Curr Genet (1990) 17:97–103

Current Genetics

Original articles

Biolistic nuclear transformation of *Saccharomyces cerevisiae* and other fungi

Daniele Armaleo[1], Guang-Ning Ye[2], Theodore M. Klein[3], Katherine B. Shark[2], John C. Sanford[2], and Stephen Albert Johnston[1]

[1] Department of Botany, Duke University, Durham NC 27706, USA
[2] Department of Horticultural Sciences, Cornell University, Geneva, NY 14456, USA
[3] Plant Gene Expression Center, USDA-ARS, 800 Buchanan St. Albany, CA 94710, USA

Received June 5/September 29, 1989

Summary. Tungsten microprojectiles coated with nucleic acid and accelerated to velocities of approximately 500 m/s, can penetrate living cells and tissues with consequent expression of the introduced genes (Klein et al. 1987). *Saccharomyces cerevisiae* is used here as a model system to define the basic parameters governing the biolistic (biological-ballistic) delivery of DNA into cells. Among the physical factors affecting the efficiency of the process in yeast are the microprojectile's constitution, size, concentration and amount, and the procedure used for binding DNA to it. The biological parameters that affect the process include the cell's genotype, growth phase, plating density, and the osmotic composition of the medium during bombardment. By optimizing these physical and biological parameters, rates of transformation between 10^{-5} and 10^{-4} were achieved. Stable nuclear transformants result primarily from penetration of single particles of 0.5–0.65 μm in diameter, delivering on average 10–30 biologically active plasmids into the cell. The tungsten particles detectably increase the buoyant density of the transformants' progenitors.

Key words: Fungal transformation – Particle gun – Microprojectiles – Biolistic gene transfer

Introduction

Metal microprojectiles coated with nucleic acid can be shot into cells with consequent expression of the introduced genes (Klein et al. 1987). This "biolistic" (biological-ballistic) transformation methodology was developed specifically to deliver nucleic acid through the wall of intact plant cells in situ (Sanford et al. 1987). Transient expression of biolistically transferred genes has been demonstrated with several mono and dicotyledonous plants including corn, tobacco, rice, wheat, and soybean (Klein et al. 1988a, b, c; Wang et al. 1988). When the present study was initiated, it was unknown whether non-plant species with smaller cells could be transformed biolistically and whether the introduced DNA could be stably inherited. Also, a versatile microorganism was needed to define the physical, cellular, and genetic aspects of the process.

For these reasons, we have tested the biolistic procedure in the fungi *Saccharomyces cerevisiae, Saccharomyces pombe,* and *Neurospora crassa.* All three are transformable by this method. Using the well-studied *S. cerevisiae* (Botstein and Fink 1988) as a model system, we demonstrate that the biolistic introduction of DNA into cells can lead to stably inherited modifications of the nuclear genome. A numer of physical and biological variables are described that modulate the efficiency of the process in yeast, as well as some of the events that, at the single cell level, may lead from a "hit" to a transformed colony. The definition of the parameters affecting nuclear transformation, as reported in this paper, was the basis for the successful transformation of yeast mitochondria (Johnston et al. 1988).

Materials and methods

Strains and plasmids. S. cerevisiae strain 947 contains the non-reverting *ura*3-52 mutation. Strain 948, bearing *ura*3-52 and the non-reverting *leu*2-3, 112 alleles, is a segregrant from a cross between strains 947 and CG61 (gift of Dr. Craig Giroux). Yeast plasmids YEp351, YIp351 (each bearing a wild type *LEU*2 gene), and YEp352, YIp352 (each bearing a wild type *URA*3 gene) have been described earlier (Hill et al. 1986). *S. pombe* strain Sp287 (h^- *ura*4$^-$ *leu*1$^-$ *ade*$^-$ ts) and Sp286 (h^- *leu*1$^-$) were transformed with plasmids pIRT-1 and pIRT-2 (gift of Dr. Howard Fried) bearing the *S. pombe* ars1 sequence and containing a *S. cerevisiae URA*3 or *LEU*2 marker, respectively, to complement the recipient's *ura*4$^-$ and *leu*1$^-$ mutations. Conidia of *N. crassa* strain *qa*-2 246, unable to catabolize quinic acid due to a non-reverting mutation in the *qa*-2 gene (Giles et al. 1985), were transformed with a plasmid bearing the wild type *qa*-2 gene. The experiments with *N. crassa* were done in collaboration with Drs. Mary Case and Robert Geever from the University of Georgia, Athens, and their detailed results will be published elsewhere. Except where indicated, culture and recombinant DNA procedures were standard.

Offprint requests to: S. A. Johnston

Preparation of cells for bombardment. Typically, stationary phase yeast cells (36–72 h cultures) are spread at 10^8 cells/plate onto selective agar media containing either 1 M sorbitol or 0.75 M sorbitol and 0.75 M mannitol. Once seeded, plates can be used directly or kept a few h at room temperature before shooting. When optimal transformation efficiencies are not nececessary, seeded plates can be stored in the refrigerator for subsequent bombardment. Transformation efficiency decreases to approximately 1/3 of its initial value after 1 day at 4 °C, to 1/10 after 2 weeks. After bombardment, plates are incubated at 30 °C. Transformed colonies (usually 200–2,000/plate) become visible after 2–3 days. To bombard yeast cells suspended in liquid, a stationary phase culture is spun down and resuspended in its own spent medium at 4×10^9 cells/ml. One volume of this suspension is thoroughly mixed with 3 volumes of regeneration medium (1 M sorbitol; 1 M mannitol; 9 mg/ml yeast nitrogen base w/o amino acids; 2.67% glucose w/v; 2.67% YEP broth v/v; 1.33 mg/ml gelatin) and kept at room temperature for at least 15 min and up to 1 h before bombardment. Following this, 10^8 cells (100 µl) are spread within a 2 inch-diameter circle around the center of a plastic dish and immediately bombarded. As soon as the plate is removed from the bombardment chamber, 1 ml of diluted regeneration medium (1 water: 3 medium) is evenly distributed onto the bombarded area to prevent drying. The covered dish is stored at room temperature for one h. The cell suspension is then gently scraped to the edge of the tilted plate, mixed with a plastic pipet and spread onto selective plates. This procedure can yield 2–5× more transformants than direct bombardment on plates.

Particle bombardment. Operationally, the biolistic process can be subdivided into two stages; coating the subcellular-sized heavy metal particles (microprojectiles) with nucleic acid, and accelerating the microprojectiles to velocities appropriate for penetration of the target cells without excessive disruption of biological integrity. The necessary momentum is transmitted to the microscopic particles by exploding a powder charge inside a specially constructed apparatus ("biolistic device") which has been described elsewhere (Klein et al. 1987; Sanford et al. 1987). Briefly, it conists of a 22 caliber, 15 cm long barrel pointing vertically downward into a sample chamber (15 × 15 × 25 cm) connected to a vacuum pump. In a typical experiment, 2.5 µl of plasmid DNA (1 µg/µl in TE buffer or water) are mixed in a microfuge tube with 25 µl of a sterile water suspension (0.05 g/ml) of tungsten particles (GTE Sylvania, Towanda, PA). Unless otherwise specified, particles with an average diameter of 1.1 µm were used. $CaCl_2$ (25 µl of a 2.5 M solution) and 5 µl of a 1 M solution of spermidine free base (the salt is not effective) are then added to the suspension and the DNA is allowed to precipitate onto the tungsten microprojectiles for 10 min. The tube is spun for 30 s in a microfuge, 25 µl of supernatant are removed and discarded, and the particles are deagglomertated by briefly (1 s) touching the outside of the microfuge tube to the probe (horn type) of a sonicator (Heat System/Ultrasonics, Plainview, NY). Two to eight µl of the resulting suspension of microprojectiles are then placed on the front surface of a cylindrical polyethylene macroprojectile. The macroprojectile is inserted into the barrel of the particle gun and a blank gun powder charge (no. 1 gray extra light; Speed Fasteners, Saint Louis) is loaded into the barrel behind the macroprojectile. Through a vacuum pump, the pressure in the sample chamber is decreased to 0.03 atm within 30 s (presumably to reduce frictional deceleration of the microprojectiles) and the powder charge is detonated with a firing pin mechanism. The macroprojectile accelerates down the barrel toward the sample chamber where it hits a stopping plate. A small aperture in the stopping plate allows the microprojectiles to be propelled from the front surface of the macroprojectile and continue their trajectory towards the cells. Air is released into the chamber restoring atmospheric pressure within 10–15 s, and the Petri dish is removed from the apparatus. Although we routinely load 5–8 µl of microprojectile suspension per shot, we have found that the number of transformants increases only with loading volumes between 0.2 and 1 µl and remains relatively constant up to 7 µl.

Visualization of microprojectiles and plasmid DNA within cells. Plasmid DNA (5 µg of YEp352 in 5 µl TE) was mixed in a microfuge tube with 0.6 µl of the fluorescent dye DAPI (4′,6-diamidino-2-phenylindole, 1 mg/ml). The microprojectile suspension (25 µl) was added, followed by the remaining components as described. Cells suspended in liquid were bombarded, harvested from the plate 10 min after shooting, stored on ice and obseved directgly at high magnification with a Leitz Dialux 20 fluorescence microscope.

Density gradient. An iso-osmotic Percoll (Pharmacia, Piscataway, NJ) gradient was prepared by delivering 9 ml of a 7:3 mixture of Percoll:sorbitol (3.33 M) into a 10 ml ultracentrifuge tube, overlayering it with 1 ml of 1 M sorbitol, and spinning in a Beckman Ti 70.1 rotor (24° angle) at 30,000 g for 15 min at 25 °C. The 1 ml of overlay was then carefully removed and substituted with 1 ml of a yeast suspension bombarded in liquid 5 min before (strain 948; plasmid YEp352). After sedimentation at 650 g in a swinging bucket rotor for 15 min at 25 °C, 500 µl fractions were collected from the bottom of the tube and stored on ice. From each fraction, 350 µl was immediately plated onto medium lacking uracil to assess the distribution of Ura^+ transformants. In addition, 10 µl was diluted 100- and 10,000-fold (in 1 M sorbitol), aliquots were plated onto YEPD medium to determine the total number of viable cells and 100 µl was weighted to measure density.

Results

Biolistic transformability of different fungi

All three fungal species tested could be transformed biolistically. The *S. cerevisiae* strains chosen for our initial trials were good transformers by the standard spheroplasting technique (Hinnen et al. 1978). Their biolistic transformation frequencies, however, were low (10^{-6}–10^{-7}, expressed as the number of transformants over the total number of cells on the plate that was bombarded) and prompted us to search for more efficient isolates from various crosses. These strains were screened for their transformability by the biolistic process. Isolates were found that yielded transformation frequencies of 10^{-3}–10^{-5} and these were used for the experiments described here. We have not yet investigated the weight and nature of the genetic determinants affecting biolistic transformation efficiency.

Without seeking to optimize the process for each species, we tested the biolistic transformability of the fission yeast *S. pombe* and of the filamentous ascomycete *N. crassa* – both also transformable by spheroplasting. In both cases, we observed transformation frequencies comparable to those in the early (non-optimized) experiments with *S. cerevisiae* (10^{-6}–10^{-7}). In *S. pombe,* uracil and leucine auxotrophies were corrected by introducing autonomously replicating plasmids bearing wild type *URA*3 or *LEU*2 genes. In the *N. crassa qa*-2-recipient, the wild type *qa*-2 gene carried by the transforming plasmid restored catabolic competency for quinic acid by stable integration into the genome, both at homologous and nonhomologous sites (Mary Case and Robert Geever, personal communication).

Table 1. Transformation efficiency of strain 948, as affected by use of an integrative versus an episomal plasmid

Plasmid	Mean # of colonies per plate
Episomal – YEp351	195 (137.5)
Integrative – YIp351-uncut	85 (30.0)
Integrative – YIp351-cut	79.5 (24.5)

The means are based on at least 5 replicates per treatment. Standard deviations are in parentheses

Table 2. Effect of growth phase on transformation efficiency

Phase	OD_{600}	H	Mean # of colonies per plates
Early-log	0.05	18	224
Mid-log	0.12	24	528
Late-log	0.50	40	439
Stationary	0.52	47	947
Late stationary	0.50	66	570

Strain 947 was bombarded on plates with YEp352 DNA, after growth in liquid YEPD medium for the times indicated. Cultures were diluted 100× for OD readings at 600 nm. The means are based on 3 replicates per time point

Biolistic transformants show stable changes in the nuclear genome

Once a stably transformed clone develops, no gross abnormalities are noted in the state of the biolistically introduced DNA. An episomal plasmid (YEp352) was retrieved from five independent *S. cerevisiae* transformants, replicated in *E. coli,* purified and restricted, without any obvious modification. Likewise, an integrative plasmid (YIp352) was found, by Southern blot analysis of five independent isolates, to be integrated normally at its homologous location, the *URA*3 gene (data not shown). In terms of differential efficiency, episomal multicopy plasmids yield 3 times as many transformants as integrative plasmids (Table 1). The same strain is transformed approximately 100-fold more efficiently by the episomal versus integrative plasmids when standard transformation protocols are used (data not shown).

The relatively high efficiency of transformation with the integrative plasmid could be due to the linearization of the plasmid (Orr-Weaver et al. 1981, 1983) in the process of coating the microprojectile with DNA, or the shooting itself. We have found that exposure of DNA to tungsten can lead to degradation (data not shown). To clarify this point we compared the relative transformation efficiency of the linearized versus circular form of the integrating plasmid, YIp351. The linearization was effected by cutting in the *LEU*2 gene. As evident in Table 1, both forms of the plasmid transformed equally well, suggesting that the relatively high transformation frequency with circular, integrating plasmids is due to the linearization of the plasmid in the biolistic process.

Effect of culture conditions on biolistic transformation

Unlike spheroplasting (Hinnen et al. 1978) and LiCl-mediated (Ito et al. 1983) transformation, which are most efficient when performed on log-phase cells, the biolistic process is most effective on mid-stationary cells (Table 2). By analogy to spheroplasting, high sugar molarity in the selective plating medium is required for optimal efficiency in biolistic transformation. In particular, we found a synergistic effect of sorbitol and mannitol together, which is most pronounced when each is present at a concentration of 0.75 M (Table 3). No improvement was obtained by pre-conditioning stationary phase yeast with sorbitol in non-selective YEP broth 3 h prior to shooting.

Conditions and components for particle coating

Different methods of DNA precipitation were tested, as well as numerous microprojectile types. As shown in Table 4, the presence of $CaCl_2$ to precipitate the DNA onto the metal particles is necessary and sufficient to yield transformants, and its effect is enhanced by spermidine. The order of component addition is important. Brief

Table 3. Effect of sugar molarity on transformation efficiency

	# of colonies per plate							
Sugar molarity	0.25	0.5	0.75	1.0	1.25	1.5	1.75	2.0
Sucrose	110	180	200		210	50		
Mannitol	220	390	390	620	770			
Sorbitol	190	340	270	430	290	320	260	390
Mannitol + sorbitol	210	350	280	250	1,300	1,580	1,250	660

Strain 947 was bombarded with YEp352 DNA on plates containing the sugars at the indicated overall concentrations. When mannitol and sorbitol were used together, each was present at equal concentration. For sucrose and mannitol, 1.5 M and 1.25 M are close to the respective solubility limits under the conditions used

Table 4. Effect of the method for coating microprojectiles on transformation efficiency

Treatment	Mean # of colonies per plate
DNA only	0 (0)
DNA + spermidine	0.3 (0.6)
DNA + $CaCl_2$	93.0 (69.9)
$CaCl_2$ + spermidine + DNA	106.0 (54.0)
DNA + spermidine + $CaCl_2$	273.0 (13.1)
DNA + $CaCl_2$ + spermidine	301.0 (10.6)

Strain 947 was bombarded on agar plates with tungsten particles coated with YEp352 DNA. Reagents are given in the order in which they were added to the microfuge tube containing the particles, with mixing after each addition. Amounts were as indicated in Materials and methods. Means are based on 3 replicates per treatment. Standard deviations are in parentheses

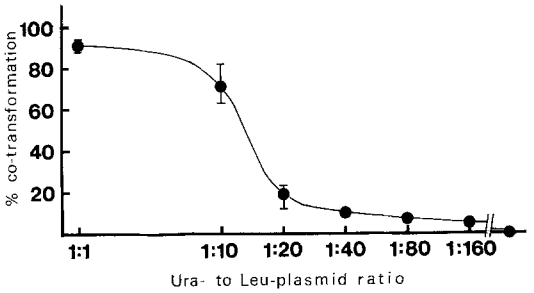

Fig. 1. Percent of *URA3*/*LEU2* co-transformation as a function of the molar ratio between the *URA3* plasmid, YEp352, and the *LEU2* plasmid, YEp351. Strain 948 was bombarded on plates lacking leucine with a series of mixtures of the two plasmids in the indicated molar ratios. Four plates were shot for each molar ratio. After 3 days, colonies were counted (200–300 per plate) and replicated onto plates lacking uracil. The % co-transformation is the ratio between the number of colonies growing without uracil and the number growing without leucine. Each value is the average of four determinations (*bars* mark the range of variation)

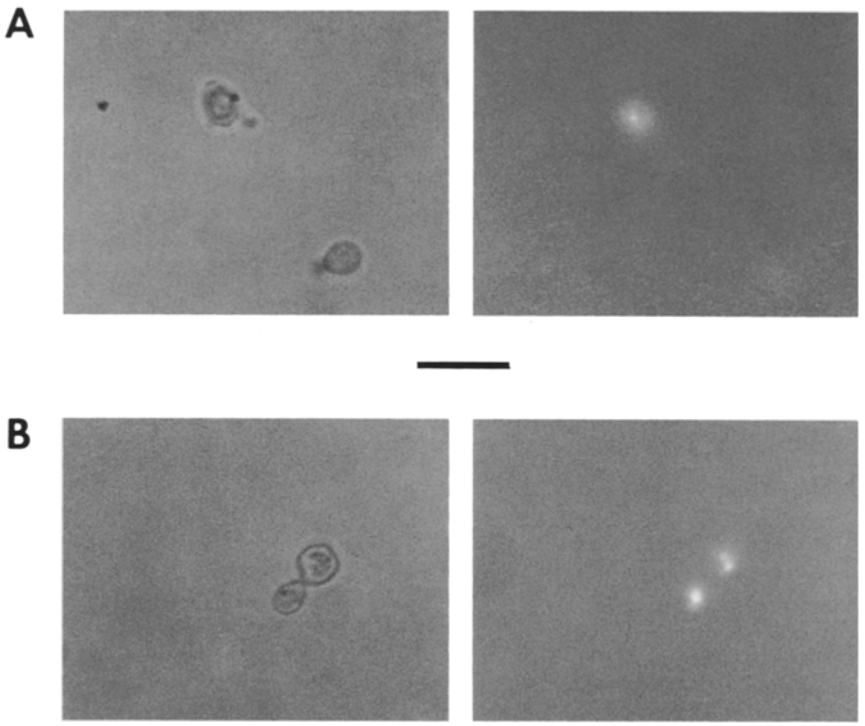

Fig. 2A, B. Visualization of microprojectiles and plasmid DNA within cells. Stationary phase yeast cells were bombarded in liquid medium with tungsten microprojectiles carrying DAPI-stained DNA as described in Materials and methods. About 30 min after shooting, photographs were taken both in visible and UV light at 630× magnification. Nuclear DNA is not stained with DAPI under these conditions. The cell in *panel A* contains a single intracellular tungsten particle and shows a small cytoplasmic extrusion. The most intense DAPI fluorescence is seen close to the center of the cell, whereas the microprojectile is located just beneath the surface. A nearby, non-fluorescent cell contains no particle. The cell in *panel B* contains four particles, two of which (out of focus) are inside the main cell body, and two are enclosed in a large cytoplasmic extrusion. DAPI staining is seen in both portions of the cell. The *bar* represents 10 μm

sonication of the microprojectile-DNA mixture after coating increases the transformation efficiency two-fold. Buffering the medium between pH 4 and 9 during coating does not improve efficiency (data not shown).

Among the metals tested [W, Pt, Fe (oxide), Au, Ir], only tungsten was significantly effective. Tungsten particles are purchased as powders of various grades, each comprising particle populations heterogeneous in size. A specific grade can be described by the average diameter of its component particles. Thus, a grade frequently used in our experiments is characterized by a 1.1 μm mean with the diameter of its constituent particles varying between 0.1 and 12 μm and with peaks around 0.6, 1.5, and 11 μm. We tested ten different tungsten powders with means of 0.6, 0.8, 1.1, 1.2, 1.4, 1.8, 2.0, 2.4, 3.4, 6.9 μm. The most effective for yeast transformation are the 0.8 and 1.1 μm powders (data not shown).

One transformant, one particle. One particle, 10–30 biologically active DNA molecules

Are transformants generated by single or by multiple penetrations per cell? In an experiment designed to address this question, 2.5 μg of plasmid YEp351 (bearing the *LEU2* gene) and 2.5 μg of plasmid YEp352 (bearing a

*URA*3 marker), each in a separate tube, were precipitated onto tungsten particles. Equal volumes from each suspension of coated microprojectiles were then mixed just before bombarding a *leu*2/*ura*3 double auxotroph (strain 948). The resulting frequency of double transformants was 1–3% of the total number of transformants. If the two plasmids are mixed in equimolar amounts *before* coating of the particles, the frequency of co-transformation approaches 100%, as described above. Therefore, when particles are coated separately, all double transformants must either arise from two or more penetrations per cell, or exchange of DNA between microprojectiles after mixing. Whatever the cause of doubles, their rarity indicates that single-particle penetrations underlie most biolistic transformation events in yeast.

Since most transformants result from single-particle penetrations, it was of interest to assess the average number of biologically productive DNA molecules per particle. To this end, we transformed the *leu*2/*ura*3 double auxotroph with varying ratios of plasmids YEp351 and YEp352, mixed before coating the microprojectiles. A series of independent coating mixtures was prepared, each with the same total amount of plasmid DNA, while the molar ratio of the *URA*3 to the *LEU*2 plasmid was decreased from 1:1 to 1:160. The frequency of *LEU*2-*URA*3 co-transformation was then measured for each molar ratio (Fig. 1). The sharp drop in co-transformation observed between the ratios 1:10 and 1:20 suggests that the average size of the biologically productive load per particle clusters around 10–20 DNA molecules. The combined data from other experiments indicate an average of 10–30 biologically active molecules per particle. The slow approach to zero co-transformation at ratios lower than 1:20 suggests that a few transformants may result from much larger productive loads.

Visualization of the tungsten particles and of the introduced DNA within individual cells

In the experiments described above, yeast was spread and bombarded on the surface of agar plates. We have also developed a method to biolistically transform yeast suspended in liquid medium. It is thus possible to observe directly, at high magnification, individual cells which have been hit by microprojectiles carrying DNA stained with DAPI (Fig. 2).

Penetrated cells were identified microscopically by the presence of one or more tungsten particles within them, by the intense DAPI fluorescence of the introduced DNA, and by extrusions of cytoplasm which sometimes included the microprojectile. These and other observations (see next paragraph) allow a description of the micro-cataclysm underlying biolistic transformation. Under standard conditions, less than 5% of the 10^8 yeast cells bombarded die and largely disintegrate (viable cell counts were determined by plating on complete medium samples from the liquid suspension before and after shooting). Most cells are viable, structurally intact, and are not transformed. A small number (about 1%) contain microprojectiles, exogeneous DNA, and often show cytoplasmic extrusions. We estimate that the potential transformants (penetrated intact cells) represent about 0.1% of the cells in this class, since the overall frequency of transformation is about 10^{-5}. Thus, most of the yeast cells containing particles and exogeneous DNA do not yield transformants even when intact in appearance. Analysis of these cells indicates that (1) projectiles larger than 1/10 of the yeast cell diameter must completely destroy structural integrity, since they are not seen inside cells; (2) multiple, non-productive penetrations are frequent; (3) penetrated cells often show cytoplasmic extrusions containing particles and/or exogenous DNA (DAPI-stained); (4) 20–30 min after shooting (the shortest time at which the pictures were taken) most DAPI-stained DNA had detached from the particles but remained clumped, without diffusing through the cytoplasm or into the nucleus. The amount and intensity of these DAPI-staining DNA clumps roughly paralleled, inside a given cell, the number of tungsten particles present. Nuclear DNA did not stain appreciably with DAPI under these conditions in any cell, whether penetrated or not.

Shortly after bombardment, density increases are detected among the progenitors of transformed colonies

Strain 948 was bombarded in liquid with YEp352 DNA and sedimented isopycnically through a Percoll density gradient. Fractions were monitored for the number of Ura^+ transformants, the number of total viable cells, and density (Fig. 3). Most viable cells are distributed smoothly around a density of 1.145 g/cc near the top of the gradient (this high baseline density is due to the high osmolarity of the gradient and of the cell suspension medium layered onto it). Transformants also peaked around 1.145 g/cc but, in addition, showed smaller but distinct peaks around 1.162, 1.166, and 1.170 g/cc. High density peaks in the transformants' distribution are reproducibly observed, although their size and exact position may vary slightly in different experiments. Virtually no transformants were recovered at densities higher than 1.170 g/cc. Peaks II, III and IV in Fig. 3 are consistent with the presence of viable yeast cells (prospective transformants) harboring single tungsten particles between 0.55 and 0.65 μm in diameter[1]. In the tungsten powder used in these experiments (mean diameter = 1.1 μm), these sizes are the most frequent class among particles smaller than 1 μm (not shown). Peak IV may also represent the rare viable double hits discussed earlier. Peak I may include cells which have rapidly extruded the microprojectiles while keeping the transforming DNA, and/or cells penetrated by particles smaller than 0.5 μm, whose density shifts would be too small to detect by this method. We think that particles smaller than 0.4 μm do not contribute significantly to

[1] The theoretical densities of yeast cells harboring tungsten microprojectiles were calculated modeling the yeast cell as a 6 μm sphere of 1.145 g/cc baseline density (top of gradient), tungsten particles (ϱ = 19.3) as spheres, and assuming no significant volume changes after particle entry

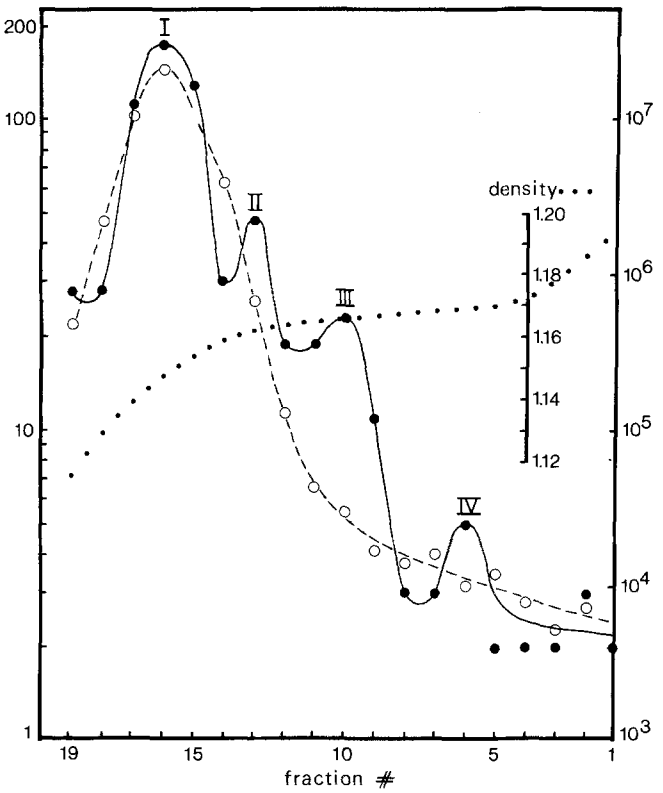

Fig. 3. Density profile of yeast cells shortly after bombardment. Strain 948 was bombarded with the *URA3* plasmid YEp352 and immediately sedimented through a Percoll density gradient. Fractions were assayed for the number of Ura^+ transformants (●–●–●), the number of viable cells (○–○–○), and density (·····). *Roman numerals* mark the peaks in the transformants' distribution. *Left ordinate,* number of transformants; *right ordinate,* number of viable cells

yeast transformation for two reasons. First, such particles are overrepresented in tungsten powders which are less effective than the 0.8 and 1.1 μm grades. Second, their kinetic energy may be too low to allow penetration into cells (see Discussion). The physical data thus suggests that, in *S. cerevisiae,* most biolistic transformants are generated by single particles not larger than 0.65 μm and not much smaller than 0.5 μm (a size range approximately equivalent to 1/10 of a cells's diameter).

Discussion

The technology used to deliver nucleic acids into cells by means of high-velocity microprojectiles is still in its infancy but has already demonstrated several advantages over preexisting transformation methods: (1) its simplicity of application allows one to target hundreds of millions of cells simultaneously, as they occur in intact tissues or in culture, without special preparatory treatments; (2) it is not limited to any particular species and has been successfully employed both in systems already transformable by other means as well as in less tractable systems; and (3) it is so far the only technique by which mitochondria (Johnston et al. 1988) and chloroplasts (Boynton et al. 1988) can be transformed.

At the time we initiated our studies, the biolistic technique had been used only with large plant cells and stable nuclear inheritance of the introduced markers had not been demonstrated. *S. cerevisiae,* molecularly perhaps the best understood and most genetically pliable eukaryote, provided an ideal model to test the process, and its underlying biology, on small cells with directly verifiable nuclear inheritance patterns. By understanding the basic principles underlying the process in yeast, applications to a variety of plant and animal species, many of which may not be transformable by other means, should be expedited. Among fungi, the biolistic transformability of species as diverse as *S. cerevisae, S. pombe,* and *N. crassa,* suggests that the method will be effective at least with Ascomycetes.

Despite profound differences between the entry mechanisms of nucleic acids in biolistic vs. spheroplasting-mediated nuclear transformation, the end-products appear the same: cells with unmodified exogenous DNA integrated at the homologous site, or replicating episomally. Both transformation methods also share the need for high osmolarity in the medium surrounding the cells during and after treatment; presumably due to the damage experienced by the cell wall in both procedures. On the other hand, a significant biological difference between the two transformation pathways is suggested by the fact that optimal genotypes differ for the two methods and that biolistic transformation works best on stationary phase cells, while spheroplasting is most effective on log-phase cells.

Single penetrations underlie the vast majority of biolistic transformation events. This conclusion is supported by two independent sets of data. First, double transformants were rarely found in the experiments in which two genetically different plasmids (YEp351 and YEp352), after separate precipitation, were mixed and delivered to the cells; while mixing the same plasmids before precipitation gave rise to nearly 100% co-transformants. Second, there were density shifts between 1.16 and 1.17 g/cc, detected in Percoll gradients among the progenitors of transformants, which were best accounted for by single intracellular particles between 0.5 and 0.65 μm in diameter. Although multiple productive penetrations by particles smaller than 0.4 μm could also be invoked to interpret the density shifts, such an explanation not only contradicts the biological data from the mixing experiments but also the following physical consideration. The particles used in the density shift experiments have a density peak at 0.55 μm; conversely, particle lots with median diameters below 0.55 μm range are much less effective in transformation. Furthermore, if particles between 0.55 and 0.65 μm have just the right kinetic energy to penetrate but not to disrupt a yeast cell, particles smaller than 0.4 μm, with less than 1/4 of the mass and kinetic energy, will probably be unable to pierce the cell wall.

Cytological observation of bombarded cells was very informative. Cells containing more than one particle are quite frequently observed under the microscope, as are cells with single particles. The vast majority of these visible penetrations, however, are not productive, attest-

ing to the narrow range of conditions allowing transformants to emerge. Microscopic observation further indicates that large (visible) amounts of exogenous DNA can be found inside cells. This DNA maintains a clumpy, unsolubilized appearance even after its initial rapid detachment from the carrier particles. A number of yeast cells quickly extrude microprojectiles and/or exogenous DNA.

The number of 10–30 plasmid molecules per transforming event should be viewed as the amount of DNA that the cell actually "sees" (i.e., processes biologically) rather than as a strict reflection of the entire load of DNA carried by a transforming particle. The actual amount of exogenous DNA originally present in a transformant may well exceed 30 molecules and remain largely silent biologically. It is, however, clear that at least 10–30 functional plasmids must be present at the initial stages of most biolistic transformation events.

In summary, we have shown that small cells can be transformed by the biolistic method. We have also defined some of the biological and physical parameters underlying this process. We expect that these studies will be helpful in applying this technique to other yeast and fungi for which an effective transformation system is not available.

Acknowledgements. We thank Scott Langdon, Chris Corton, John Salmeron, Jaqueline Bromberg and Rodney Rothstein for their ideas and criticisms and Alan Shore for technical assistence. This work was supported by grants from the North Carolina Biotechnology Center and N.I.H. to S.A.J., and from AMOCO Corporation to J.C.S. The biolistic device was provided on loan by Biolistics, Inc.

References

Botstein D, Fink GR (1988) Science 240:1439–1443
Boynton JE, Gillham NW, Harris EH, Hosler JP, Johnson AM, Jones AR, Randolph-Anderson BL, Robertson D, Klein TM, Shark K, Sanford JC (1988) Science 240:1534–1538
Giles NH, Case ME, Baum J, Geever R, Huiet L, Patel V, Tyler B (1985) Microbiol Rev 49:338–358
Hill JE, Myers AM, Koerner TJ, Tzagoloff A (1986) Yeast 2:163–167
Hinnen A, Hicks JB, Fink GR (1978) Proc Natl Acad Sci USA 75:1929–1933
Ito H, Fukuda Y, Murata A (1983) J Bacteriol 153:163–168
Johnston SA, Anziano PQ, Shark K, Sanford JC, Butow RA (1988) Science 240:1538–1541
Klein TM, Wolf ED, Wu R, Sanford JC (1987) Nature 327:70–73
Klein TM, Fromm M, Weissinger A, Tomes D, Schaaf S, Sletten M, Sanford JC (1988a) Proc Natl Acad Sci USA 85:4305–4309
Klein TM, Fromm ME, Gradziel T, Sanford JC (1988b) Biotechnology 6:559–563
Klein TM, Harper EC, Svab Z, Sanford JC, Fromm ME, Maliga P (1988c) Proc Natl Acad Sci USA 85:8502–8505
Orr-Weaver TL, Szostak JW, Rothstein RJ (1981) Proc Natl Acad Sci USA 78:6354–6358
Orr-Weaver TL, Szostak JW, Rothstein RJ (1983) In: Wu R, Grossman L, Moldave K (eds) Methods in Enzymology, vol. 101. Academic Press 228–245
Sanford JC, Klein TM, Wolf ED, Allen N (1987) Particul Sci Technol 5:27–37
Wang YC, Klein TM, Fromm M, Cao J, Sanford JC, Wu R (1988) Plant Mol Biol 11:433–440

Communicated by R. R. Rothstein