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Nucleic Acids Research, 1994, Vol. 22, No. 23 5011–5015

Introduction of YACs into intact yeast cells by a procedure which shows low levels of recombinagenicity and co-transformation

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Received August 8, 1994; Accepted October 14, 1994

ABSTRACT

Yeast artificial chromosomes (YACs) enable the cloning and analysis of large segments of genomic DNA and permit the isolation of sequences which are impossible to maintain in *Escherichia coli*. However, the construction of genome libraries in YAC vectors is beset by a number of technical problems, not least of which is the creation of cloned fragments which are not true representatives of the donor genome. These artefactual clones arise mainly due to intra-fragment rearrangements or inter-fragment chimaera formation, both phenomena resulting from the activity of the host yeast's mitotic recombination system. We demonstrate that this system is significantly stimulated by the spheroplasting step of the standard YAC transformation system. In contrast, the transformation of intact yeast cells by either the lithium method or a new lithium-free protocol is much less recombinagenic. It is not possible to introduce high molecular weight YACs into yeast using the lithium protocol, but we find that such molecules may be introduced into *pde2*$^-$ mutants using the lithium-free approach. Since intact cells are transformed by this method, automation of post-transformation steps in the construction of YAC libraries is facilitated. Moreover, the frequency of co-transformation (and, therefore, chimera formation) is significantly reduced. However, these advantages do incur a penalty. Yields of YAC transformants by this simplified intact cell approach are reduced some 25- to 30-fold compared to those obtained by the spheroplast transformation route. Nevertheless, the considerable advantages of the new system recommend it for a number of applications.

INTRODUCTION

Early reports told of the successful construction of libraries from a variety of different genomes using the YAC cloning system (1). The fact that YAC clones behaved similarly to normal yeast chromosomes and were stably maintained through both mitotic and meiotic nuclear division (2) was encouraging. Moreover, YAC clones were found to obey the usual rules of yeast recombinaton, enabling the integration of novel restriction sites or marker genes into the insert sequence and permitting the assembly of complete genes via homologous recombination between YACs whose inserts represented overlapping segments of the donor genome (see ref. 3). However, as more detailed analysis of these libraries progressed, a number of problems were revealed. Foremost amongst these was the presence of artefactual chimeric YACs in virtually all of the libraries; these chimeras were thought to have arisen via recombination between and within YACs (4).

Several studies have investigated the effect of yeast genotype on the structural stability of YAC inserts. The *rad52* mutation has been shown to increase stability of some YACs containing tandem repeated sequences from the human Y chromosome (5). However, there is very little one can do with YACs contained in such hosts. Most, if not all, of the DNA manipulations described in yeast (6) are not possible in *rad52* strains (7) Similarly, attempts to construct libraries from hybrid-cell lines have also failed to eliminate chimeras, and there is a possibility that the proportion of artefactual clones in such libraries may have been underestimated (8). It seems desirable, therefore, to reduce the probability of chimera formation at the very start of the cloning process. However, YACs are introduced into yeast by the transformation of spheroplasts (3). This is a harsh procedure involving enzymatic removal of the cell wall followed by treatment with toxic chemicals. As a variety of other 'insults' to the yeast cell have been found to stimulate genetic recombination (9,10) it is possible that the transformation procedure may have a similar effect. We have examined the effect of three different yeast transformation protocols for their effect on mitotic recombination, their ability to deliver high molecular weight YACs to the host cell, and their frequency of uptake of more than one YAC molecule (co-transformation).

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MATERIALS AND METHODS

Yeast strains

AB1380 *MAT***a** *ade2-1 trp1 ura3-52 his5 lys2* [ψ^+]
NSS1 A *pde2* ::*TRP1* derivative of AB1380
SY1 *MAT***a** *ade2*-101 *lys2*-801 *his3*-Δ200 *trp1*-Δ1 *ura3*-52 *leu2*-Δ1 *can1*^{R}
SY2 A *pde2* ::*TRP1* derivative of SY1 (see ref. 11)

YAC clones

sLLP1, sLLP2 and sLLP3 represent pJS97/98 containing inserts of 23.5 kb (*Nru*I), 28.5 kb (*Sac*II) and 48.5 kb (*Nhe*I) of bacteriophage λ DNA. Y43F10 represents an insert of ca. 680 kb of *C.elegans* DNA in pYAC4 (see ref. 12). In the co-transformation experiments, 'YAC Pair 1' comprised Y72A10 and Y12C1, which are two overlapping clones from the *C.elegans* genomic library which contain DNA inserts of 340 and 300 kb, respectively, inserted into the *Eco*RI site of pYAC4. This pair of clones was kindly provided by Alan Coulson (Sanger Centre, Hinxton Hall, Cambridge). 'YAC Pair 2' consisted of two differently marked YAC vectors (YAC12-UT and YAC12-LH; ref. 2) which each contain an identical 350 kb insert of human DNA. These two clones were a gift from Phil Hieter (Johns Hopkins Medical School, Baltimore). 'YAC Pair 3' involved two YAC clones (YAC12-UH; ref. 13 and YPH607; ref. 2) which each bear non-homologous inserts of human DNA of approximately equal size (ca. 360 kb); these YACs were provided by Phil Hieter and John Riley (Zeneca plc, Alderley Park), respectively.

Growth conditions

Procedures for the growth, mating and sporulation of yeast strains were as described by Sherman *et al.* (14).

Transformation of yeast

The lithium acetate intact cell procedure of Schiestl and Gietz (15) or the spheroplast method of Burgers and Percival (16), as modified by McCormick *et al.* (17), were employed to transform wild-type (*SRB1 PDE2*) strains.

Intact cells of *pde2* mutant strains were transformed using a no-lithium procedure based on that of Philipova (18). Cells were grown in YEPD until an OD_{600nm} value of 0.55. A 4 ml aliquot of cells was harvested, washed in 4 ml of TE buffer (10 mM Tris, 1 mM EDTA) and, finally, resuspended in 200 μl of TE. Approximately 10 μg of transforming DNA was added to the cells in a volume of not more than 20 μl and the mixture incubated at 30°C for 30 min, without agitation. Following this incubation, 70% w/v PEG-4000 was added, to give a final concentration of 35% w/v. The suspension was gently mixed and a further incubation of 15 min was carried out at 30°C. The cells were then recovered by centrifugation, washed twice in TE, and resuspended in 1 ml of the same buffer. Dilutions of this mixture were plated out onto selective plates. Viability of the cells was determined by plating cells on YEPD. The plates were incubated at 30°C and transformants appeared after 3−7 days.

RESULTS

Stimulation of mitotic recombination by transformation procedures

It has been shown previously that electroporation of intact yeast cells, even in the absence of DNA, stimulates mitotic interchromosomal recombination between heteroalleles in a diploid strain (10). We used this same tester strain, GM1, to determine whether the generation of spheroplasts was similarly recombinagenic. The system is based on the insertion of 2 genes (*trp1* and *his3*) into chromosome III between *CRY1* and *MAT*. The diploid strain is heteroallelic for the *trp1* and *his3* genes and can give rise to Trp^+ and His^+ prototrophs by gene conversion or crossing-over. We found that the spheroplast transformation procedure had a dramatic effect on the production of both types of prototrophs, stimulating the appearance of Trp^+ colonies by 1500-fold and of His^+ colonies by 3000-fold (Table 1). Thus ~1 in every 60−150 surviving cells had a recombination event at one or both of these loci.

We examined next whether treatment of intact GM1 cells with lithium acetate and PEG, as in the Ito *et al.* (19) transformation procedure, had a similar recombinagenic effect at the *trp1−his3* locus as spheroplast generation. The results (Table 2) showed that mitotic recombination was stimulated only 5- to 10-fold by

Table 1. Effect of spheroplast formation on recombination in GM1

Experiment	% Survival	Spontaneous mitotic rate[a]	Induced mitotic rate[b]	Fold increase
A. His$^+$ recombinants				
1	0.14	61.5	3.2×10^5	5200
2	0.18	21.7	8.4×10^4	3870
3	0.11	58.0	1.1×10^5	1900
4	0.13	45.5	1.3×10^5	2800
Average	0.14	46.7	1.6×10^5	3443
B. Trp$^+$ recombinants				
1	0.14	75.0	1.1×10^5	1520
2	0.18	20.5	4.1×10^4	2000
3	0.11	46.0	3.5×10^4	760
4	0.13	34.7	6.3×10^4	1800
Average	0.14	44.1	6.3×10^4	1529

GM1 cells were converted to spheroplasts and subjected to a 'mock' transformation as described in ref. 17, but without DNA. Spontaneous and induced intragenic recombination are compared. Experiments 1−4 represent the same experiment in each part of the table (i.e. cells in experiment 1 were assayed for both tryptophan and histidine prototrophy, and so on). Number of His^+ or Trp^+ prototrophs per either [a]10^7 total cells or [b]10^7 surviving spheroplasts.

this procedure. Intact yeast cells may be transformed without recourse to lithium pre-treatment if they carry lesions in the *SRB1* (20,21,22), *PDE2* (11,22) or *RAS2* (22) genes, all of which cause an increase in cell permeability. This simple, no-lithium procedure produced the lowest increase in the level of mitotic recombination of the three regimes examined: just 3- to 5-fold (Table 2). A comparison of the levels of cell survival shown in Tables 1 and 2 suggests that the two intact cell techniques damage cells to a lesser extent and so, presumably, do not induce the repair-recombination response to the same degree as the spheroplast procedure.

Introduction of high MW YACs into intact yeast cells

It is known that the lithium acetate transformation procedure (19) is unable to mediate the uptake of high molecular weight YACs (23, and see below). We had previously demonstrated

Table 2. Effect of intact cell transformation regimes on recombination in GM1

Experiment	% Survival	Spontaneous mitotic rate[a]		Induced mitotic rate[b]		Fold increase	
		His^+	Trp^+	His^+	Trp^+	His^+	Trp^+
A. Lithium transformation							
1	72	56.0	34.7	511	225	9.1	6.5
2	63	46.5	48.4	473	190	10.2	3.9
Average	68	51.2	41.5	492	207	9.6	5.0
B. Lithium-free transformation							
1	85	43.8	47.8	269	135	6.1	2.8
2	79	61.6	38.9	299	120	4.9	3.1
Average	82	52.7	43.4	284	128	5.4	3.0

Cells of GM1 were put through a 'mock' transformation using the lithium and simple, no-lithium techniques described in methods, but without the addition of DNA.
[a,b]See footnote to Table 1.

Table 3. Comparison of the different transformation procedures with YACs of different sizes

YAC (Insert size)	Transformants/μg DNA/10^6 viable cells		
	AB1380 Lithium	Spheroplast	NSS1
sLLP1 (23.5 kb)	722	nd	2207
sLLP2 (28.4 kb)	578	nd	1408
sLLP3 (48.5 kb)	439	nd	1031
Y43F10 (ca. 680 kb)	< 1	2000	63

Transformation frequencies (using the spheroplast or the lithium procedure for AB1380 and the simple no-lithium procedure for NSS1) are the average of five separate transformations carried out in two parallel series of experiments. Series 1 comprised the sLLP1, sLLP2 and sLLp3 transformations using the lithium procedure for AB1380 and the no-lithium procedure for NSS1. Cell viabilities were 61 and 26% for AB1380 and NSS1, respectively. Series 2 comprised the Y43F10 transformations using the spheroplast procedure for AB1380 and the no-lithium method for NSS1; spheroplast/cell viabilities were 0.15% for AB1380 and 24% for NSS1. In Series 3 (not shown), AB1380 cells were transformed by the no-lithium procedure. Cell viabilities were higher than for NSS1 (72%) and no transformants were obtained, with any of the YACs, in three separate experiments.

Table 4. Comparison of co-transformation frequencies with the two methods

	YAC Pair 1	YAC Pair 2	YAC Pair 3
SY1 (*PDE2*)			
Transformants/μg/10^6 viable cells	2100	2570	1700
No. transformants analysed	1000	1000	860
% Co-transformants	10.5	9.1	5.9
SY2 (*pde2 ::TRP1*)			
Transformants/μg/10^6 viable cells	80	105	71
No. transformants analysed	168	148	98
% Co-transformants	2.4	1.4	1.0

The transformation frequencies given are the average for the two YACs in each pair. SY1 was transformed using the spheroplast method and SY2 by the simple no-lithium method. Viabilities were 0.17% for SY1 spheroplasts and 23.5% for SY2 cells.

that the simple no-lithium procedure (11,18) enabled a *pde2::TRP1* strain to take up small circular (22) or linear (24) plasmids at frequencies comparable to those achieved by the spheroplast transformation procedure (15). The ability of such a mutant to be transformed with YAC molecules bearing large inserts of heterologous DNA was now examined (Table 3). It can be seen that transformation frequencies with YACs bearing inserts of bacteriophage λ DNA of 30 to 60 kb in size are at least as high as those obtained by the lithium acetate procedure (19). It proved impossible to introduce YACs containing larger inserts of either human (13) or *Caenorhabditis elegans* (12) DNA into the standard host yeast strain AB1380 using the lithium method. However such YACs could be transformed into the *pde2*$^-$ derivative, NSS1, albeit at a frequency reduced by some 25- to 30-fold as compared to the values obtained using the spheroplast procedure on the parent strain (Tables 3 and 4). Moreover, perhaps as a consequence of this reduced level of competence, the proportion of co-transformants was reduced from 5–10% for spheroplast transformation to just 1–2% for the no-lithium, intact cell regime (Table 4). Control experiments (not shown) demonstrated that, once strain SY2 had taken up two YAC molecules, these were just as likely to recombine as in the Pde2$^+$ parent strain, SY1. In addition, both the *pde2::TRP1* and *PDE2* strains gave similar numbers of transformants with small circular plasmids when transformed by the spheroplast procedure (data not shown).

DISCUSSION

In order for the YAC cloning system to realize its full potential in the analysis of large genomes, it is essential that the overwhelming majority of YAC clones are faithful representatives of the genomes from which they are derived. We have demonstrated that the standard sphaeroplast transformation procedure designed to introduce YACs into yeast causes a massive stimulation of yeast mitotic recombination (the very system responsible for the generation of chimaeras and other kinds of rearrangement) at the most critical stage of the generation of a YAC genomic library: the initial introduction of the clones into the host cells. It should, however, be noted that we cannot, at the moment, exclude the possibility that the elevated levels of recombination at *trp1* and *his3* are due to an allele-specific effect rather than genome-wide changes. Surviving sphaeroplasts could be committed to higher levels of recombination due to at least two different reasons. First, the extensive damage to the cell wall caused by lytic enzymes, coupled with damage to the cell as a result of this (caused by chemicals used in the transformation procedure such as β-mercaptoethanol, PEG and $CaCl_2$), may trigger a general SOS-like response. In *E.coli*, the SOS-repair response is triggered by a variety of chemical and physical insults to the cell (25) leading to increased production of the RecA protein, involved in general genetic recombination. In yeast, the *RAD51* and *RAD54* gene transcripts are induced by cellular damage and mutations in these genes reduce both spontaneous mitotic recombination and induced recombination (16). Yeast sphaeroplasts are immersed in a PEG-containing salt solution for up to 20 min before being placed in regeneration agar. During this time, the highly porous sphaeroplasts may be flooded with chemicals which could damage the cells' DNA. In this model, recombination would be required to repair genetic damage and additional recombination events might be a consequence of the SOS-like induction of recombination enzymes such as *RAD51* and *RAD54*.

The results in Table 2 offer some support for this theory. Following lithium acetate treatment, recombination was rarer at the *trp1–his3* locus, and induction of recombination was lower still in cells treated with the simple, lithium-free, procedure. These two techniques presumably damage cells to a lesser extent and, therefore, may not induce the repair-recombination response as much as the sphaeroplast technique. Finally, the viability data showed a larger proportion of the cells survived lithium acetate and no-lithium treatments as compared to the sphaeroplast technique.

An alternative explanation is that sphaeroplasts have to undergo recombination to survive, regardless of genetic damage. The assay employed here specifically selects for recombination events at the *trp1–his3* locus. In all probability, there will be many additional, unselected, events occurring elsewhere. Higgins and Strathern (10) showed the viability of a GM1 derivative bearing *rad52::hisG/rad52*::YIp5 following electroporation was about the same as that for GM1. In addition, they concluded that electroporation-stimulated events they observed were the result of the predominant *RAD52*-dependent pathway in yeast. It seems likely that the recombination described here is also the result of that same pathway (as *RAD52* is absolutely required for gene conversions; 7). An isogenic *rad52/rad52* GM1 derivative would therefore be predicted to show little or no recombination at this locus following sphaeroplast treatment. However, the viability of an isogenic *rad52* derivative of SY1 was not significantly affected by treatment with the sphaeroplast procedure (data not shown).

The employment of an alternative transformation system which involves the use of intact yeast cells with a Pde2$^-$ phenotype obviates this stimulation of host recombination and, moreover, reduces the level of the co-transformation events which are an obligatory precursor to chimaera formation. The new system has the further advantage that transformants grow as normal colonies on the agar surface which should simplify their subsequent manipulation by either manual or automated methods. The main drawback to the procedure is that the YAC transformation frequencies are ca. 30-fold lower than those obtainable by sphaeroplast transformation. However, it should be pointed out that we have routinely used the no-lithium, intact cell system to transfer YACs of up to 700 kb in size and that no attempt has so far been made to optimize the system even for plasmid transformation, still less for the uptake of large YAC clones. We believe that, even at its current stage of development, the new system may offer advantages over the traditional sphaeroplast transformation protocol for certain applications and we would encourage its evaluation by laboratories committed to the detailed analysis of complex genomes.

ACKNOWLEDGEMENTS

We are grateful to Grant Hamilton, Simon Tracy and Richard Walmsley for useful discussions, and to Phil Hieter, Jeff Strathern, Rakesh Anand, John Riley and Alan Coulson for provision of strains or clones. This work was supported by a grant from the SERC Biotechnology Directorate to SGO and by an Ear-Marked Studentship, from the same source, to SMH.

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