Transformation of yeast using calcium alginate microbeads with surface-immobilized chromosomal DNA

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Yeast artificial chromosomes (YACs) are useful cloning vectors that have the capacity to carry large DNA inserts. The largest barrier to using such large DNA molecules in transformation experiments has been their physical instability in solution. We developed a new method of transforming yeast using chromosome-sized DNA. The method uses calcium alginate microbeads to immobilize high-density yeast chromosomal DNA. Chromosomal DNA immobilized on microbeads is physically stabilized compared with naked chromosomal DNA. The microbead-mediated transformation performed well, not only with respect to the transformation frequency with large DNA molecules (>100 kb) but also in successful transformation using split chromosome DNA that exceeded 450 kb.

INTRODUCTION

Transformation methods are essential to genetic engineering. Various methods for transforming eukaryotic cells have been developed. Transformation methods using budding yeast *Saccharomyces cerevisiae* have been improved since the first successful reports (1,2). Electroporation (3) and exposure to lithium acetate (4,5) are used to introduce plasmid-sized DNA. Further development of the spheroplast method has allowed for the introduction of large DNA constructs (6).

Although these improvements allow for efficient transformation using plasmid-sized DNA, it is still difficult to introduce chromosome-sized DNA. Because chromosome-sized DNA such as yeast artificial chromosomes (YACs) can carry large amounts of genetic information, it would be a tremendous advantage if YACs could be efficiently manipulated and then introduced into yeast cells. A YAC cloning system has been used to construct genomic libraries by a method that has been previously described (6). In addition to its usefulness in library construction, the chromosome-splitting technique of fragmenting yeast chromosomes enables us to gener-

ate chromosomes of any size and retrofit YACs (7,8). However, the physical instability of such large DNA molecules in solution poses a technical problem in transformation experiments. Although promoting the compaction of YAC DNA with polyamines confers physical stability and reduces the size bias in the transformation frequency to some extent (9,10), large DNA molecules are inevitably degraded, even when handled extremely gently.

We developed an efficient gene delivery system in plants using calcium alginate microbeads (11). Protoplasts of tobacco BY-2 were efficiently transformed with alginate microbeads containing plasmid DNA in combination with polyethylene glycol (PEG) treatment. Here we report the application of alginate microbeads to the transformation of *S. cerevisiae* with large DNA molecules such as chromosomal DNA. To take advantage of this transformation system, we evaluated the physical stabilization of yeast chromosomal DNA immobilized on alginate microbeads. The feasibility of alginate microbeads for yeast transformation was investigated by the actual transformation of yeast split chromosome DNA.

MATERIALS AND METHODS

Yeast Strain and Preparation of Chromosomal DNA

SH4965 [*MATa ura3-52 leu2-3, 112 trp1Δ his3Δ ade2-101 lys2-801* (XIR-185 kb:*URA3*; IVR-468 kb:*TRP1*; VIIIIR-283 kb:*HIS3*; IIR-389 kb:*ADE2*; and XIIL-1489 kb:*LEU2*)] corresponding to YMH471 (12); SH5962 [*MATa ura3-1 leu2-3, 112 trp1 his3-11, 15 ade2-1* (IXR-106 kb:*URA3 ADE2*)]; AB1380 (*MATa ura3-52 trp1-289 ade2-1 can1-100 lys2-1 his5*); and SH964, equivalent in size to YPH149 (13), were used throughout this study. Agarose gel plugs were prepared according to the protocol described by McCormick et al. (14). The density of yeast cells in agarose plugs was adjusted to approximately 5×10^7 cells/plug.

Contour-clamped homogeneous electric field (CHEF) gel electrophoresis was used to separate the chromosomes. The agarose plugs were loaded onto 1% low melting point agarose (SeaPlaque® GTG®; CAMBREX, Rockland, ME, USA) gels in 0.5× TBE buffer (45 mM Tris-borate, pH 8.0, 1 mM EDTA), and CHEF gel electrophoresis was performed for 15.5 h at 6 V/cm at 14°C with a 60-s switching interval, followed by a 5.5-h run with a 90-s switching interval. The CHEF gel was stained with ethidium bromide solution (0.5 µg/mL), exposed to ultraviolet light, and the target bands were quickly excised.

Agarose gel melting and digestion were carried out as previously described (9), with minor modifications. Agarose gel plugs or gel slices excised from the CHEF gel were soaked with equilibration buffer [1× TAE (40 mM Tris-acetate, pH 8.0, 1 mM EDTA), 100 mM NaCl, 300 µM spermine, 750 µM spermidine], heated to 70°C, and digested with β-agarase I (Wako, Osaka, Japan).

Preparation of Chromosomal DNA Immobilized Using Calcium Alginate Microbeads

A solution of chromosomal DNA, either from melted plugs or gel-purified, was gently transferred to a new 1.5-mL tube using truncated microtips. DNA was mixed with polyamine solu-

tion (300 μ M spermine, 750 μ M spermidine) and 50 μ g salmon sperm DNA (10 mg/mL; Wako) as the carrier DNA, and the volume was adjusted to 450 μ L. The final concentration of CaCl_2 was adjusted to 100 mM with 50 μ L 1 M CaCl_2 solution.

To form a water-in-oil type emulsion, 100 μ L 0.5% sodium alginate (100–150 cP; Wako) solution were mixed with 900 μ L of isoamyl alcohol in a microtube and emulsified with an ultrasonic disrupter (Handy Sonic UR-20P; Tomy Seiko, Tokyo, Japan). To solidify the sodium alginate solution in the emulsion, the entire CaCl_2 solution containing the chromosomal DNA was added using truncated microtips. To eliminate the isoamyl alcohol, the microtube was centrifuged at 1900 $\times$ g for 3 min, and the supernatant was removed. The microtube was filled up with 100 mM CaCl_2 solution, and the resuspended alginate microbeads were harvested by centrifugation at 1900 $\times$ g for 3 min. This washing step was repeated at least twice. The chromosomal DNA immobilized on the surface of the alginate microbeads was stained with a

DNA binding fluorescent dye, YOYO-1[®] (Molecular Probes, Eugene, OR, USA), and observed under an Axioplan 2 Fluorescence Microscope (Carl Zeiss, Thornwood, NY, USA).

Verification of the Physical Stabilization of Chromosomal DNA

To isolate chromosomes VI (280 kb) and I (260 kb), 10 SH5962 plugs were loaded onto the CHEF gel and electrophoresed. The chromosomal DNA solution was prepared following the procedure described earlier. In 1.5-mL aliquots, approximately 3 mL of the chromosomal DNA solution corresponding to 10 plugs were gently transferred to the sample reservoir of a Centricon[®] YM-30 centrifugal filter device (Millipore, Bedford, MA, USA) and centrifuged at 1300 $\times$ g until the concentration was increased 10-fold. The volume of the retentate was checked every 10 min.

To immobilize the chromosomal DNA on alginate microbeads, we used 50 μ L of the concentrated chromosomal DNA solution. The alginate microbeads were resuspended in 250 μ L 100 μ M CaCl_2 solution. The alginate microbead suspension and 250 μ L of the concentrated chromosomal DNA solution were stirred simultaneously with a microtube mixer (model CST-

040; Asahi Technoglass, Tokyo, Japan) for 15 s at the maximum speed of 3000 rpm. The alginate microbead suspension was harvested by centrifugation at 700 $\times$ g, and the volume was adjusted to 50 μ L with 100 μ M CaCl_2 solution. The agarose gel plugs were produced for CHEF analysis with the use of all of the alginate microbead suspension or 50 μ L of the chromosomal DNA suspension.

Yeast Transformation via Spheroplasting

Transformation using spheroplasts was performed as previously described (15), with some modifications. Approximately 8×10^8 competent spheroplast cells were resuspended in 100 μ L STC (1.2 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM CaCl_2). For transformation with naked DNA, 1 μ L 100 $\times$ polyamine solution (30 mM spermine, 75 mM spermidine) and 5 μ L salmon sperm DNA solution (10 mg/mL) were mixed, and then 12 μ L plug DNA solution were added with gentle mixing. After 15 min, 1.9 mL 20% PEG4000 (Wako) solution containing polyamines (300 μ M spermine, 750 μ M spermidine) were added and mixed. For transformation using the alginate microbead system, the alginate microbeads resuspended in 20 μ L 100 mM CaCl_2 solution were added to the spheroplast suspension, followed by 20% PEG4000 solution without polyamines. As a negative control, 20 μ L distilled water were treated with PEG. The cells were cultured on selection plates for 6 days following transformation.

RESULTS AND DISCUSSION

To verify the physical stabilization of chromosomal DNA (280 and 260 kb) immobilized on alginate microbeads, a solution of gel-purified chromosomal DNA and a suspension of alginate microbeads immobilizing gel-purified chro-

Figure 1. Contour-clamped homogenous electric field analysis to verify the physical stabilization of yeast chromosomal DNA immobilized on calcium alginate microbeads. Lane SH5962 includes the bands of chromosomes VI (280 kb) and I (260 kb). Lane 1, the solutions of gel-purified chromosomal DNA; lane 2, calcium alginate microbead suspension was stirred with a microtube mixer and electrophoresed. Faint smear bands were detected in lane 1, whereas visible bands corresponding to 280 and 260 kb were seen in lane 2.

Figure 2. Yeast spheroplasts and calcium alginate microbeads immobilizing yeast chromosomal DNA. Yeast chromosomal DNA was immobilized without carrier DNA. The DNA molecules immobilized on calcium alginate microbeads were stained with YOYO-1. The phase contrast and fluorescent images were merged. The white arrow indicates the site of physical attachment between a yeast spheroplast and an alginate microbead. The green color indicates condensed yeast chromosomal DNA. The bar represents 5 μ m.

Table 1. The Number of Transformants Obtained with Melted Plug DNA from Donor Strain SH4965

	Plugs (n)	Plates (n)	185 kb	389 kb	468 kb
Naked DNA	1	7 ^a	1/4	1/5	0/6
Calcium alginate microbeads	1	1 ^b	2/2	2/2	0/1
	5	1 ^b	9/9	3/4	1/2
Negative control	0	1 ^b	0	0/1	0/1

The numerator indicates the number of clones carrying extra chromosomes corresponding to a split chromosome of SH4965. The denominator indicates the number of colonies obtained from selection plates.

^aApproximately 5.6×10^8 cells were treated with polyethylene glycol (PEG).

^bApproximately 8×10^7 cells were treated with PEG.

mosomal DNA were stirred simultaneously in the microtube mixer. CHEF gel electrophoresis was performed for the DNA solution and microbead suspension (Figure 1). No visible bands were detected in the lane containing naked chromosomal DNA (lane 1), indicating the complete destruction of the chromosomal DNA. By contrast, distinct 280- and 260-kb bands were detected in the lane containing chromosomal DNA immobilized on the alginate microbeads (lane 2). These results indicate that the chromosomal DNA immobilized on alginate microbeads was physically more stable than the naked chromosomal DNA in solution. Because the solidifica-

tion of alginate is a reversible reaction, EDTA should be excluded completely to stabilize the chromosomal DNA immobilized on the alginate microbeads. With the addition of EDTA, the chromosomal DNA molecules were released from the alginate microbeads, and the DNA fibers could be observed under a fluorescent microscope (data not shown).

To confirm whether gel-purified DNA could be used for transformation, we first performed yeast transformation via spheroplasting using gel-purified naked DNA. After the confirmation of successful yeast transformation using the gel-purified naked DNA, gel-purified 106-kb minichromosome DNA (*URA3*)

immobilized on alginate microbeads was mixed with yeast spheroplasts. The physical attachment of the spheroplasts and alginate microbeads was frequently observed in the suspension (Figure 2), which suggests that the high density of DNA molecules attached to and concentrated at the surface of the alginate microbeads provided the proper conditions for introducing chromosomal DNA into yeast cells. Subsequently, 16 colonies of *Ura*⁺ transformants were observed 6 days following the transformation. CHEF gel electrophoresis was used to study the chromosomes of the transformants (Figure 3A). The CHEF gel image showed an extra chromosome band that corresponded to the 106-kb minichromosome from the donor strain. This indicates that gel-purified chromosomal DNA immobilized on alginate microbeads can be introduced into yeast cells in the presence of PEG, and it functions as an independent chromosome. Using the alginate microbead method, 14 of the 16 clones obtained from a selection plate carried the 106-kb minichromosome, whereas such transformants were rarely obtained from only one selection plate with the PEG treatment of naked minichromosome DNA. Although the transformation efficiency using a concentrated minichromosome DNA solution was also evaluated, no significant difference was observed. This is presumably due to the degradation of DNA molecules during the process of centrifugal concentration. However, the addition of 50 μ g carrier DNA was essential for effective transformation using alginate microbeads. When the carrier DNA was not added, the transformation efficiency and reproducibility decreased drastically. A certain number of DNA molecules are necessary for the effective immobilization of large DNA molecules on alginate microbeads. Carrier DNA may assist both the introduction of DNA molecules into yeast cells and the effective immobilization of large DNA molecules present in small numbers.

Although 106-kb minichromosome DNA was successfully introduced into yeast cells, we believed that it would still be difficult to introduce larger chromosomal DNA. Therefore, we examined the maximum size of chromosomal DNA that could be transformed using alginate microbead-mediated

Figure 3. Contour-clamped homogenous electric field analysis of transformants obtained using calcium alginate microbead-mediated chromosome transfer. (A) Donor strain SH5962 contains a clear minichromosome band. The white arrows indicate the 106-kb minichromosome. Recipient strain AB1380 has a different karyotype from SH5962. The identical karyotype is seen in the transformant lane with an extra band for the 106-kb minichromosome. (B) Donor strain SH4965 carries 185- (*URA3*), 389- (*ADE2*), and 468- (*TRP1*) kb split chromosomes. The white arrows indicate each chromosome. Compared with recipient strain AB1380, the *Ura*⁺ transformant has an extra 185-kb chromosome. Extra chromosome bands are also seen in the lanes containing the *Ade*⁺ and *Trp*⁺ transformants, which indicates the existence of 389- and 468-kb exogenous chromosomes, respectively. SH964 is used as a size marker.

transformation. Donor strain SH4965 carries three split chromosomes (185 kb:*URA3*, 389 kb:*ADE2*, and 468 kb:*TRP1*) that complement the gene deficiency of recipient strain AB1380. To perform experiments under the identical conditions, melted plug DNAs containing the whole SH4965 genome (all the chromosomal DNAs) were used for the transformation without purification. As a result, we obtained positive clones for each selection marker. CHEF analysis showed the definite transfer of chromosomal DNA into the yeast cells (Figure 3B). An extra chromosome band, identical to the split chromosome band from the donor strain, was observed in the lane of each transformant. This confirmed that chromosomal DNA of up to 450 kb was successfully transformed using alginate microbeads.

We also compared the transformation efficiency of the new method with the conventional PEG treatment of naked DNA. One of the most significant advantages of alginate microbead-mediated transformation is that a large amount of fragile DNA can be used without scaling up the transformation because it is possible to control the density of DNA molecules immobilized on alginate microbeads without physical damage. Similar to how a higher DNA concentration at the target cell surface enhances DNA uptake (16), the very dense chromosomal DNA on alginate microbeads is preferable for transformation. In our procedure, five plugs, corresponding to approximately 425 μ L DNA solution, was the maximum volume immobilized on alginate microbeads. Although some loss of DNA molecules is inevitable during the preparatory steps, the suspension of alginate microbeads can be adjusted to less than 20 μ L for a 2-mL scale transformation. For the conventional method, at least a 7-fold increased volume must be prepared to use just one plug to avoid hypo-osmosis of the spheroplasts. This requires a larger number of cells and many selection plates.

Table 1 shows the number of transformants obtained with melted plug DNA. When using one plug, we found no distinct difference between the conventional method (naked DNA) and the alginate microbead-mediated transformation (alginate microbeads) for the number

of clones carrying extra chromosomes that corresponded to a split chromosome of the donor strain. However, it is worth noting that only one selection plate was required for the alginate microbead-mediated transformation, while seven plates were required for the naked plug DNA. Using five plugs, we obtained a larger number of transformants with the alginate microbeads. In addition to the high frequency of transformation, there were remarkably fewer false-positive clones with our method. Five plugs of naked plug DNA might generate more transformants; however, it is difficult to concentrate the solution without degrading the DNA and scaling up the transformation, generating more false-positive clones. False-positive clones can be generated in two ways. First, there might be spontaneous mutations, such as suppressor mutations. In fact, we even obtained false-positive clones from the negative control PEG treatment with distilled water. In our study of AB1380, at least one revertant was generated from 3×10^8 cells for *Ura*⁺ and *Ade*⁺, and one *Trp*⁺ revertant was generated from 1.5×10^8 cells. Therefore, small-scale transformation is preferred to avoid the generation of revertants. The other possibility is the integration of chromosome DNA into the host genome by recombination. We sometimes observed a change in the band pattern in the CHEF gel image, indicating homologous recombination after introduction.

We developed a new method for transforming yeast using alginate microbeads. Alginate microbead-mediated transformation is especially effective for chromosome-sized DNA and reduces the labor and time needed for transformation. In conjunction with genomic YAC libraries and the yeast chromosome-splitting method, this technique will pave the way to stable and effective transfer of quantitative trait loci into plant and mammalian cells.

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Selection of candidate housekeeping controls in tomato plants using EST data

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Because the expression levels of housekeeping genes are relatively constant in most tissues, they are often useful controls when quantifying gene expression. We present an analytical method for identifying candidate housekeeping controls using expressed sequence tags (ESTs) from The Institute for Genomic Research Tomato Gene Index. We found relative expression levels for a collection of 127 transcripts and calculated the percentage of cDNA libraries that had expression levels (for a given transcript) within 2-fold through 10-fold ranges. When all libraries were considered together, the highest ranked housekeeping controls included transcripts for a DnaJ-like protein, translationally controlled tumor protein, two α -tubulins, cyclophilin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For each organ (leaf, root, fruit, and flower), at least one transcript was found that occurred within a 2-fold range of expression in all respective libraries. These included transcripts for α -tubulin, DnaJ-like proteins, phosphoglycerate kinase, and GAPDH, although different transcripts appear better suited than others for different tissues. This analytical method is useful for identifying candidate housekeeping controls in particular tissues and at particular levels of expression and would be relevant for any species for which significant EST data exist.

INTRODUCTION

Expressed sequence tags (ESTs) are partial, single-pass sequences from randomly selected cDNA clones. ESTs in public databases are a useful tool for identifying novel genes and assembling contigs (1,2), as well as for preliminary analysis of gene expression (3,4). Previous studies have investigated methods of detecting significant differences in the relative expression levels of particular genes using EST databases. For example, statistical tests have been devised to compare the abundance of individual genes between two cDNA libraries (5) and between any number of libraries (6,7). These methods focus on detecting genes that are up-regulated or down-regulated, and not on identifying genes whose relative expression levels are constant. The current study demonstrates a method to address the latter.

Housekeeping genes encode proteins that typically function in basic cell metabolism/upkeep. Because the expression levels of housekeeping genes are relatively constant in most tissues, they are often useful controls

when quantifying gene expression. Housekeeping controls allow the consistency of tissue collection, mRNA extraction, and other procedures to be verified, thus supporting conclusions about differentially expressed genes. They also help to show what levels of variation are to be expected in particular experiments and, therefore, how to distinguish differentially expressed genes from those oscillating due to random chance (8). Even though control transcripts have been used for the last quarter century, they have inherent limitations (9,10). Suzuki et al. (11) reviewed the pitfalls of the two most popular controls in mammals, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and actin, while Lee et al. (12) observed that 12 housekeeping genes (GAPDH, actin, phosphoglycerate kinase, tubulin, etc.) typically used as controls show considerable variability in microarray data sets.

In the current study, we investigated whether or not a database of tomato ESTs supports the use of housekeeping control genes and then ranked candidate control genes in leaves, roots, fruits, and flowers.