Interaction of plasmid DNA with yeast protoplasts and a mechanism of genetic transformation

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DNA of plasmid pBB 29 is bound to protoplasts of *Saccharomyces cerevisiae* by a time-dependent, energy-independent process requiring Ca^{2+}. It is not degraded during binding but most of the bound DNA can be degraded by exogenously added DNase. Foreign DNA competes for the binding. Genetic transformation of protoplasts depends on the same conditions as does the binding of DNA but, in addition, it also requires conditions allowing fusion of the protoplasts. Transformation may consist of firm adsorption of plasmid DNA to the protoplast surface, mediated by Ca^{2+}, and of internalization of the DNA entrapped between two aggregated protoplasts as a suit of their fusion.

Cell fusion *Plasmid DNA* *Transformation* S. cerevisiae

1. INTRODUCTION

Genetic transformation of yeast has been possible since the discovery of Hinnen et al. [1] that protoplasts of *Saccharomyces cerevisiae* become competent for transformation with plasmid DNA when incubated with Ca^{2+} and PEG. The manner in which transforming DNA enters the protoplasts has not been elucidated. The elucidation is relevant for optimization of the method of yeast transformation and, more importantly, for general understanding of processes which enable uptake of macromolecules into cells. Here, interactions of plasmid DNA with yeast protoplasts are described. They lend support to the idea of Harashima et al. [2] that transformation of yeast protoplasts is directly associated with cell fusion and suggest a mechanism of the transformation.

2. MATERIALS AND METHODS

Plasmid pBB 29, containing, within 6.6 kilobase pairs, bacterial A^r and T^r genes from pBR 322, yeast LEU2 gene and a fragment of yeast 2 μm DNA from pJDB 219 (kindly provided by Dr B. Berse, University of Warsaw) was prepared from the plasmid-bearing strain *E. coli* C 600 OTC^- (thi hsdR hsdM Δpro-lac argI-1) by a clear lysate procedure [3]. For binding studies, the plasmid was labelled by nick translation [4] using [α-^{32}P]dCTP (Institute of Isotopes, Budapest) and DNA polymerase I (Boehringer). Yeast protoplasts were prepared by snail-gut treatment of exponentially grown cells (in a medium containing 1% yeast autolyzate, 1% peptone, 2% glucose, pH 5.5) after alkaline preincubation with mercaptoethanol [5] and standard transformation performed according to Hinnen et al. [1]. Unlabelled plasmid was used for transformation. Interaction of DNA was done with protoplasts prepared from either wild-type strain DT XII or from strain GRF 18 (MATα his 3–11,15 leu 2–3,11; obtained from Dr G. Dujardin). The interactions were similar with the two strains. The latter strain was used in transformation.

Salmon sperm DNA and pancreatic DNase were from Sigma, other chemicals from Lachema. Radioactivity was measured by scintillation counting.

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Abbreviation: PEG, polyethylene glycol 6000

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3. RESULTS AND DISCUSSION

Fig.1 shows the main characteristics of plasmid interaction with yeast protoplasts. When radioactively labelled plasmid was incubated with protoplasts in a solution containing slightly buffered isotonic sorbitol and Ca^{2+}, radioactivity of the solution diminished during incubation and an equivalent amount of radioactivity became bound to the protoplasts. The process was not active since it was the same at 0 as at 30°C and also was not dependent on metabolism (no inhibition with antimycin A + 2-deoxyglucose; not shown in the fig.). Unlabelled plasmid competed with the labelled plasmid for binding, but so did sperm DNA as well, indicating that the binding was not specific. The binding was not affected by elevating the pH to 9.5, nor by subsequent addition of PEG, but it was virtually nil if Ca^{2+} had been omitted from the solution. Also, no time-dependent binding took place if, instead of protoplasts, intact cells were used.

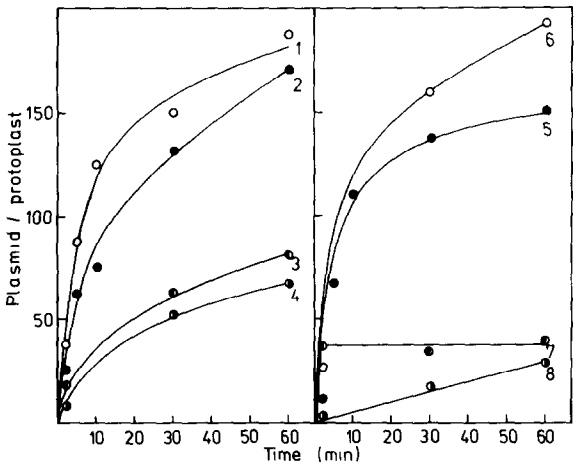

Fig.1. Time course of binding of labelled plasmid to yeast protoplasts. Protoplasts (10^9/ml) of strain GRF 18 were suspended in 1 M sorbitol containing buffer and additions as indicated below and incubated with labelled plasmid pBB29 (2.4 μg DNA/ml, 5×10^4 cpm/ml) at 30°C. At time intervals, aliquots of the incubation mixture (150 μl) were centrifuged at 14000 × g for 2 min and radioactivities of both the supernatant and the sediment (washed twice with 1 M sorbitol and dissolved in 150 μl 2% SDS) were determined. 1, 10 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$; 2, as in 1, but incubation at 0°C; 3, as in 1, protoplasts preincubated for 10 min with unlabelled plasmid (100 μg/ml); 4, as in 1, protoplasts preincubated for 10 min with sperm DNA (500 μg/ml); 5, 30 mM Tris (pH 9.5), 10 mM $CaCl_2$; 6, as in 1, after incubation for time indicated on the abscissa protoplasts were suspended in 40% PEG, 10 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$ and incubated 20 min at 30°C before centrifugation and determination of radioactivity; 7, as in 1, instead of protoplasts, intact cells were used; 8, as in 1, but $CaCl_2$ omitted.

Binding of plasmid to protoplasts was not accompanied by its degradation as no trichloroacetic acid-soluble radioactivity was found in the solution after the incubation of radioactive plasmid with protoplasts. However, 90% of the plasmid which had been bound to protoplasts could afterwards be degraded by DNase (table 1). The remaining 10% represented intact, non-degraded plasmid (not shown), but only about a third of it was found to be associated with membranes after lysis of protoplasts and a tiny fraction of the membrane-bound plasmid remained DNase-resistant (table 1).

It is thus obvious that most of the plasmid bound to protoplasts corresponded to molecules attached to the surface of protoplasts and accessible to added DNase. The rest of it may not necessarily have crossed the plasma membrane and become internalized but rather it may have become confined to a compartment in the plasma membrane where the bulky DNase molecule could not reach it.

If the majority, or even all, of the plasmid molecules do not penetrate into the interior of the protoplasts how can genetic transformation take place? The efficient binding of plasmid DNA to protoplasts is apparently a prerequisite for efficient transformation as both the binding and transformation required Ca^{2+} and were inhibited when foreign sperm DNA was present (fig.2). On the other hand, transformation was dependent, and affected in a similar manner, by those factors which determine fusion of protoplasts (fig.2): both transformation [1] and fusion [6,7] are dependent on the presence of PEG; and both transformation (fig.2) and fusion [8] are more efficient at alkaline pH. When protoplasts were preincubated with plasmid for 20 min and then DNase (+ $MgCl_2$) was added and further incubated for 30 min, no transformation occurred. This shows that the tiny fraction of DNase-resistant plasmid which had been found attached to the membrane fraction of

Table 1

Location of plasmid bound to protoplasts

Sample	Radioactivity (%)	
	− PEG	+ PEG
Plasmid used, total	100	100
Plasmid bound to protoplasts	25.8	28.9
Fraction of bound plasmid resistant to DNase	1.6	2.3
Plasmid in the supernatant of the lysate	0.8	1.2
Plasmid in the membrane fraction, total	0.7	0.7
Plasmid in the membrane fraction, resistant to DNase	0.1	0.1

Labelled plasmid pBB 29 (50 μg DNA/ml, 7 × 10^6 cpm/ml) was incubated with protoplasts of strain GRF 18 for 20 min under standard conditions of fig.1, curve 1 (− PEG) and then PEG was added to an aliquot and further incubated as in fig.1, curve 6 (+ PEG). The protoplasts were then washed 3 times with 1 M sorbitol, resuspended in 1 M sorbitol and treated with 0.1 mg/ml DNase in the presence of 10 mM $MgCl_2$ for 30 min at 30°C. The protoplasts were washed again twice with 1 M sorbitol and lysed by vortex-mixing in 0.4 M sorbitol, 10 mM Tris-HCl (pH 7.5), 20 mM EDTA. Membranes were separated by centrifugation at 14000 × g for 5 min, resuspended in 1 M sorbitol and an aliquot was then incubated with DNase as above. The membrane fractions, as well as the supernatant, which had been precipitated with 70% ethanol, were dissolved in 1% SDS, 100 mM NaCl, 50 mM Tris-HCl (pH 7.5), and extracted twice with phenol and 3 times with ethyl ether. DNA was precipitated with ethanol, dissolved in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and its radioactivity determined

protoplasts (table 1) does not take part in transformation.

The data are consistent with the following mechanism of genetic transformation: Plasmid DNA is firmly attached to the surface membrane of protoplasts by an intermediary of Ca^{2+} in a time-dependent process. Neither specificity of interaction nor any arrangement for DNA transport across the plasma membrane are necessary. PEG induces aggregation and fusion of protoplasts. Plasmid DNA, adsorbed at the surface, becomes entrapped between two aggregated and fusing protoplasts and will appear in the interior of the final

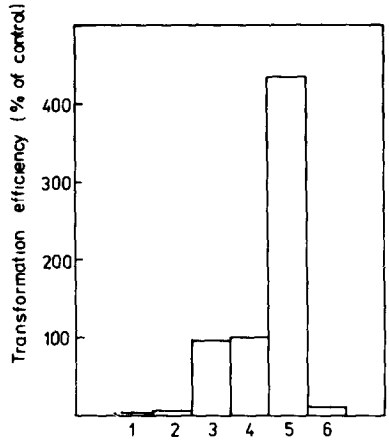

Fig.2. Efficiency of protoplast transformation under different conditions. Transformation of protoplasts of strain GRF 18 (1.3 × 10^8/ml) with plasmid pBB 29 (33 μg DNA/ml) was done according to Hinnen et al. [1]. Protoplasts were suspended in 1 M sorbitol, 10 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, plasmid was added, the suspension incubated for 20 min, then mixed with 40% PEG and further incubated for 20 min (standard conditions). Changes in the standard conditions are indicated below. The yield of transformants under the standard conditions was 1.6 × $10^3/\mu$g DNA. 1, Without $CaCl_2$ and PEG; 2, without PEG; 3, standard conditions; 4, incubation at 0°C; 5, buffered with 10 mM Tris at pH 9.5; 6, protoplasts preincubated for 10 min with 500 μg/ml sperm DNA before addition of plasmid.

fusion product. Such a mechanism would account for the high frequency of cotransformation with different plasmids [2,9]. An association of transformation with protoplast fusion has been proposed to explain the observation that most of the transformants issued from haploid protoplasts were found to be polyploid [2]. Similar data were reported by other investigators [9–11]. The mechanism of transformation of intact yeast cells [12,13] in which the cell wall prevents both the binding of DNA to the protoplast surface and cell fusion must be different and requires an independent study.

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