Christian P. Miller. FACTORS AFFECTING THE ELECTRO-
TRANSFORMATION OF INTACT YEAST CELLS WITH A YEAST ARTIFICIAL
CHROMOSOME VECTOR. (Under the direction of W. James Smith)
Department of Biology, April 1992.

Electroporation, the brief exposure of cells to a
controlled electric field, was first reported to induce
uptake and expression of exogenous DNA molecules in 1982.
Since that time, the technique has been used in the
transformation of a wide variety of cell types, including
yeast cells. The current work describes several factors
that were found to affect the introduction of the yeast
artificial chromosome vector pYAC4 (11.1 kb) into intact
yeast cells by electroporation. Factors that were found to
exhibit a positive effect on transformation efficiency
included the use of high cell concentrations (efficiency
increased linearly with cell concentration up to 5×10^8
cells/ml), the addition of carrier DNA (5-10 fold increase)
and polyethylene glycol (4 fold increase) to the
transformation media, and the use of a recovery period after
electroporation (150% increase).

Treatment of the cells with the reducing agent
dithiothreitol was found to have the greatest positive
effect on transformation efficiency (up to a 9,000 fold
increase). Based on the results of experiments described in
the present work, an optimized transformation protocol has
been developed that has resulted in up to 5.8×10^5
transformants/ug pYAC4 DNA. This figure is 100-500 times
higher than the transformation efficiencies reported in two
other papers describing electroporation with pYAC4-based
constructs of similar size.

It was also determined that it is possible to transform
yeast cells with plasmid DNA that is suspended in melted
agarose. This finding may be beneficial when working with
large YACs, which are often prepared in agarose in order to
prevent shearing of the DNA molecule. At the present time,
no methods have been described for the introduction of large
YACs into yeast cells by electroporation. Several
experiments that were conducted in an attempt to accomplish
this feat are described.

FACTORS AFFECTING

THE ELECTRO-TRANSFORMATION OF INTACT YEAST CELLS

WITH A YEAST ARTIFICIAL CHROMOSOME VECTOR

A Thesis

Presented to

the Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment

of the Requirements for the Degree
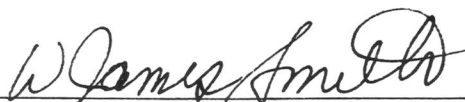
Master of Science in Biology

by

Christian P. Miller

April 1992

FACTORS AFFECTING

THE ELECTRO-TRANSFORMATION OF INTACT YEAST CELLS

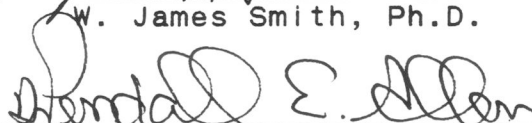WITH A YEAST ARTIFICIAL CHROMOSOME VECTOR

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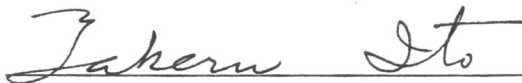APPROVED BY:

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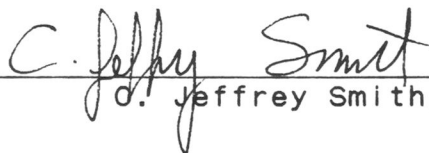
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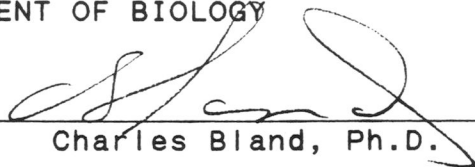
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DEDICATION

This work is dedicated to my loving wife, Gwendolen and my children. The unwavering support of my family made the completion of this thesis less difficult, and their efforts will not be forgotten.

ACKNOWLEDGMENT

TABLE OF CONTENTS

LIST OF GRAPHS

Introduction

The yeast *Saccharomyces cerevisiae* has been a valuable tool in biochemical research for some time. It has served as a key experimental organism in the elucidation of the glycolytic pathway, and the characterization of many enzymes, coenzymes and vitamins. Yeast have a rapid generation time and are easily grown in the laboratory on inexpensive media. While yeast cells are more complex than bacterial cells, they are the least complex eucaryotic organism and thus serve as a simple model for the study of a wide range of topics in cell biology that are unique to eucaryotes.

Recently, yeast have been used extensively in the investigation of a variety of topics in the fields of general and molecular genetics. The ability to induce mutations in yeast DNA has allowed researchers to produce strains that are deficient in some biological function. These mutants have been used to study the structure and function of many proteins involved in multiple processes in yeast cell biology. The ready availability of nutritional mutants in conjunction with the discovery by Hinnen and co-workers (1978) that yeast could be transformed by exogenous DNA laid the groundwork for a new biochemical application, the cloning of DNA fragments in yeast. The present work explores some of the factors affecting yeast transformation.

Cellular Biology of Yeasts

Unless otherwise noted, the term yeast in this paper
refers to the budding yeast, *S. cerevisiae*. Yeast cells are
ovoid in shape and have a diameter of approximately 3 *um*.
They are facultative anaerobes, and under optimal conditions
have generation time of 90 minutes. Yeast can exist in
either a haploid or diploid state. Haploid cells exist in
two different forms that are characterized by their mating
type. The two mating types are designated **a** and alpha, and
are determined by the alleles of the mating type locus *MAT* a
or *MAT* alpha. These alleles are involved in the control of
a variety of cellular processes including pheromone
production, mating, and sporulation (Herskowitz and Oshima,
1981). In homothallic yeast strains the mating-type genes
of haploid cells interconvert very frequently (approximately
once every other generation), with the cells rapidly
alternating between the **a** and alpha mating types. The
haplophase is much more stable in heterothallic strains, and
can be maintained indefinitely through multiple mitotic
cycles. When cells of opposite mating types come in contact
mating can occur, resulting in the formation of the
diplophase. Under favorable nutritional conditions the
diploid cells will undergo mitosis and maintain the diploid
state. When the growth media is nutritionally deficient,
the diploid cells undergo meiosis resulting in four progeny

haploid spores contained in an ascus. The spores are then released from the asci, germinate, and begin a new phase of haploid existence. The haploid state is useful in genetic studies as it allows for the examination of changes in phenotype resulting from recessive mutations.

Yeast cells contain all of the internal organelles found in eucaryotes including mitochondria, the endoplasmic reticulum, the Golgi apparatus, vacuoles, peroxisomes (Watson *et al.*, 1987), and cytoskeletal structures (Solomon, 1991). One characteristic that sets yeast cells apart from other eucaryotic cells is the fact that the nuclear envelope of yeasts does not break down prior to the initiation of mitosis. Each yeast cell contains one spindle pole body (SPB) within the nuclear envelope. The SPBs are microtubule organizing centers similar to centrosomes in animal cells. Late in the G_1 phase of the cell cycle, the SPB duplicates itself and chromosome replication commences (Pringle and Hartwell, 1981). Some time after SPB duplication, a chitin ring forms in the wall of the mother cell and bud emergence begins. As the SPBs migrate along the nuclear envelope the microtubules extending from them elongate, eventually forming a complete spindle as the SPBs reach their final positions on opposite sides of the nuclear membrane. One of the SPBs moves into the bud, carrying with it the daughter cell's chromosomes. Following nuclear cleavage and

cytokinesis, the bud becomes a free living cell.

The Yeast Cell Wall

The plasma membrane of yeast is surrounded by a cell wall that is made up of mannoproteins along with the polysaccharides glucan and chitin. The highly immunogenic mannoproteins comprise the outermost layer and consist of an inner core and an outer chain. The inner core is made up of 15-17 alpha (1->6) linked mannose units that are linked to an asparagine residue of the peptide via a di-N-acetyl-chitobiose unit (Cabib *et al.*, 1982). The outer chain consists of 100-200 alpha (1->6) linked mannose units with branching side chains that are stabilized by occasional intra-chain phosphodiester bonds. There are also some shorter mannose chains that are attached to serine or threonine residues of the peptides.

The mannoproteins can be subdivided into a sodium dodecyl sulfate (SDS)-soluble fraction and an SDS-insoluble, glucanase-soluble fraction (De Nobel *et al.*, 1990). The SDS-soluble mannoproteins comprise approximately 80% of the cell wall protein. It has been demonstrated that the mannoprotein layer determines the porosity of the cell wall (Zlotnik *et al.*, 1984; De Nobel *et al.*, 1989). De Nobel and co-workers (1990) further determined that cell wall porosity is limited by the glucanase-soluble

mannoproteins rather than the SDS-soluble mannoproteins.

Beneath the mannoprotein layer of the cell wall is the glucan layer. Glucan is a polymer of predominantly $B(1->3)$ linked D-glucopyranose units with some branching via $B(1->6)$ linked units. The chain is thought to be in the form of a long twisted ribbon that has both a hydrophilic and a hydrophobic side (Ballou, 1982). It has been suggested that two glucan chains could wrap around each other to form a double helix. The glucan layer is thought to impart rigidity to the cell wall (Cabib *et al.*, 1982), and is apparently anchored to both the plasma membrane and the mannoprotein layer.

The third major constituent of the yeast cell wall is chitin. Chitin is a linear $B(1->4)$ polymer of N-acetyl-glucosamine that is found predominately in bud scars and as a component of the primary septa (Farkas, 1989). According to Cabib and co-workers, the chitin in yeast cell walls is alpha chitin, a form in which maximal hydrogen bonding makes chemical extraction difficult.

Together, chitin, glucan, and mannoproteins make up over 90% of the yeast cell wall. Between the plasma membrane and the glucan layer is the periplasmic region. This space contains several degradative enzymes, some of which break down nutrients to facilitate their passage through the cell membrane.

The Yeast Genome

The haploid genome of *S. cerevisiae* contains approximately 14,000 kilobase pairs (kb) of nuclear DNA arranged into 16 individual chromosomes ranging in size from 150 kb to 2500 kb (Newlon, 1989). The chromosomal DNA has a 60% adenine + thymine base composition and a buoyant density in cesium chloride of 1.699 g/cm^3 (Moustacchi and Williamson, 1966). There is considerable variation in chromosome size between different yeast strains. The yeast genome contains very little repetitive DNA compared to the higher eucaryotes, with up to 95% of the nuclear DNA being present as single copy sequence (Lauer *et al.*, 1977). Another unusual feature of yeast DNA is its near total sensitivity to DNase I digestion (Lohr and Hereford, 1979).

The chromatin of *S. cerevisiae* is arranged into nucleosomal subunits with the nucleosome core containing about 140 base pairs (bp) of DNA. The 20 bp region between the cores is smaller and more uniform than the linker DNA found in higher eucaryotes. The histones H2A, H2B, H3 and H4 are present in yeast, while histone H1 has not been demonstrated. Yeast chromatin also contains several proteins that are similar in electrophoretic mobility and amino acid composition to the high mobility group (HMG) proteins found in other eucaryotes.

Yeast chromosomes are structurally similar to other eucaryotic chromosomes. Each chromosome has a single centromere that serves as the site for spindle attachment during mitosis. The presence of a centromere assures that each daughter cell will receive the correct complement of chromosomes. There are three essential elements required for centromeric function. Centromere DNA element (CDE) I (8bp) and CDE III (25bp) are two highly conserved sequences that have been found in all yeast centromeres sequenced to date (Schulman and Bloom, 1991). CDE II lies between CDE I and CDE III and consists of a 78-86 bp region that varies between centromeres but always contains greater than 90% adenine + thymine nucleotides. CDE III appears to be especially important in centromere function, as single base changes in this region result in extreme reductions in chromosome stability (Newlon, 1989).

Another important structural feature of yeast chromosomes are telomeres. Telomeres stabilize the ends of linear chromosomes by protecting essential genetic information from digestion by nucleases, and also prevent recombination between broken ends of chromosomes (Blackburn, 1991). Telomeres are also necessary for the complete replication of the ends of linear chromosomes. The form of telomeric DNA is highly conserved among eucaryotes and consists of tandemly repeated sequences with one strand

containing clusters of guanine residues. In yeast, the telomeric repeat sequence consists of 1-3 guanines followed by a thymine, or in the conventional shorthand, $G_{1-3}T$ (Blackburn, 1991).

Yeast chromosomal DNA replication is known to be initiated at multiple sites in the DNA molecule. There is a great deal of indirect evidence that *autonomously replicating sequences* (ARSs) serve as the replication origins in yeast (Newlon, 1989). Newlon has demonstrated that the number of ARS elements in the yeast genome is consistent with estimates of the number of replication origins as determined by electron microscopy. ARS elements were discovered when it was found that plasmids that contained certain sequences of yeast DNA transformed yeast cells at a much higher frequency than plasmids that lacked these sequences. It has been estimated that there is one ARS for every 32-40 kb of DNA (Newlon, 1989), a number that correlates well with the 36 kb spacing between origins that has been determined by electron microscopy (Newlon and Burke, 1980). The analysis of several ARS sequences from yeast have revealed that the ARS is contained within a 100-120 bp region, and that there is an 11 bp core consensus sequence, 5'(A/T)TTTAT(A/G)TTT(A/T)3' (Fangman and Brewer, 1991) that is critical.

In addition to the chromosomal DNA, yeast cells contain about 10-40 copies of an AT-rich, 75 kb circular mitochondrial DNA molecule. The copy number varies between strains and with carbon source and growth conditions (Fangman and Zakian, 1981). Most yeast strains also harbor 50-100 copies of a 6 kb, double-stranded, circular DNA molecule known as the 2um plasmid. The presence of the 2um plasmid confers no known growth advantage.

Yeast Plasmids

There are several types of artificial plasmids available that are used in the transformation of yeast. These plasmids differ in their mitotic stability, copy number per host cell, ability to self-replicate, and ability to integrate into the host cell genome. Most yeast plasmids are shuttle vectors that carry an *E. coli* replication origin and an antibiotic resistance gene in order to allow propagation and selection in *E. coli*. Yeast integrative plasmids or YIp plasmids consist of pBR322 DNA and a yeast gene, usually the *URA3* gene. YIp plasmids lack a yeast replication origin and can only transform yeast by homologous recombination into the recipient cell genome, either at the *URA3* locus or at the locus of the cloned insert if it is of yeast origin (Rine and Carlson, 1985). Subsequently, transformation efficiencies with YIp plasmids

are quite low (1-10 transformants/ug DNA). The efficiency can be increased up to 1000-fold by linearizing the plasmid within the intended integrating sequence.

A second type of plasmid that transforms yeast at efficiencies several orders of magnitude higher than YIp plasmids are the ARS plasmids or yeast replicating plasmids (YRp plasmids). ARS plasmids contain an autonomously replicating sequence that enables the plasmid to self-replicate as an episome. The ARS plasmids replicate once per generation (Fangman *et al.*, 1983), but do not segregate randomly during mitosis and have a strong tendency toward remaining in the mother cell. As a result, even under selective pressure up to 90% of the cells lack the plasmid after a single generation. Cells that do retain the plasmid often contain up to 50 or more copies per host cell (Murray and Szostak, 1983).

The stability of ARS plasmids can be significantly increased by the addition of a centromeric sequence (Clark and Carbon, 1980) to create a yeast centromeric plasmid (YCp plasmid). The centromeres from several yeast chromosomes have been identified and cloned. The presence of a centromere on an ARS plasmid increases the mitotic stability such that 90% of the cells grown selectively retain the plasmid, and the copy number decreases to one to two per cell (Murray and Szostak, 1983).

Yeast Artificial Chromosomes

In 1983, Murray and Szostak added *Tetrahymena* rDNA termini to the ends of a linear ARS-bearing centromeric plasmid. The *Tetrahymena* rDNA termini function as telomeres in yeast, and were demonstrated to be structurally similar to cloned yeast telomeres (Szostak and Blackburn, 1982). This construct contained replication origins, a centromere, and telomeres, and thus was called a yeast artificial chromosome (YAC). Murray and Szostak found that small (7-15 kb) linear YACs were less stable than circular centromeric ARS plasmids and were also present at higher copy numbers. However, when the size of the linear YACs was increased to 55 kb the stability exceeded that of the circular plasmids and the copy number was restored to one per cell. This suggests that a chromosome must be of a certain length in order for its centromere to function (Blackburn, 1985).

Burke and co-workers (1987) were the first to report the utilization of yeast artificial chromosomes in the cloning of large DNA molecules. These YAC clones contained inserts of up to 400 kb, a dramatic improvement over the 50 kb that can be packaged into conventional cosmid vectors. The functional genetic units in eucaryotic organisms can span up to millions of base pairs. YACs with inserts of up to 800 kb have been reported (Little *et al.*, 1989). The

ability to clone DNA fragments of this size facilitates the
isolation of complete genes, and greatly reduces the number
of individual clones needed to obtain single hit coverage in
a library. YACs have recently been used in the cloning of
genes involved in the human leukocyte antigen system (Little
et al., 1989), Huntington disease (Bates et al., 1990), and
cystic fibrosis (Green and Waterston, 1991). YACs are also
being utilized in the ongoing effort to sequence the human
genome (Green and Waterston, 1991).

Genetic Transformation of Yeast Cells

The cell wall presents a formidable barrier to the
introduction of large molecules such as DNA into the yeast
cell. It has been reported that only molecules with a
molecular weight of 700 Daltons or less are able to diffuse
freely through the yeast cell wall (Scherrer et al., 1974).
In 1978, Hinnen and co-workers reported the first successful
protocol for the transformation of yeast. Their method, and
similar protocols described by other workers (Beggs, 1978;
Hsiao and Carbon, 1979; Sherman et al., 1983) involved the
removal of the cell wall by degradative enzymes such as
glusulase or zymolyase. The resulting spheroplasts were
then mixed with DNA in the presence of calcium ions and
polyethylene glycol (PEG). Throughout the process the
spheroplasts must be maintained in osmotically stable media,

usually 1 M sorbitol.

While methods utilizing spheroplasts have historically yielded higher transformation frequencies (10^4 to 10^5 transformants/ug DNA) than methods employing intact cells, there are several drawbacks to these methods. The spheroplasts are susceptible to lysis and must be handled gently. It is also necessary to suspend the spheroplasts in top agar before selective plating to allow cell wall regeneration. In addition to the longer time required for growth of colonies from spheroplasts, there is the added inconvenience of having the colonies embedded in agar, which makes their processing more tedious. A further disadvantage is that these protocols often result in polyploids due to cell fusion. For these reasons, attempts were made to improve the efficiency of methods of intact cell transformation.

Conventional protocols for transforming intact yeast cells involve pretreatment of the yeast cells with a lithium salt, usually lithium acetate, prior to the DNA/PEG incubation (Ito *et al*., 1983a). Other treatments that have been found to render intact yeast cells competent for transformation include exposure to thiol compounds (Ito *et al*., 1983b, 1984) and freezing and thawing of yeast cell suspensions (Klebe *et al*., 1983). Brzobohaty and Kovac (1986) reported large increases in the number of

transformants obtained following a preincubation with proteolytic enzymes. Exposure of yeast cells to proteases or thiol compounds (dithiothreitol, 2-mercaptoethanol) has been shown to modify a mannan-protein complex within the cell wall (Brzobohaty and Kovac, 1986). The mannoprotein component of the yeast cell wall is the portion that determines cell porosity. Brzobahaty and Kovac maintain that all the treatments that augment transformation of intact yeast cells act by increasing the porosity of the cell wall, as was demonstrated in their experiments by the leaking of cellular RNAs into the medium.

The lithium acetate protocol is relatively simple and requires less time for growth of transformants than spheroplasting methods. However, the intact cell methods generally have a much lower transformation efficiency (10^2 to 10^3 transformants/ug DNA) than the spheroplasting methods. In addition, it has been reported that treatment of yeast cells with lithium acetate may induce deletions of unselected markers on the transforming plasmid (Clancy *et al.*, 1984).

Electroporation

In the past ten years a new technology has been developed that has been useful in the genetic transformation of a wide range of organisms including plant cells,

trypanosomes, bacteria, fungi, and mammalian cell lines (Potter, 1988). The technique is known as electroporation or electro-transformation, and involves the treatment of the cells with a controlled electric pulse that causes a brief hyper-permeable state. Treatment of cells with a high voltage shock was first used to induce cell fusion. In 1982, Neumann and his colleagues reported that the electroporation of mouse fibroblasts caused them to take up and express exogenous DNA. This discovery inspired Neumann and others to investigate the ability of electroporation to induce transformation in other cell types.

During the electroporation process, the cell membrane behaves as an electrical capacitor that is not capable of passing current except through ion channels. Subjecting the membranes to a voltage above a certain threshold results in a reversible electrical breakdown of the membrane, during which pores or cracks are formed in the membrane (Sugar *et al*., 1987). Some of the pores are large enough to allow the entry of macromolecules, including DNA, into the cell. The pores then reseal, enclosing the macromolecule within the cell.

The pores are thought to be aqueous in nature, with the polar heads of the membrane lipid molecules lining the inner wall of the pore (Weaver and Powell, 1989). Chang and Reese (1990) used rapid freezing electron microscopy to study

changes in membrane structure induced by electroporation. They determined that in human erythrocytes, volcano shaped pores appeared on the membrane in as little as 3 milliseconds (ms) after the pulse. The pores were found to expand in size during the first 20 ms after the pulse, with some of the pores reaching a diameter of 120 nm (range=20-120 nm). In 1977, Kinosita and Tsong reported that higher field strengths, longer pulse duration, and the use of lower ionic-strength medium (e.g., isotonic sucrose) favored the formation of larger pores in human erythrocytes. Zimmerman and Benz (1980) demonstrated that electroporation at higher temperatures may result in larger pores in *Valonia urtricularis*. As individual pores increase in size they may coalesce to form crack-like openings in the membrane (Sugar *et al.*,1987).

In order for a cell to survive the exposure to a high voltage electric field the membrane must reseal before excessive loss of cytoplasmic contents or cell lysis occurs. The resealing of pores begins almost immediately after the pulse, but apparently complete resealing of the membrane can take up to several minutes. Chang and Reese (1990) reported that by 5 seconds after the pulse there was a visible reduction in the diameter of most of the pores, and by 10 seconds the pore-like structures had disappeared and were replaced by pit-like membrane indentations. By 20 seconds,

the indentations had disappeared and except for a small
number of pores that were found in a few cells, the
membranes had regained their normal appearance. Xie and co-
workers (1990) provided further evidence that some pores can
remain open for a considerable amount of time after the
pulse. They worked with the electroporation of *E. coli* and
found that transfection still occurred when the transforming
DNA was added up to 10 minutes after the pulse was
delivered. It is possible that these long-lived pores are
stabilized by interactions with membrane proteins. In
eucaryotic cells, interactions of the pore with cytoskeletal
elements may prolong pore life span. Membranes tend to
complete the resealing process faster at higher temperatures
than at lower temperatures.

The conditions must be optimized in order to achieve
efficient transformation by electroporation. Excessive
field strengths result in irreversible breakdown of the
membrane and cell death, usually due to lysis resulting from
cell swelling that occurs as a result of the colloidal
pressure exerted by cytosolic macromolecules (Tsong, 1989).
On the other hand, insufficient field strength or exposure
time fails to induce a hyper-permeable state. The goal is
to apply a sufficient electric field to induce pore
formation without killing the cells.

There are a number of factors that contribute to successful transformation by electroporation. Increasing the applied field strength increases both the number and size of the pores (Tsong, 1989). Typically, smaller cells require stronger field strengths than larger cells. Other important factors include the duration of the pulse (time constant), and the electroporation medium (Potter, 1988). The time constant is an expression of the pulse length, and is determined by the capacitance setting and the resistance of the electroporation medium. The electroporation medium is important, as some of its components may enter the cells and have adverse effects. Furthermore, the presence or absence of monovalent or divalent cations in the media can influence the efficiency of transformation. Some organisms show an increase in transformation efficiency when cations are present, while in others the efficiency is decreased. Cations may facilitate the adsorption of DNA onto the cell surface. Finally, the ionic strength of the electroporation media must be kept low enough to prevent arcing in the electroporation chamber.

Electroporation of Yeast

Electroporation offers several advantages over other methods of yeast transformation. Most of the published protocols are relatively simple, and do not require time

consuming preparation steps. Electroporation may be successful in transforming strains that are resistant to chemical transformation, and avoids the use of chemicals that may be toxic to cells. In addition, electroporation tends to introduce plasmids in low copy number, which is a desirable result in many genetic studies (Evans, 1986).

Karube and co-workers reported the first successful transformation of yeast by electroporation in 1985. They obtained 945 transformants/ug DNA using spheroplasts, which is considerably less transformants than conventional spheroplast transformation protocols yield. Later in 1985, Hashimoto and co-workers achieved the transformation of intact yeast cells by electroporation. However, only 100 transformants/ug of DNA were obtained by their method.

Several protocols for intact yeast cell electroporation have been published in the last few years, and the efficiency has been improved tremendously (Delorme, 1989; Simon and McEntee, 1989; Meilhoc *et al*., 1990; Becker and Guarente, 1991). None of these methods utilize PEG in the electroporation buffer, and only one of the methods call for a chemical pretreatment of the cells prior to electroporation (Meilhoc *et al*., 1990). Meilhoc and co-workers achieved transformation efficiencies of up to 10^7 transformants/ug DNA, and report that pretreating the cells with the reducing agent dithiothreitol increased the

efficiency by a factor of 5. The transformation efficiencies reported for the other protocols are as follows:

Becker and Guarente	2-5 X 10^5 transformants/ug
Delorme	1-4.5 X 10^3 transformants/ug
Simon and McEntee	3 X 10^3 transformants/ug

There are only two protocols in the literature concerning the electroporation of YACs into AB1380, which is the yeast strain that is most often used in YAC cloning. Optimal conditions for transformation differ among strains of yeast. Bell and Mortimer (1991) followed the protocol described by Becker and Gaurente to transform AB1380 and achieved up to 700 transformants/ug using a 14.4 kb "mini-YAC". In the other YAC protocol, Rech and co-workers (1990) obtained 300-500 transformants/ug using linearized pYAC4 that contained a short oligonucleotide inserted into the cloning site. Their procedure included a lengthy PEG incubation, and utilized a field strength of 854 volts/cm and a capacitance setting of 500 $u$farads. Neither of these protocols resulted in efficiencies that could rival that obtained by standard spheroplast methods that are commonly used in YAC cloning. Burke and Olson reported in 1991 that to date, no protocols for the preparation of YAC clones by lithium acetate transformation have been published.

Furthermore, neither of these protocols examined the possibility of transforming yeast cells with large YACs.

The development of a method for the electro-transformation of intact yeast cells with large YACs would simplify YAC cloning. The first step in that process is to develop a protocol for the reliable transformation of intact AB1380 cells. We report on several factors that affect the efficiency of electro-transformation of *S. cerevisae* strain AB1380. Our optimized protocol has resulted in up to 5.8 X 10^5 transformants/ug of pYAC4 DNA. In addition, we describe our attempts to construct large YACs and introduce them into intact yeast cells by electroporation.

Materials and Methods

Media, Chemicals and Enzymes

Components used in the preparation of yeast growth media (yeast extract, bacto-peptone, dextrose, yeast nitrogen base, and amino acids) were obtained from Difco. YPD (1% yeast extract, 2% peptone, 2% dextrose) was used as a complex medium for routine growth. SD ura-, a synthetic minimal medium, was used for the selection of transformants. Zymolyase 20-T was from ICN Biomedicals. Promega was the commercial source for the restriction enzymes, T7 DNA polymerase and T4 DNA ligase. Proteinase K and dithiothreitol were purchased through Sigma. The Gibco BRL Nick Translation System was used to radioactively label pBR 322 DNA in hybridization experiments. Yeast chromosomal size markers for field inversion gel electrophoresis were obtained from Bio-Rad. Sea-plaque low melting temperature agarose was purchased from FMC Bioproducts.

Yeast Strains and Plasmids

Saccharomyces cerevisiae AB1380 (*MATa, psi+, ura3, trp1, ade2-1, can1-100, lys2-1, his5*) was obtained from Michael Turnage (Department of Biology, East Carolina University) and was used as the host strain for all experiments involving yeast artificial chromosomes. The ura and trp mutations were used in the selection of transformed

cells and as an indication that both arms of the artificial chromosome were present. The *ade2-1* mutation results in the accumulation of a red intermediate in the adenine biosynthetic pathway which causes colonies of this strain to be red when grown under limiting adenine concentrations. The *psi*+ factor is a cytoplasmic determinant that enhances suppression.

The plasmid pYAC4 (Burke *et al.*, 1987) was used in the transformation experiments and for construction of yeast artificial chromosomes (YACs). This 11.1 kb shuttle vector contains the yeast genes *TRP1, URA3*, and *HIS3*, as well as the *ARS1* replication origin, the *CEN4* centromeric segment, and two telomeric sequences derived from *Tetrahymena*. In addition, pYAC4 has an ampicillin resistance gene and an origin of replication that allow for growth and selection in *E. coli*. The *Eco*R1 cloning site in pYAC4 is located within the *SUP4* gene, an ochre suppressing allele of a tyrosine tRNA. When a plasmid containing the *SUP4* gene is transformed into an *ade2-1* yeast strain such as AB1380, the *ade2-1* mutation is suppressed and the colonies are white instead of red. The location of the cloning site within the *SUP4* gene provides a useful tool for selecting transformants that contain an insert, since the insert will inactivate the *SUP4* gene and result in the formation of red colonies, while cells transformed by the plasmid alone will give rise to

white colonies.

The pYAC4 plasmid was grown in *E. coli* C600 cells, extracted using an alkaline-SDS lysis protocol, and purified on a Quiagen 500 anion exchange column. Following elution, the DNA was precipitated with isopropanol, washed with 70% ethanol, dried, and dissolved in deionized water.

The plasmid was checked by restriction enzyme digestion and gel electrophoresis, and exhibited the expected banding pattern. The supercoiled plasmid was used in most of the transformation experiments. *Bam*HI digestion of the plasmid frees the telomeres, resulting in a linear 9.6 kb artificial chromosome. After digesting with *Eco*RI, the plasmid is ready for ligation.

Electroporation Protocol

Preliminary electroporation experiments were done using modifications of several published protocols (Delorme, 1989; Rech *et al.*, 1990; Meilhoc *et al.*, 1990; Becker and Guarente, 1991). The best initial results were obtained using a modification of the method described by Meilhoc and co-workers, and this method was adopted as the standard protocol. In order to measure the effect of different factors on transformation efficiency, some parameter of the standard method was modified, and the number of transformants obtained was compared to the number obtained

using the unmodified, standard protocol. The standard
protocol is described in detail below.

Yeast cells were grown in YPD broth (1 % yeast extract,
2 % peptone, and 2 % dextrose) to early log phase
(approximately 1 x 10^7 cells/ml). A cell count was
performed and the cells harvested by centrifugation at room
temperature. The cells were washed once in electroporation
buffer (270 mM sucrose, 10 mM Tris-HCl pH 7.5, and 1 mM
$MgCl_2$) and then resuspended in 2 ml of a dithiothreitol
solution (25 mM DTT in YPD, 20 mM HEPES pH 8.0) and
incubated for 10 minutes at 30°C. The cells were washed in
1-2 ml electroporation buffer and then resuspended in the
same buffer to a concentration of approximately 5 x 10^7
cells/50 ul aliquot (resuspended in 1/100 original culture
volume). The transforming DNA was added to 50 ul aliquots
of the cell suspension and gently vortexed. Sonicated calf-
thymus DNA (50 ug) was added as a carrier. The cell-DNA
suspension was then transferred to a 0.2 cm cuvette and
immediately subjected to electroporation. A Bio-Rad Gene
Pulser apparatus, complete with the Pulse Controller, was
used to deliver the pulse. Electroporation was typically
carried out at a setting of 600 volts (3,000 volts/cm) with
a capacitance setting of 25 ufarads and a resistance of 200
ohms. These settings resulted in a time constant of
approximately 4 ms.

Immediately after delivering the pulse, 1 ml of YPD was added and the cell suspension was incubated at 30°C for 30-60 minutes. Following the recovery period, the cells were pelleted and resuspended in minimal media lacking uracil (SD ura-). An appropriate volume was then plated onto SD ura- plates and incubated at 30°C for 3-4 days. When necessary, colonies were then streaked onto SD trp- plates to screen for the presence of both arms of the artificial chromosome.

Examination of Factors Affecting Electroporation

One of the primary goals of the current research was to determine the effect of numerous factors on the transformation efficiency of the standard electroporation protocol. Experiments were conducted in which one variable was modified while all other conditions were maintained as described in the standard protocol. We measured the effects of cell concentration and plasmid DNA concentration on electroporation efficiency. The impact of making various changes in the way the cells were treated before, during and after the pulse was also examined.

The effect of treating the cells with various chemicals and enzymes prior to electroporation was determined. The cells were treated with the enzymes proteinase K or zymolyase, the reducing agent DTT, or one of several

alcohols before carrying out electroporation. All other variables were kept as close to the standard conditions as possible. For convenience, the detailed descriptions of the chemical pretreatments have been placed in the results section of this work.

PEG is commonly used in transformation protocols, and experiments were conducted to examine the effect of including various concentrations of PEG in the transformation buffer. A stock solution of PEG 8000 was prepared in electroporation buffer, and cells were suspended in appropriate dilutions immediately prior to the addition of DNA. Following the addition of PEG and DNA the standard protocol was followed except that two wash steps were included after the recovery period to remove the PEG prior to plating.

The success of an initial experiment in which DNA suspended in an agarose solution was used to transform yeast cells inspired further experiments to examine whether the agarose concentration influenced the number of transformants that could be obtained. Agarose solutions (0.5%-1.2% w/v) were prepared in electroporation buffer, dissolved in a microwave oven, and maintained at 45°C. Aliquots of 5×10^7 cells were resuspended in 50 ul of agarose solution and electroporation was performed as described.

The addition of carrier DNA has been found to increase

transformation efficiencies. While calf-thymus DNA was utilized as a carrier in the majority of the described experiments, it was omitted in several experiments in order to determine if the addition of carrier DNA actually influenced the number of transformants obtained.

In the transformation of bacterial cells it is usually necessary to include a recovery period before plating onto selective media. This allows the transformed cells time to begin synthesizing the required gene product, usually an enzyme that confers resistance to an antibiotic. In most protocols for the electroporation of yeast cells a recovery period is not included. However, the procedure that was adopted as our standard protocol does utilize a recovery period. Experiments were performed in order to determine whether YPD recovery had a beneficial effect on transformation efficiency. In these experiments, the standard protocol was followed throughout, but some cells were diluted in SD ura- and plated on selective media immediately following electroporation.

Preparation of Large YACs

Two approaches were employed to produce large DNA fragments with EcoRI compatible ends, one using phage Lambda DNA and the other using herring sperm DNA (hsDNA). The desired goal with the Lambda DNA was to methylate the EcoRI

sites to protect them, ligate concatemers of the 48.5 kb
Lambda genome, and then fill in the twelve nucleotide
single-stranded overhangs (cohesive ends) on either side of
the molecule to prevent recircularization. An *Eco*RI linker
was then ligated onto the resulting blunt-ended, linear
molecule. Following digestion with *Eco*RI endonuclease to
free the linker ends, the modified Lambda DNA was ready for
ligation into the vector.

Ninety-three ug of Lambda DNA was treated with 2,000
units of *Eco*RI methylase in the suggested methylase buffer
(100 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 uM
s-adenosylmethionine and 100 ug/ml bovine serum albumin) for
60 minutes at 37°C. The reaction was stopped by heating to
65° C for 20 minutes. An aliquot of 500 ul (approximately
46 ug DNA) was transferred to a microcentrifuge tube, and
200 ul of 10X ligase buffer (250 mM Tris-HCL pH 7.5, 100 mM
$MgCl_2$, 100 mM dithiothreitol), 100 ul of 10 mM ATP and 20
units of T4 DNA ligase were added. The ligation reaction
was carried out at 37°C for 45 minutes, and was followed by
a 10 minute, 75°C enzyme inactivation step. The four
deoxynucleotides (ATP, CTP, GTP, and TTP) were added to a
final concentration of 7 uM, along with 50 units of T7 DNA
polymerase. The mixture was incubated at 37°C for 20
minutes. Following another enzyme inactivating step, *Eco*RI
linkers were ligated onto the blunt ends of the Lambda

concatemers by adding a twenty-fold molar excess of linker,
3 ul of 0.1 M ATP, and 100 units of T4 ligase and incubating
overnight at 8°C. The ligase was inactivated by heating to
75°C, and EcoRI was used to create compatible ends for
ligation into the pYAC4 cloning site.

Herring sperm DNA was partially digested with EcoRI by
treating it with varying concentrations of the enzyme in
EcoRI restriction buffer. The EcoRI digestion was carried
out at 37°C for 30-60 minutes.

Size Fractionation of DNA

Gel permeation chromatography, sucrose gradient
centrifugation, and electrophoresis were employed in
attempts to isolate large DNA fragments for subsequent use
in YAC cloning experiments.

Forty seven ug of partially digested hsDNA in 250 ul
was layered onto the top of a 12 ml Bio-Gel A-150m agarose
gel bead column that had been equilibrated with TE buffer
(10 mM Tris-HCl, 1 mM EDTA pH 8.0). The estimated nucleic
acid exclusion limit for this type of bead was 70,000 base
pairs. The flow rate was adjusted to approximately 9
ml/hour and 30 (1 ml) fractions were collected. The DNA
concentration of the fractions was assayed by absorbance at
260 nm, and selected fractions were concentrated in a
speed-vac and analyzed by field inversion gel

electrophoresis (FIGE).

A 5-25 % exponential sucrose gradient was prepared in 0.8 M NaCl, 20 mM Tris-HCl pH 8.0 and 10 mM EDTA as described by Burke and Olson (1991). Approximately 75 ug of partially digested hsDNA in 50 ul was loaded onto an 11 ml gradient and centrifuged for 21 hours at 10,300 rpm in a SW41 rotor (r=11.02). A total of 38 (0.5 ml) fractions were collected, starting from the bottom of the tube. Every fourth fraction was analyzed by FIGE.

Conventional DNA electrophoresis was used as an additional method of isolating large DNA fragments. A 0.8 % FMC low melting temperature agarose gel was cast in 1X TBE buffer (90 mM Tris, 90 mM boric acid and 1 mM EDTA). Partially digested hsDNA was loaded into the wells using wide-tipped pipette tips to minimize shearing of the DNA molecules. Electrophoresis was at 100 volts for 60 minutes. A control lane containing Lambda DNA, and one lane each of digested and undigested hsDNA were cut out and stained with ethidium bromide. The location of the Lambda DNA was marked on the gel, and the entire unstained portion of the gel from this mark back to and including the sample wells, was cut out for further processing. The volume of the excised gel was approximately 2 ml when melted. The gel slice containing the DNA was soaked overnight in 1X TBE at 4°C, then equilibrated for 30 minutes at room temperature (20-

25°C). The gel was then melted at 68°C for 10 minutes, and cooled to 37°C. Thirty ul of *Eco*RI and *Bam*HI digested pYAC4 (3.5 ug), 200 ul of 10X ligase buffer, 25 ul of 0.1 M ATP and 15 ul of T4 DNA ligase (45 units) were added and the mixture was incubated overnight at 31°C. The mixture was allowed to solidify at 4°C, then was equilibrated overnight at 4°C in electroporation buffer. The ligation mixture in agarose was melted and kept at 45°C prior to transformation experiments.

Field Inversion Gel Electrophoresis

FIGE was used to screen for the presence of YACs in the transformed cells. The PPI-200 programmable electrophoresis controller, from MJ Research Inc., was connected to the power supply and inverted the electric field at programmed intervals. Gels were usually 1% agarose in 0.5X TBE. Electrophoresis was carried out at 150 volts (4.3 volts/cm) at room temperature (20-25°C) with run times of 12-15 hours. Satisfactory resolution of DNA molecules up to 700kb in size was obtained using a built-in program on the PPI-200. A linear time ramp with a constant forward-to-reverse ratio of 3 was used, with a maximum reverse time of 10 seconds and a maximum forward time of 30 seconds.

Yeast DNA samples were prepared for FIGE essentially as described by Sheehan and Weiss (1990). Yeast were grown

to saturation in broth media, washed in ET buffer (10mM
Tris-HCl, pH 7.5, 50mM EDTA), and converted to spheroplasts
with Zymolyase 20-T (2-3 mg/ml) in a 0.9 M sorbitol-1X ET
solution containing 75 ul/ml mercaptoethanol. The
spheroplasts were suspended in 1% Incert agarose (FMC) and
the suspension was poured into moulds and allowed to
solidify. The plugs were placed into a 1X ET-1% SDS
solution and incubated at 60°C overnight. The ET-SDS
solution was replaced and the plugs could be stored at room
temperature in this solution. For storage at 4°C, the plugs
were placed in 0.05 M EDTA. Concatemers of Lambda DNA were
also prepared for use as size markers for FIGE.

Hybridization

    In order to demonstrate that the transformants actually
contained pYAC4 DNA, hybridization experiments were carried
out on several gels. The method described by Smiley and co-
workers (1983) for direct hybridization of nucleic acids in
agarose gels was followed. pBR322 DNA was labeled with [32]P
via nick translation (Gibco BRL) and was used to probe the
gels. After washing, the gels were dried and exposed to
Kodak XAR-5 film. The film was developed in the Radiology
department at Pitt County Memorial Hospital.

Results

Several factors were found to have an effect on the ability to transform *S. cerevisiae* strain AB1380 by electroporation and are discussed below. These factors included plasmid DNA concentration, cell concentration, age of the yeast culture, pretreatment of the cells with DTT or proteinase K, the addition of PEG and carrier DNA to the electroporation medium, and recovery of the cells in YPD following electroporation. Some early experiments were performed before the electroporation protocol was optimized. While these experiments did not result in high numbers of transformants, they did provide data concerning specific factors that affect the transformation of yeast cells.

Survival Studies

The effect of the field strength on cell survival was determined by pulsing 50 ul aliquots containing 5×10^7 cells in a 0.2 cm electroporation cuvette at voltage settings ranging from 0.4 KV to 1.6 KV. The capacitance setting was maintained at 25 $u$farads. Immediately after delivering the pulse, the cells were recovered in electroporation buffer, and appropriate dilutions were plated onto YPD plates and incubated at 30°C. Colonies were counted after 48-72 hours. The number of cells that survived electroporation decreased as the voltage applied

increased (Figure 1). Approximately 85% of the cells survived electroporation at the field strength used in the standard protocol.

In some early experiments, sorbitol was used as an osmotic stabilizer in the electroporation buffer instead of sucrose. In these experiments, it was found that increasing the sorbitol concentration from 0.4 M to 1.0 M increased the percentage of cells that survived a 4 KV/cm pulse from 2-4% up to 20-24%.

Plasmid DNA Concentration

The effect of plasmid DNA concentration on transformation efficiency was measured by electroporating the cells under standard conditions and varying the amount of DNA added from 0.05 ug to 1.9 ug. The number of cells transformed increased as the DNA concentration increased. However, the transformation efficiency as expressed by the number of transformants/ug DNA decreased as more plasmid DNA was added (Figure 2).

Cell Concentration

The effect of cell concentration was determined by suspending increasing numbers of DTT-treated cells in 50 ul electroporation buffer and pulsing under the standard conditions. The cell concentrations ranged from 2.5 X 10^7
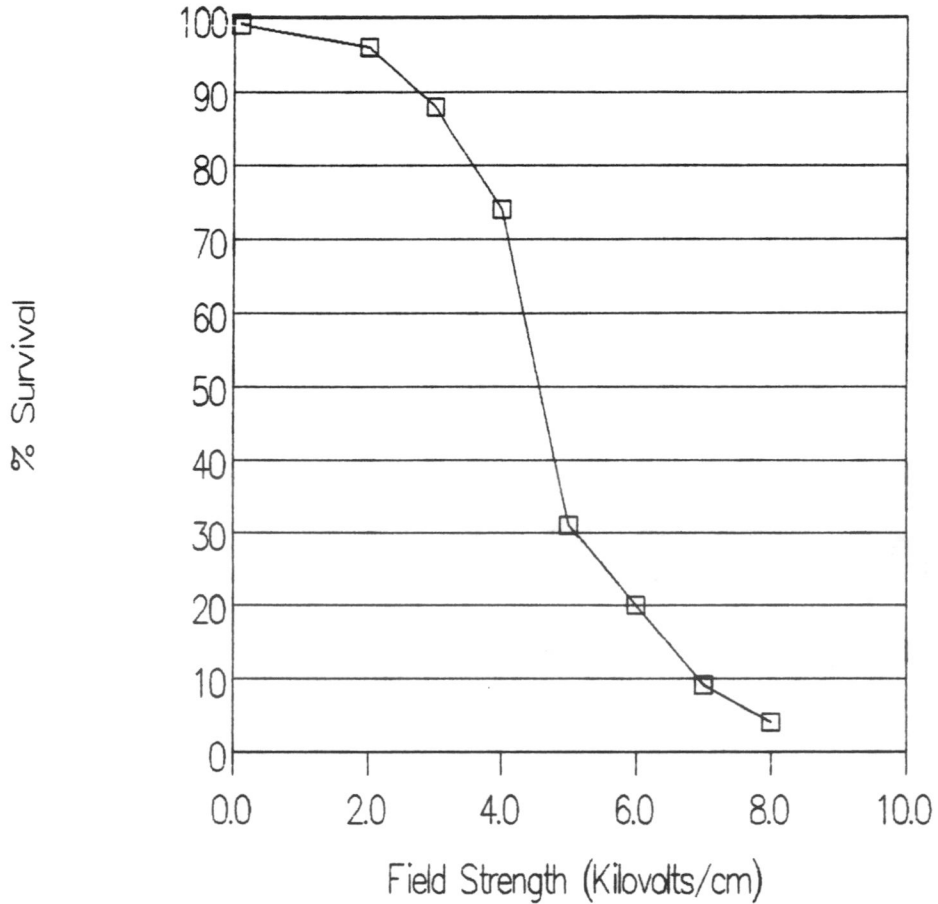
Survival Study

Figure 1 Cell survival as a function of field strength. 50 *u*l aliquots containing 5×10^7 cells were added to a 0.2 cm cuvette and electroporated at various voltage settings. The capacitance was maintained at 25 *u*farads, resulting in time constants of approximately 4 msec. Immediately following electroporation, the cells were recovered and appropriate dilutions were plated onto YPD plates.
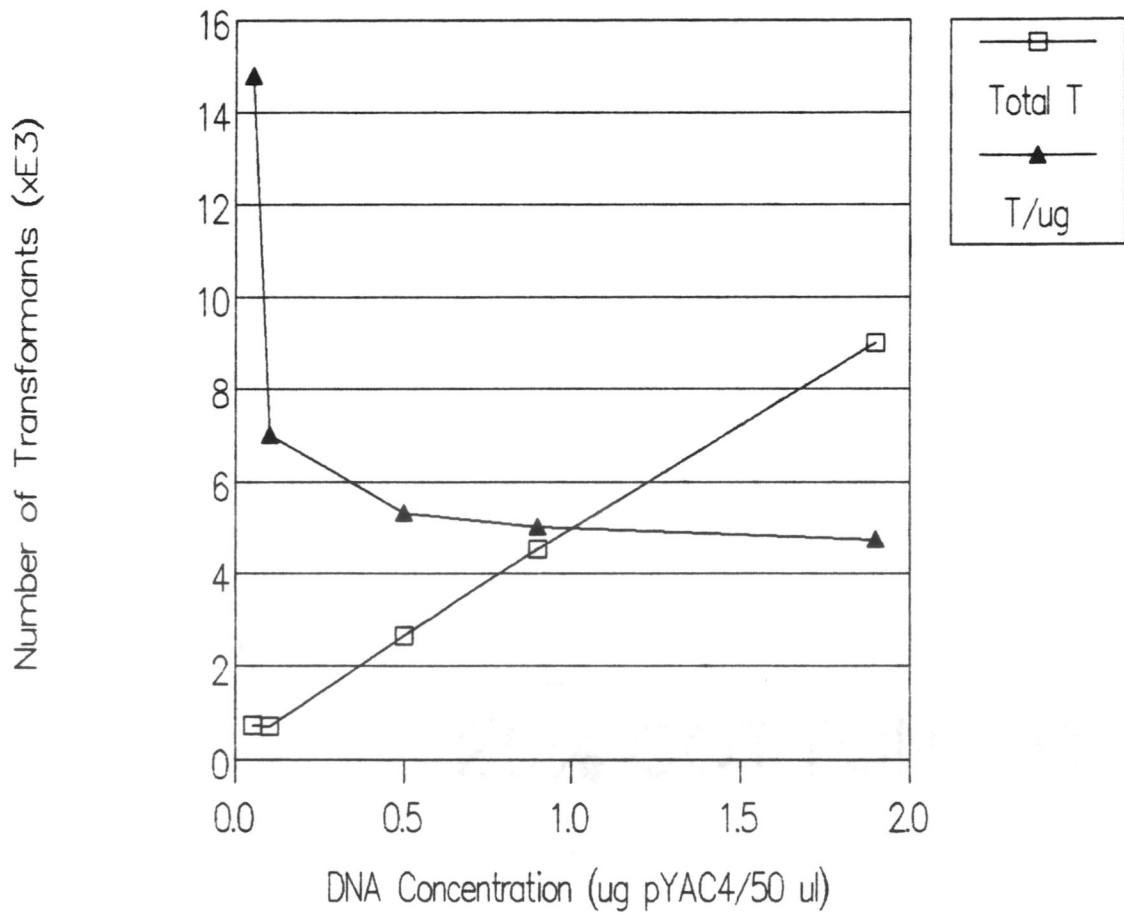
Effect of Plasmid DNA Concentration

Figure 2 Transformation efficiency as a function of plasmid DNA concentration. The cells were electroporated as described in the standard protocol, and the amount of pYAC4 added was varied. Both the total number of transformants obtained and the transformation efficiency (the number of transformants/ug DNA) were measured (see legend).

cells/50 ul to 10^9 cells/50 ul. The number of transformants obtained increased as the number of cells in the transformation mix increased. Transformation efficiency was maximal when the cell concentration in the electroporation cuvette was 5×10^8 cells/50 ul (Figure 3).

Polyethylene Glycol Concentration

A stock solution of PEG 8000 (50% weight/volume) was prepared in electroporation buffer and filter sterilized. Cells were centrifuged, resuspended in 50 ul aliquots of PEG solution and electroporated. A range of PEG concentrations from 2.5% to 50% was tested. The addition of 10% PEG to the electroporation buffer resulted in a four-fold increase in the number of transformants obtained (Figure 4). While a final PEG concentration of 25% (w/v) was found to be optimal in an initial experiment, in later experiments better transformation efficiencies were obtained using 10% PEG. Transformation efficiency declined when PEG concentrations above 25% were used.

Concentration of Low Melting Temperature Agarose

A preliminary experiment demonstrated that DNA suspended in liquified low melting temperature (LMT) agarose could be used to transform cells by electroporation. Following this finding a series of experiments were
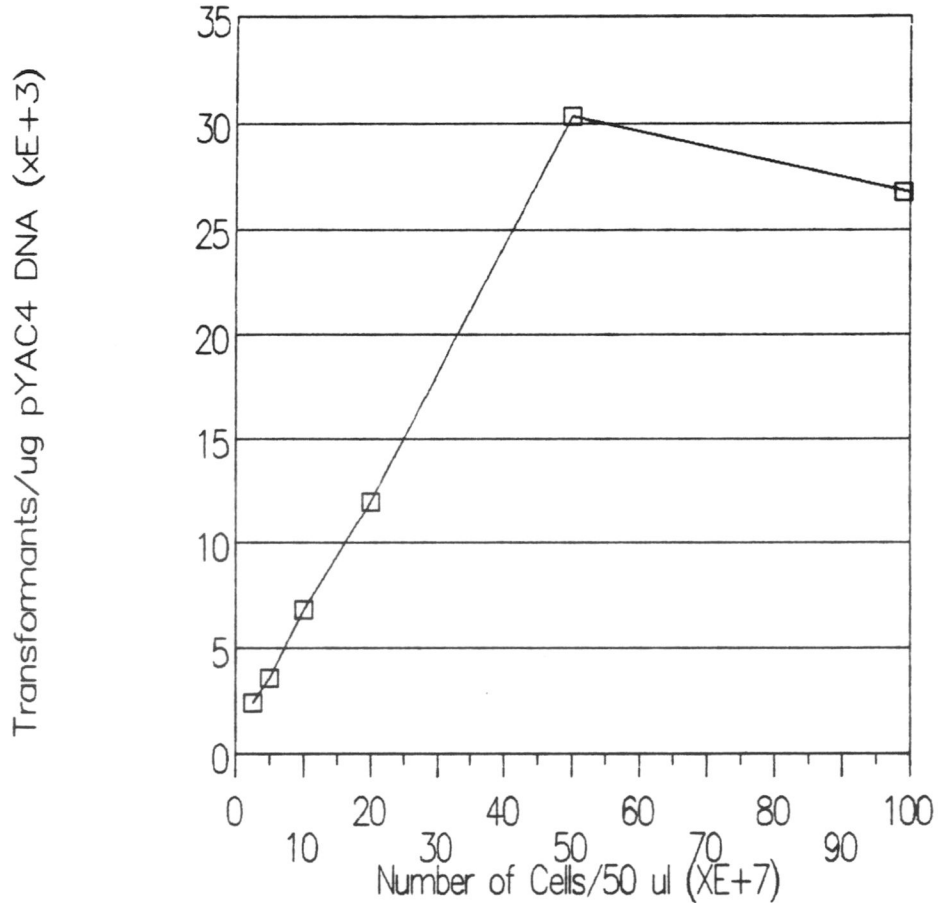
Effect of Cell Concentration

Figure 3 Transformation efficiency as a function of cell
concentration. Electroporation was carried out as described
in the standard protocol, and the number of cells added to
the cuvette was varied. In all cases, the cells were
suspended in 50 *ul* of electroporation buffer before being
transferred to the cuvette.

Effect of PEG Concentration
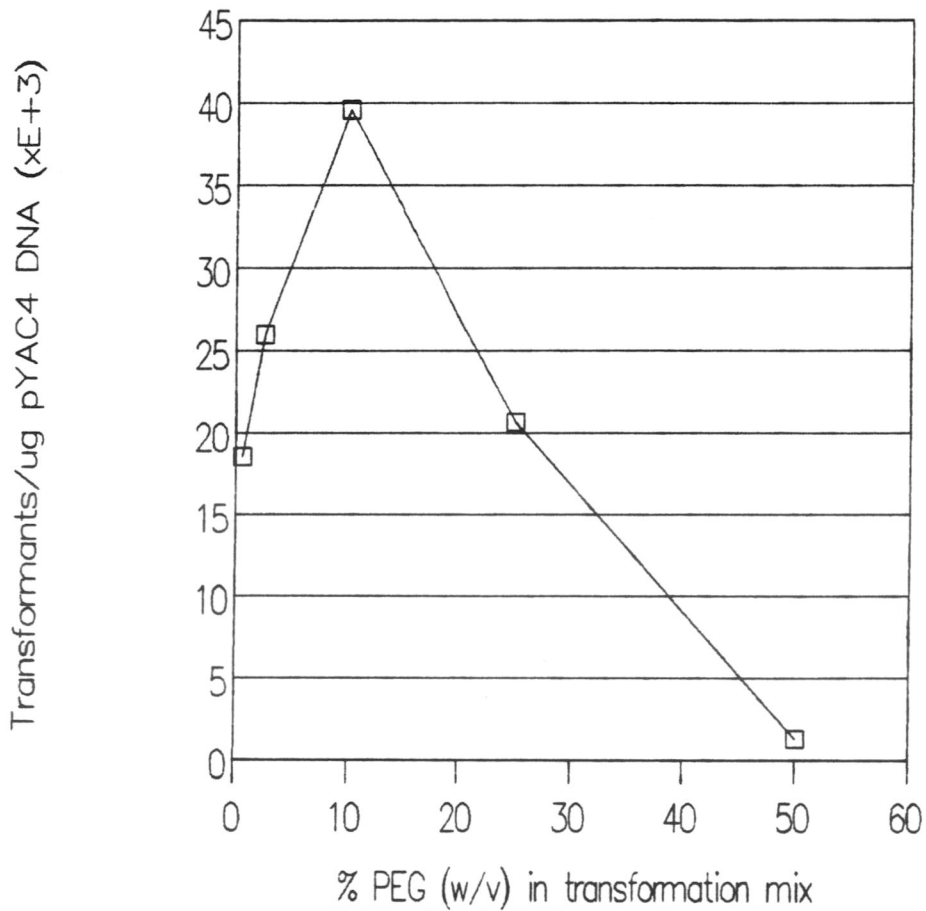

Figure 4 Transformation efficiency as a function of PEG
concentration. DTT-treated cells were suspended in 50 ul of
electroporation buffer containing various concentrations of
PEG and transformed as described in the standard protocol.
The values recorded on the graph represent the average
number of transformants obtained at each PEG concentration
in three replicates of the experiment.

conducted to determine if the agarose concentration had an effect on the number of transformants that could be obtained by this method. A series of LMT agarose solutions ranging in concentration from 0.5% to 1.2% (w/v) were prepared in electroporation buffer. The agarose solutions were melted in a microwave oven and placed in a 45°C water bath. Cells were centrifuged, resuspended in 50 ul of melted agarose solution and transformed using the standard protocol. The presence of 0.5-1.0% melted LMT agarose in the electroporation media reduced transformation efficiency slightly. As Figure 5 illustrates, increasing the concentration of agarose to 1.2% resulted in a dramatic reduction in the number of transformants.

Carrier DNA

In most experiments 50 ug of sonicated calf-thymus DNA in a volume of 10 ul was used as carrier. Three experiments were performed without adding calf-thymus DNA in order to compare the efficiency of electroporation in the presence and absence of carrier DNA. The addition of sonicated calf-thymus DNA resulted in a 5-10 fold increase in transformation efficiency. The use of larger amounts of carrier DNA did not produce a further increase in transformation efficiency.

Effect of Agarose Concentration
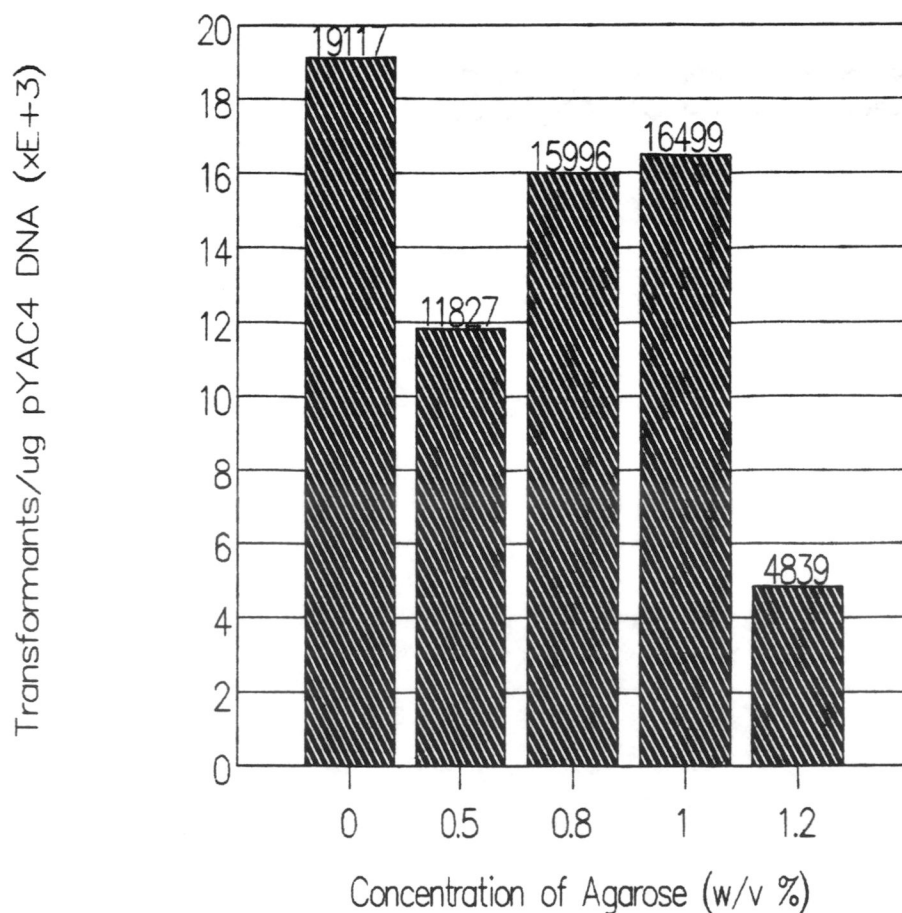
Figure 5 The effect of the presence of melted agarose on transformation efficiency. LMT agarose solutions of various concentrations were prepared in electroporation buffer, melted in a microwave oven, and kept at 45°C. The cells were resuspended in 50 ul aliquots of the agarose solutions and electroporated as described in the standard protocol. The experiment was performed twice, and the average number of transformants obtained at each agarose concentration was determined.

Recovery in YPD

The protocol described by Meilhoc and co-workers (1990) calls for a 30-60 minute recovery incubation in YPD following the pulse. Most protocols for the electroporation of yeast suggest that the cells can be plated onto selective media immediately after electroporation. Five experiments were performed in order to determine if the recovery period was beneficial to transformation efficiency. Following the pulse, cells were either recovered in YPD and incubated at 30°C as described, or recovered in 1 ml of SD ura- and plated immediately. Allowing the cells a 30-60 minute recovery period in YPD prior to plating resulted in an average increase in transformation efficiency of 150%.

Pretreatment of Cells With Proteinase K

The effect of treating the cells with proteinase K prior to electroporation was examined. Cells were washed and centrifuged as in the standard protocol. The cell pellet was resuspended in 1 ml of electroporation buffer containing 2 mg/ml proteinase K and incubated for 10 minutes at 30°C. The cells were washed twice in electroporation buffer and then electroporated. Exposure of the cells to 2 mg/ml proteinase K prior to electroporation resulted higher transformation efficiencies than could be obtained using untreated cells (Figure 6). However, the increase in

transformation efficiency associated with proteinase K pretreatment was much lower than the increase that was observed following treatment of the cells with DTT.

Pretreatment With Zymolyase

Zymolyase is an enzyme preparation isolated from *Arthrobacter luteus* cultures that is used in spheroplast production to lyse yeast cell walls. The essential enzyme present in zymolyase is B-1,3-glucan laminaripentao-hydrolase, although the preparation also contains B-1,3-glucanase, mannanase and protease activities. Experiments were conducted to investigate whether partial digestion of the yeast cell wall prior to electroporation would have an effect on transformation efficiency. Stock solutions of Zymolyase 20-T were prepared in electroporation buffer, and cells were treated with enzyme concentrations ranging from 0.4 ug/ml to 4 mg/ml. Incubation was at 30°C for 10 minutes. The cells were then washed and electroporated as usual. The effect of treating the cells with zymolyase in conjunction with DTT was also examined. These experiments were carried out as described above, but the cells were treated with 25 mM DTT either before or during the zymolyase treatment. Initial experiments in which cells were treated with 2-4 mg/ml Zymolyase 20-T resulted in very few transformants. When the enzyme concentration was decreased to 0.4-40 ug/ml a slight increase in the number of

transformants was noted (in comparison to the number obtained using untreated cells). The exposure of the cells to DTT either prior to or in conjunction with zymolyase treatment greatly increased transformation efficiency, although the number of transformants obtained was less than the number obtained following DTT-treatment alone (Figure 7).

Pretreatment With Alcohols

Experiments were conducted to observe whether treating the cells with various alcohols prior to electroporation would have an effect on transformation efficiency. Immediately before applying the pulse, the alcohol was added to a final concentration of 5% (v/v). The alcohols tested were methanol, ethanol, n-propanol, n-butanol, n-pentanol and n-hexanol. Erratic results were obtained in the experiments involving alcohol pretreatment, although in two out of three cases treatment of cells with 5-10% ethanol prior to electroporation resulted in slight increases in transformation efficiency (360 and 378 transformants/ug DNA).

Application of an Electric Field Following Pulse

The possibility that plasmid DNA may be driven in to existing pores by electrophoresis was the basis for a series

Efficiencies Without DTT Treatment

Figure 6 Average transformation efficiencies obtained using methods in which cells were not treated with DTT. In all cases, cells were treated as described in the text. The number of experimental replicates used in determining the average transformation efficiency for each condition is noted. In order to normalize the transformation efficiencies with respect to cell concentration, the results have been expressed as transformants/ug DNA/5 X 10^7 cells.

Efficiencies Using DTT–Treated Cells

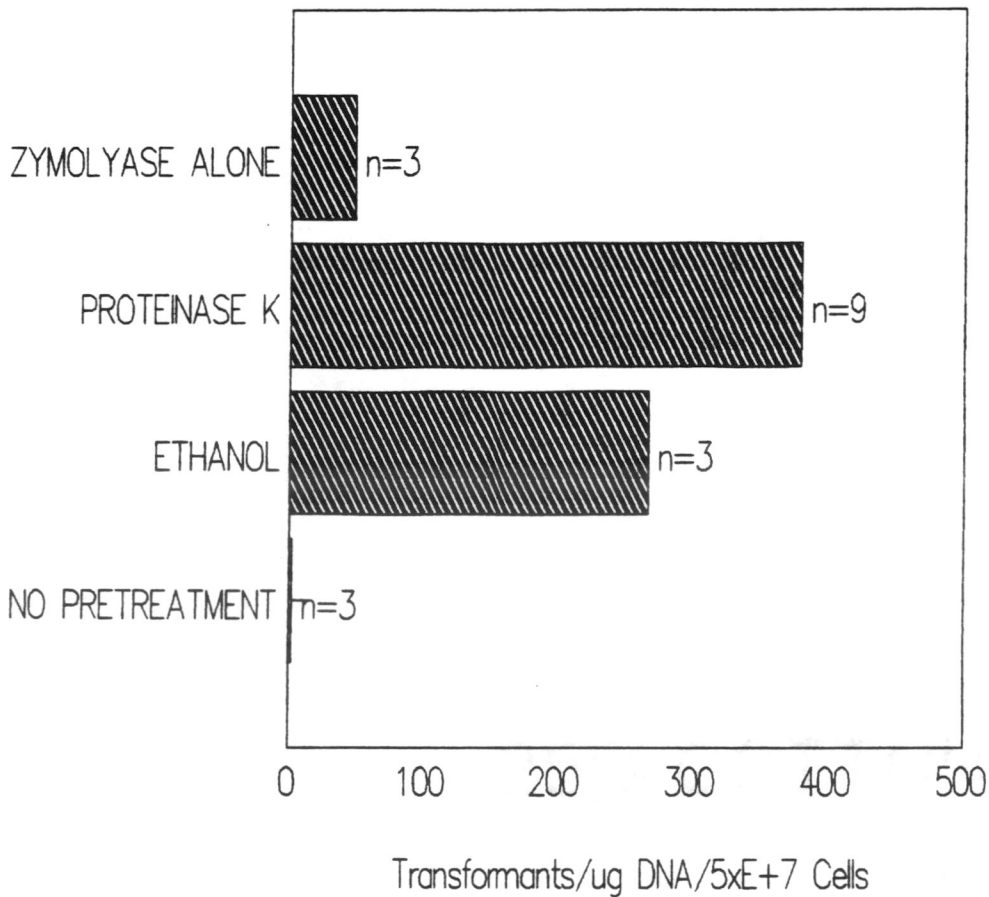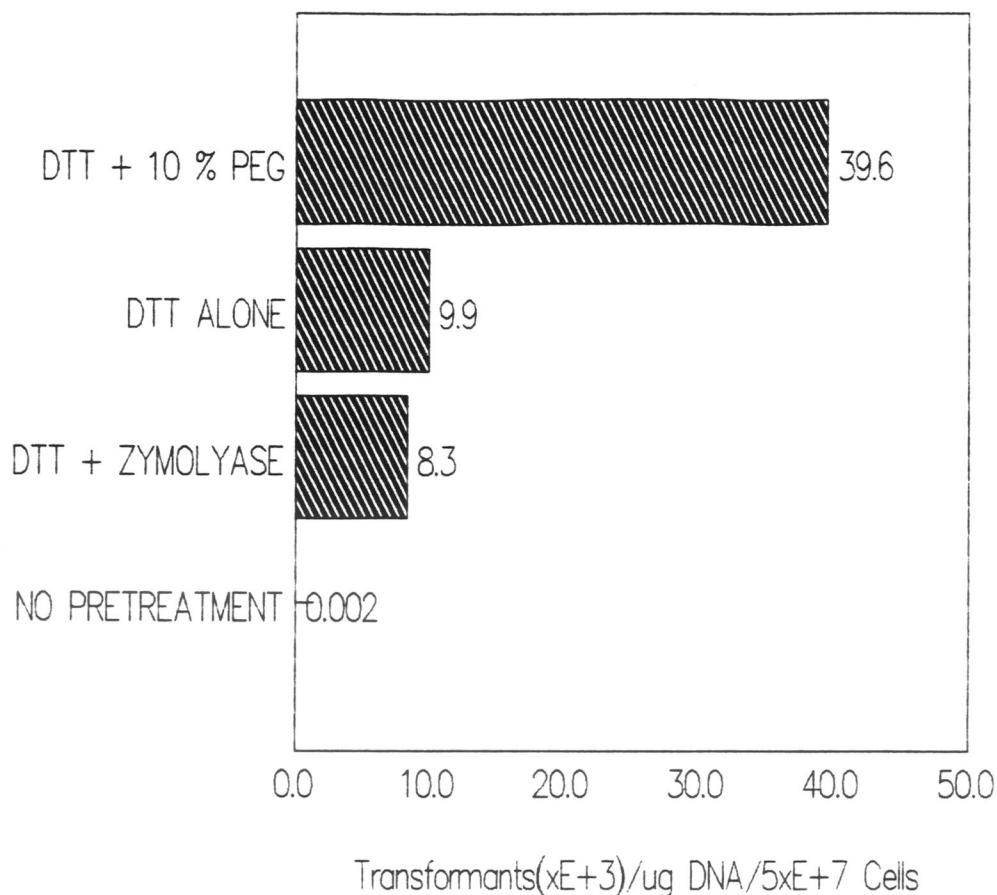**Figure 7** Transformation efficiencies obtained using methods in which cells had been treated with DTT. The cells were treated as described in the text, electroporated, and recovered in YPD. For each method, the average of the efficiencies obtained in three replicates of the experiment are reported. In order to normalize the transformation efficiency with respect to cell concentration, the results have been expressed as transformants/ug DNA/5 X 10^7 cells. Note that in this figure, the number of transformants expressed on the X-axis are multiplied by a factor of 10^3.

of experiments that examined the effect of applying an electric field to the electroporation cuvette. Immediately after electroporation the leads were disconnected from the pulse controller and connected to a 1.5 volt battery. The application of 1.5 V across a 0.2 cm cuvette results in an electric field strength of 7.5 V/cm (DNA electrophoresis is typically carried out at 4-5 V/cm). After 60 seconds the cells were recovered in YPD as described. Application of an electric field immediately after electroporation reduced the number of transformants obtained by an average of 55%.

Electroporation of YACs

The attempts at isolating large fragments of hsDNA by the methods employed were for the most part unsuccessful. The gel permeation column did not appear to separate the large and small hsDNA fragments based on analysis by FIGE. When sucrose gradient centrifugation was used to separate the hsDNA most of the hsDNA ended up in the pellet, where it was contaminated with uncut hsDNA that could not be ligated into the vector.

Yeast cells were then transformed with YACs that were prepared using partially digested hsDNA or lambda DNA that had not been size fractionated prior to ligation. A total of 50 colonies that exhibited the expected phenotype (ura +, trp +, red color) were screened by FIGE. Agarose gels

containing 18 of the samples were further analyzed by ^{32}P hybridization. Most of the YACs appeared to contain only small inserts based on their migration characteristics in agarose gels. The largest YACs that were demonstrated were approximately 20-25 kb in size.

A final attempt to isolate large DNA fragments by gel electrophoresis was apparently successful, *i.e.*, the DNA excised from the gel appeared to be larger than 50 kb. Following ligation, electroporation in agarose yielded a total of 1.0×10^4 transformants containing inserts. However, FIGE analysis of 30 transformed colonies failed to reveal any large YACs.

Optimized Transformation Protocol

Based on the results of the experiments conducted an optimized protocol for the electroporation of intact cells of *S. cerevisiae* strain AB1380 with pYAC4 DNA has been developed. The entire protocol requires only 1-2 hours to complete (not including time required for cell growth). Using the procedure described below we have achieved up to 5.8×10^5 transformants/*u*g of pYAC4 DNA.

1. Grow cells in 200 ml YPD at 30°C to early log phase (approximately 1×10^7 cells/ml). Perform a microscopic cell count to determine cell concentration and check for

possible bacterial contamination.

2. Harvest cells by centrifugation at ambient temperature. Wash cells one time in electroporation buffer (270 mM Sucrose, 10 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$).

3. Resuspend the cell pellet in 2 ml of DTT solution (25 mM DTT in YPD, 20 mM Hepes pH 8.0) and incubate at 30°C for 10 minutes. Wash the cells twice with 1 ml of electroporation buffer. From this point on the manipulations can be carried out in microcentrifuge tubes.

4. Resuspend the cells to a concentration of 10^{10} cells/ml in a 10% PEG solution prepared in electroporation buffer. Transfer 50 ul aliquot to a microcentrifuge tube.

5. Add 10 ul calf-thymus DNA (50 ug) and the desired amount of plasmid DNA. Mix and transfer entire contents to a 0.2 cm electroporation cuvette.

6. Place the cuvette in the Bio-Rad Gene Pulser apparatus and deliver a single pulse at the following settings:

> Voltage = 600 volts
>
> Capacitance = 25 $u$farads
>
> Resistance = 200 ohms

7. Immediately after the pulse recover the cells in 1 ml of YPD broth media. Incubate at 30°C for 30-60 minutes. Spin in a microcentrifuge, wash one time with SD ura- broth

(or other suitable selective media), and resuspend in 1 ml
SD ura-. Plate appropriate dilutions onto selective media
and allow 3-4 days growth at 30°C.

Discussion

The use of YACs in the cloning of large DNA fragments is becoming more common. Until recently, the only published methods for the transformation of yeast with YACs have required the removal of the cell wall. Two recent papers describe the electro-transformation of intact cells of *S. cerevisiae* strain AB1380 with small YACs (Rech *et al.*, 1990; Bell and Mortimer, 1991). However, these protocols are plagued by low transformation efficiencies. Indeed, the cell wall would seem to be a significant barrier to the introduction of large DNA molecules such as YACs. The development of a simple procedure for the efficient electroporative transfer of large YACs into intact yeast cells would simplify YAC cloning. The current work has identified several factors that influence the efficiency of electro-transformation of strain AB1380 with pYAC4 DNA (11.1 kb).

Pretreatment With DTT

Treatment of the cells with DTT prior to electroporation was found to be the most important factor influencing transformation efficiency. De Nobel and co-workers (1990) reported that DTT treatment resulted in an opening up of cell wall disulfide bridges (measured as an increase in free sulfhydryl groups). The opening up of

disulfide bridges was accompanied by an increase in cell
wall porosity as measured by increased uptake of 500
kilodalton (kDa) diethyl amino ethyl-dextran (DEAE-dextran)
molecules and increased release of the periplasmic enzyme
invertase. Furthermore, it has been demonstrated that DTT
treatment of yeast cell walls results in an increase in the
release of cell wall mannoproteins. The loss of cell wall
mannoproteins, in conjuction with an opening up of the cell
wall by reduction of disulfide bonds, may result in the
formation of openings in the cell wall that allow the entry
of macromolecules (De Nobel et al, 1989).

Pretreatment With Proteinase K

Treatment of yeast cells with proteases has also been
reported to result in an opening of cell wall mannoprotein
structure (Zlotnik et al., 1984; Brzobohaty and Kovac,
1986). In the current study, treatment of the cells with
proteinase K resulted in a modest increase in transformation
efficiency. It appears that in the case of yeast strain
AB1380, DTT-induced modifications of cell wall mannoprotein
structure are more amenable to entry of pYAC4 DNA than
modifications resulting from proteinase K treatment.

Pretreatment With Zymolyase

Treating the cells with zymolyase prior to

electroporation resulted in only a slight increase in transformation efficiency unless DTT treatment was also included. Reducing agents and proteases have been demonstrated to facilitate glucanase digestion of the glucan component of the yeast cell wall (Scherrer et al., 1974; Kitamura, 1982; Zlotnik et al., 1984). In the current study, the number of transformants obtained following zymolyase/DTT treatment was always less than the number obtained following DTT treatment alone, which suggests that the increase resulting from combined zymolyase and DTT treatment may actually be attributed solely to the effects of DTT. Treating cells with both zymolyase and DTT may result in the conversion of some cells into spheroplasts, which would not survive unless they were suspended in top agar before plating. A zymolyase-induced reduction in the number of surviving cells could explain the reduction in transformation efficiency seen when zymolyase is included in the DTT-pretreatment.

Growth Stage of Yeast Culture

It has been noted in the literature that yeast cells harvested in early log phase are more readily transformed than cells from older cultures (Delorme, 1989 and Meilhoc et al., 1990). In the current study, experiments in which cells were harvested in early log phase resulted in the

highest number of transformants. It is possible that the entry of DNA is hindered as the cell walls grow thicker with age. Older cells that have experienced numerous budding cycles have more abundant chitin deposits in their cell walls, which may render them more resistant to transformation. It is also possible that cell size or cell cycle stage may play a role in determining whether a cell is likely to be transformed by electroporation (Meilhoc *et al.*, 1990). The majority of the factors discussed above concern some aspect of the yeast cell wall, which acts as an initial barrier to the entry of exogenous DNA molecules.

Pretreatment With Alcohols

The yeast cell membrane presents a second barrier that must be penetrated by plasmid DNA molecules in order for transformation to occur. It has been demonstrated that ethanol increases the fluidity of cell membranes (increases the lateral mobility of lipid molecules within the membrane). This increase in fluidity is accompanied by an increase in membrane permeability (Jones and Greenfield, 1987). Lauermann (1991) has reported that treatment of intact yeast cells with ethanol prior to lithium acetate transformation increases the transformation efficiency. In the current study, the addition of ethanol to the transformation mixture resulted in modest increases in the

number of transformants obtained by electroporation in two out of three cases. The other alcohols tested failed to show a positive effect on the efficiency of electro-transformation. While ethanol may alter cell membrane structure in a manner that facilitates the entry of exogenous DNA, the ethanol-induced increase in transformation efficiency observed in the current study was minor in comparison to the large increase seen following DTT treatment.

Plasmid DNA Concentration

As might be expected, the number of transformants obtained was found to increase with the amount of DNA added. However, transformation efficiency was highest when lower amounts of plasmid DNA was used (0.1 ug). Thus, when transforming cells with valuable DNA it would be beneficial to use small amounts of DNA in multiple transformation experiments rather than using large amounts of DNA in fewer transformation experiments. For example, if one had 10 ug of DNA, it would be more efficient to perform 100 attempts using 0.1 ug DNA each than it would be to perform 10 attempts using 1.0 ug DNA each.

Cell Concentration

The current study also demonstrated that transformation efficiency increased as the cell concentration increased. The optimum number of cells was found to be 5 X 10^8 cells/50 ul (10^{10} cells/ml). It was reported by Meilhoc and co-workers (1990) that cell concentrations above 4 X 10^9 cells/ml resulted in erratic experimental results that were attributed to increases in conductivity or microheterogeneity of the applied electric field. Since the current study found transformation efficiency to be optimal at cell concentrations twice the reported level, it is possible that optimal yeast cell concentrations for electroporative gene transfer are strain specific. One of the experimental modifications (addition of ctDNA, PEG) employed in the current study may also have caused this difference. It should also be noted that Meilhoc and co-workers used square wave pulses while the current study utilized capacitance discharge (exponentially decaying) pulses.

Effect of PEG

PEG promotes cell aggregation and has been used to induce cell fusion and enhance the genetic transformation of many cell types. The presence of 10% PEG was found to increase electroporation efficiency up to four-fold in the

current study. It is possible that PEG exerts this positive
effect by promoting adsorption of DNA molecules to the cell
membrane (Bruschi *et al.*, 1987). Once the application of a
pulse has resulted in DNA entry through a pore, PEG may
facilitate the resealing of the pore by inducing contact
between adjacent regions of the cell membrane. The
transforming DNA would then be trapped within the yeast
cell.

Effect of YPD Recovery

Allowing the cells a 30-60 minute recovery period in
YPD resulted in nearly a two-fold increase in the number of
transformants. While protocols for the transformation of
bacteria with antibiotic resistance genes typically call for
a recovery period, most yeast transformation protocols do
not. A recovery period in a nutrient-rich medium such as
YPD may allow the cells to rapidly synthesize components
needed in the repair of cell membranes damaged by
electroporation. Rapid repair of cell membranes may enable
a higher percentage of the cells to survive electroporation,
resulting in a higher number of viable transformants.

When cells are plated directly onto selective media,
they must begin to synthesize an new enzyme in addition to
having to repair any cell membrane or cell wall damage
induced by the electroporation process. The combined

requirements may be too much for some cells to overcome.
The recovery of cells in YPD supplies the uracil needed for
rapid RNA synthesis, which may allow the cells to synthesize
a pool of the required enzyme before selective plating.
Allowing the cells time to repair membrane damage and
manufacture necessary enzymes may increase the number of
cells that survive the process.

Exposure to an Electric Field Following Electroporation

It has been proposed that electrophoresis of DNA
molecules across cell membranes may play a role in
electroporative gene transfer (Chernomordik *et al.*, 1989;
Xie *et al.*, 1990). In the current study, the application of
a 7.5 V/cm electric field immediately after electroporation
resulted in a reduction in transformation efficiency. The
application of the electric field resulted in the formation
of cell aggregates that were not present following normal
electroporation. The electric field may have caused
movement of the cells and/or the DNA molecules such that the
DNA was no longer aligned with the pore in the cell
membrane, preventing its entry into the cell.

Transformation in Agarose

Large DNA molecules used in YAC cloning are easily
broken during laboratory processing and are often prepared

and manipulated in agarose blocks in order to minimize

shearing. YACs prepared in LMT agarose have been used to

transform spheroplasts without removal of the agarose (Bates

et al., 1990; Chimini *et al.*,1990; Grill and Somerville,

1991). There are no reports in the literature concerning

the electro-transformation of yeast cells with DNA contained

in melted agarose solutions. The current study has

demonstrated that LMT agarose plugs containing YACs can be

melted in a water bath and used to transform *S. cerevisiae*

cells by electroporation without excessive loss of

efficiency as long as the agarose concentration is

maintained at 1.0% or less. Using this procedure, a YAC can

be separated by FIGE, isolated from the agarose gel, and

used to transform yeast cells without the need for ethanol

precipitation. The ability to transform without having to

precipitate the DNA is advantageous, as large DNA is often

difficult to redissolve following ethanol precipitation, and

may become sheared during the process. The agarose plug

containing the DNA must be equilibrated with electroporation

buffer before it can be used in electroporation. In the

current study, equilibration was accomplished by soaking the

agarose plug in sterile electroporation buffer for 18-24

hours at 4°C.

Electroporation of Large YACs

The electroporative transfer of large YACs into yeast cells has not been reported. While no transformants containing large YACs have been detected in the current study, this may be due in part to the methods used to isolate large DNA fragments. It is possible that there is a limitation on the size of DNA molecules that can be introduced through the transient membrane pores induced by electroporation. While the width of a DNA molecule may be small enough to initiate entry through a pore, the enormous length of large YACs may not allow entry of the entire molecule prior to resealing of the pore. The presence of the yeast cell wall may hinder the adsorption of a large YAC to the cell membrane. It is also possible that high voltage electroporation causes shearing of large DNA molecules. However, when lambda concatemers were electroporated under standard conditions (3,000 V/cm) and analyzed by FIGE, no gross degradation of molecules up to 350 kb in size was discernible.

The protocol developed in the current study can be used as a basis for examining the possibility of introducing large YACs into intact yeast cells by electroporation. Additional studies need to be carried out to determine if larger pores produced by electroporation at higher voltages would allow larger YACs to enter yeast cells. It would also

be useful to alter the electroporation medium in ways that
may promote larger or more long-lived pores. Efforts to
increase the percentage of cells that are transformed by
electroporation would also be worthwhile. In the current
study, approximately 0.1% of the cells that would be
expected to survive the procedure were transformed.

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