 M-mannitol. After washing, protoplasts (about 10⁶ of each auxotroph) were mixed in an appropriate combination (Table 1) and centrifuged. The pellet was resuspended in 1 ml of 30 % (w/v) polyethylene glycol (PEG; mol. wt 4000; Lachema, Brno, Czechoslovakia) containing 0.01 M-CaCl₂. After incubation for 20 min at 37 °C the suspension was slowly diluted with minimal medium containing 0.7 M-mannitol, and then washed twice with similar medium.

Growth and regeneration of fusion products. PEG-treated and control protoplasts were diluted in stabilized minimal medium, streaked by means of a capillary on a minimal agar-coated microscope coverslip, and then incubated in a moist chamber. The chamber provides excellent conditions for prolonged cultivation of protoplasts. The preparations were incubated at 28 °C and observed microscopically at regular intervals.

To observe regeneration of the wall and reversion to cells, the pellet of mixed protoplasts was embedded in 2 % (w/v) agar minimal medium in Petri dishes. The number of reverting protoplasts was determined microscopically. A group of at least ten cells around the regenerated protoplast was taken as indicating reversion.

Staining of nuclei. Nuclei in cells and protoplasts were counted in Feulgen-stained preparations.

RESULTS

Growth and reversion of untreated protoplasts

Both *S. cerevisiae* and *H. wingei* protoplasts require gel media for wall regeneration and reversion to cells. On the surface of agar or in liquid media they only grow and do not revert, forming an incomplete fibrillar wall (Nečas, 1971). However, protoplasts of *Sch. pombe* may revert under these conditions (Svoboda, 1966, 1967).

When spread on to the surface of solid minimal medium the protoplasts of all *S. cerevisiae* and *H. wingei* auxotrophs, either singly or in complementary mixtures (Table 1), failed to grow or grew to a limited extent only (Fig. 1). The slight increase in volume was apparently due to osmotic enlargement of the vacuole. Some protoplasts of *Sch. pombe* regenerated the walls but did not develop further.

Within solid minimal medium the auxotrophic protoplasts retained their spherical shapes (Fig. 2), but no reversion was observed in an inoculum of about 10⁴ protoplasts. In similar experiments with normal cells no colonies arose on minimal medium plates indicating a good stability of auxotrophic markers. With *Sch. pombe*, some revertants appeared within 2 d of cultivation as groups of one or two cells around the regenerated protoplasts. No visible colonies were found when an inoculum of more than 10⁴ protoplasts was used.

Growth and reversion of PEG-treated protoplasts

The addition of PEG to the protoplast suspension resulted in intensive agglutination which led to formation of large aggregates (Fig. 3). The number of protoplasts in the aggregates depended mainly on the density of the pellet: the more diluted the pellet, the less protoplasts in the clumps. During incubation at 37 °C the aggregated protoplasts were stable without any visible lysis. On subsequent dilution with stabilized medium, the clumps were disrupted into single protoplasts among which some larger bodies still persisted. In these treated and washed protoplasts one to five nuclei were revealed by Feulgen staining (Fig. 4), while controls (untreated protoplasts) were regularly uni-nucleate. Evaluation of samples of PEG-treated and stained protoplasts showed up to 10% of bi- and multi-nucleate bodies.

All bar markers represent 10 μm .

Fig. 1. Mixture of *S. cerevisiae* 291 α +9 α protoplasts grown for 24 h on the surface of solid minimal medium.

Fig. 2. One of the protoplasts from the same mixture grown for 24 h in solid minimal medium. Note the regenerated wall on the protoplast.

Fig. 3. Agglutination of *S. cerevisiae* 291 α +9 α protoplasts induced with 30 % (w/v) PEG. Phase contrast microscopy.

Fig. 4. Nuclei in the treated and washed protoplasts as revealed after Feulgen staining

Fig. 5. PEG-treated mixture of *S. cerevisiae* 291 α +9 α protoplasts grown for 24 h on the surface of solid minimal medium. Note one growing vacuolated protoplast; the others are non-growing.

Fig. 6. Reverting protoplast from a PEG-treated mixture of *S. cerevisiae* 291 α +9 α protoplasts after 24 h incubation in solid minimal medium. Normal cells arise by budding from the regenerated protoplast.

Table 1. *Efficiency of protoplast fusion after treatment with 30% (w/v) PEG*

Fusion treatment was carried out as described in Methods. After washing free from PEG, the protoplasts were transferred on to minimal agar film or into minimal agar medium. The preparations were incubated at 28 °C and after 24 h the numbers of growing or reverting protoplasts were determined microscopically. The efficiency of fusion was expressed as a percentage of growing or reverting protoplasts from an inoculum of about 10⁴ protoplasts. The same mixtures of complementing protoplasts but untreated with PEG were used as controls.

Strains hybridized	Growing protoplasts on the surface of minimal medium (%)		Reverting protoplasts in minimal medium (%)	
	Control	PEG	Control	PEG
<i>Saccharomyces cerevisiae</i>				
9 his α + 5 trp a	0	2.7	0	0.1
9 his α + rec 291 leu trp ura met tyr α	0	3.2	0	0.1
390 1 C his α + 5 trp a	0	1.3	0	0.08
8282 ade α + 9 his α	0	2.1	0	0.1
<i>Schizosaccharomyces pombe</i>				
leu h ⁻ + ade h ⁻	0	0.5*	0	0.09
<i>Hansenula wingei</i>				
5 cyh lys + 21 ade his	0	0.01	0	—†

* Occasional reversion.

† Contamination with prototrophic cells.

For hybridization experiments, the suspension of mixed protoplasts (Table 1) was diluted so that aggregates of two to four protoplasts were formed after PEG addition.

When the PEG-treated and washed protoplast suspensions were seeded on to minimal agar films, some of them grew into large vacuolated formations (Fig. 5). It should be stressed that this was active growth with a progressive increase in the cytoplasmic volume. The frequency of growing protoplasts varied from one experiment to another and typical results are shown in Table 1. Similar results were found when the protoplasts were grown in liquid medium but under these conditions most lysed after 8 to 16 h. In *Sch. pombe* auxotrophic combinations, growth of about 0.5% of the protoplasts was observed and occasionally some reversion occurred (one to three microcolonies per slide preparation).

The growing protoplasts were found not only in the aggregates but also singly, without any contact with other bodies. On the surface of solid minimal medium lysis of the protoplasts occurred only rarely, so the lysed material could hardly supplement the medium with substances that were lacking. These results, together with those showing the stability of auxotrophic markers, suggest that the growing protoplasts originate from successfully fused complementary protoplasts.

When the PEG-treated and washed protoplasts were embedded into solid minimal medium, some of them increased in volume, regenerated walls and reverted to cells (Table 1). Microcolonies of normal cells were formed in 12 to 16 h (Fig. 6). With further cultivation the colonies became visible and the cells could be transferred on to fresh minimal medium. Here again, these cells can be regarded as revertants of protoplasts which have arisen by fusion, as confirmed by a series of control experiments. As well as the controls already mentioned, these included: cultivation of PEG-treated non-complementing protoplast mixtures, cultivation of PEG-treated complementing protoplasts after lysis with distilled water and cultivation of PEG-treated cells in complementary combinations.

Other PEG concentrations (20 to 50%), PEG solutions with pH values ranging from 5.4 to 10.5, temperatures of less than 37 °C and incubation times greater than 20 min generally gave lower yields of prototrophic protoplasts than the procedure described in Methods.

All bar markers represent 10 μ m.

Fig. 7. Large hybrid cells of *Sch. pombe* $leu^- + ade^-$, prepared by fusion, and their parent forms (smaller cells).

Fig. 8. Sporulating asymmetric zygotes prepared by crossing *S. cerevisiae* ($291\alpha + 9\alpha$) $\times$ *5a*.

Fig. 9. Spores in *Sch. pombe* zygotes ($leu^- + ade^-$) $\times$ *his^+*. Note the apparent asymmetry of the zygotes.

Fig. 10. Feulgen staining of hybrid cells *Sch. pombe* $leu^- + ade^-$. All the cells contain one nucleus only.

Analysis of fusion products

Hybrid cells were regularly larger than the parent strains (Fig. 7) and in their size they resembled diploid cells. The reverted *S. cerevisiae* cells originating from fusion of $\alpha + a$ protoplasts were typical diploids capable of sporulation. The cells from $\alpha + \alpha$ combinations were of α mating-type, incapable of sporulating and showed a haploid type of budding. Hybrids $291\alpha + 9\alpha$ were flocculent like the parent strain 9α and could conjugate with strain *5a*. Hybrid (larger) and haploid (smaller) cells formed asymmetric zygotes. The zygotes and cells arising from them sporulated, showing one to six spored asci with the maximum of four spores in an ascus (Fig. 8). Orientation genetical analyses using mitotic segregation in hybrids $291\alpha + 9\alpha$, and random spore analysis in hybrids $(291\alpha + 9\alpha) \times 5a$, revealed that auxotrophic colonies requiring leucine, uracil, methionine, histidine, histidine plus uracil and tryptophan were segregated. No quantitative screening of segregants was made, however.

Hybrid cells originating from the fusion of *Sch. pombe* $h^- + h^-$ protoplasts were of h^-

mating-type and non-sporulating. They could conjugate with h^+ cells giving asymmetric zygotes with four spores (Fig. 9), but zygotes with six spores were also present. No detailed analysis of spores was performed.

The hybrid cells reverted from the fused protoplasts did not lose their prototrophic character after several passages on minimal media. They contained only one nucleus, as revealed by Feulgen staining (Fig. 10).

DISCUSSION

Artificially induced fusion has been demonstrated in a number of animal cells and plant and bacterial protoplasts (Harris & Watkins, 1965; Power *et al.*, 1970; Fodor & Alföldi, 1976). It was reasonable to expect that yeast protoplasts could also be fused without any difficulties. The results [obtained here, like] those reported recently (Sipiczki & Ferenczy, 1977; van Solingen & van der Plaat, 1977; Yamamoto & Fukui, 1977), confirmed this assumption. The appearance of prototrophic growing and reverting protoplasts from mixtures of complementary auxotrophs is believed to result from protoplast fusion. Control experiments showed that cross-feeding, enrichment of the medium by components from disintegrated protoplasts and eventual reverse mutations can be excluded as possible causes of protoplast growth and reversion.

The frequencies of fusion as determined by hybrid colony formation were low but comparable with results obtained in mould protoplasts (Anné & Peberdy, 1975, 1976). Very high frequencies of fusion, however, have been reported (Ferenczy *et al.*, 1975). The low yield of resultant hybrids does not correspond with the observation of about 10% of bi- and multi-nucleate protoplasts after PEG treatment. This apparent discrepancy could be caused by disruption of some fused protoplasts during washing from PEG and, mainly, by insufficient embedding of protoplasts in gel which, at least in budding yeast, is the most important requirement for successful regeneration of the cell wall (Nečas, 1971). For comparison, not more than 10% of wild-type *S. cerevisiae* protoplasts reverted and produced colonies under the same cultivation conditions. It should also be mentioned that not all microcolonies manifest themselves into visible colonies and this may explain the relatively higher yield of hybrids in comparison with other published results (Sipiczki & Ferenczy, 1977; van Solingen & van der Plaat, 1977; Yamamoto & Fukui, 1977).

The estimation of fusion rate based on the frequency of growing protoplasts on the surface of minimal agar media proved more reliable than that based on colony counts, although in the former case – except when *Sch. pombe* was studied – no cells were recovered. One can suggest that the growth of hybrid protoplasts is induced when the necessary genetic information is somehow introduced into a common cytoplasm, while regeneration of the cell wall and reversion to normal cells requires entire genome integration. In spite of these limitations the technique of cultivation of hybrid protoplasts on the surface of agar media can be recommended, at least for orientation experiments, as a test of whether fusions have occurred.

The finding of only a low frequency of growing hybrid protoplasts of *H. wingei* was not analysed further and its cause remained unclear. Due to persistence of some non-converted cells in protoplast preparations, which give rise to prototrophic colonies by conjugation, one cannot determine the exact number of reverted hybrid protoplasts, even when they are present.

These cytological and genetical studies have demonstrated that the integration of both parental genes into one synkaryon occurs in hybrid cells. This has also been demonstrated more precisely by Sipiczki & Ferenczy (1977) in the case of *Sch. pombe* protoplasts.

The low yield of prototrophic regenerated protoplasts has not allowed a more detailed study of their morphology and morphogenesis which is our main objective. The final result obtained in this study was the production of large cells which were possibly diploid. How-

ever, the regenerated protoplasts are likely to segregate not only diploids, but also dikaryons, polykaryons, polyploids or some parental forms. These problems can only be solved if the fusion frequency and the efficiency of reversion are improved.

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