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Transformation of Protoplasted Yeast Cells Is Directly Associated with Cell Fusion

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The frequency of cell fusion during transformation of yeast protoplasts with various yeast plasmids with a chromosome replicon (YRp or YCp) or 2μ DNA (YEp) was estimated by two methods. In one method, a mixture of protoplasts of two haploid strains with identical mating type and complementary auxotrophic nuclear markers with or without cytoplasmic markers was transformed. When the number of various phenotypic classes of transformants for the nuclear markers was analyzed by equations derived from binominal distribution theory, the frequency of nuclear fusion among the transformants was 42 to 100% in transformations with the YRp or YCp plasmids and 28 to 39% with the YEp plasmids. In another method, a haploid bearing the *sir* mutation, which allows a diploid (or polyploid) homozygous for the *MAT* (mating type) locus to sporulate by the expression of the silent mating-type loci *HML* and *HMR*, was transformed with the plasmids. Sporulation ability was found in 43 to 95% of the transformants with the YRp or YCp plasmids, and 26 to 31% of the YEp transformants. When cytoplasmic mixing was included with the nuclear fusion, 96 to 100% of the transformants were found to be cell fusants. Based upon these observations, we concluded that transformation of yeast protoplasts is directly associated with cell fusion.

DNA uptake by living cells is essential to establish genetic transformation. The conditions for cellular uptake of DNA, however, vary in different organisms. For example, cells of *Bacillus subtilis* (1), *Streptococcus pneumoniae* (23), and *Haemophilus influenzae* (28) become physiologically competent to take up DNA at a certain stage of cell growth. In contrast, *Escherichia coli* cells are able to bind DNA in the presence of a high concentration of $CaCl_2$ at low temperature (25). The bound DNA is then taken up in a subsequent heat pulse at 42°C (19). It has been suggested that some protein components are required for either the binding or transport of DNA or both in the *E. coli* transformation (37). In animal cells, DNA coprecipitated with calcium phosphate is adsorbed onto the cells and taken up into the cells in the continued presence of excess $CaCl_2$ (12). On the other hand, protoplasting is essential for plant cells to take up DNA (26, 31, 35).

In *Saccharomyces cerevisiae*, Hinnen et al. (18) first succeeded in transformation of protoplasted cells by using a polyethylene glycol-$CaCl_2$ medium which was originally developed for promoting protoplast fusion in plant cells (36). However, little is known about the mechanism by which DNA is taken up into yeast cells. During the course of tetrad analysis of yeast transformants obtained under similar conditions, however, we noted that almost all transformants were diploids or polyploids in their cell size, shape, and segregation patterns of genetic markers after crossing with a standard haploid strain, although the strains used as the recipients in the transformation were haploids as described by Hicks et al. (17). It was considered that the increase of ploidy arose as a result of cell fusion concomitant with transformation.

This communication describes the frequency of cell fusion during the transformation of protoplasted cells of *S. cerevisiae* with plasmids in the polyethylene glycol-$CaCl_2$ medium. Results indicated that the transformation in yeasts is directly associated with cell fusion.

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MATERIALS AND METHODS

Strains and plasmids. The *S. cerevisiae* and *E. coli* strains and the plasmids used in this study are described in Table 1. Plasmid 43A (Fig. 1), constructed by ligating the yeast *Sau*3AI fragment bearing the *HO* gene into the *Bam*HI site of YEp13 (9), was supplied by J. B. Hicks. YCp19 (Fig. 1), a YRp plasmid connected with a functional centromere (*CEN4* [29]), which greatly increases the mitotic and meiotic stability of the plasmid, was provided by R. W. Davis. pSH15 and pSH19 were constructed by inserting a *Hin*dIII fragment bearing the replication origin of 2μ DNA and the *LEU2* gene from pJDB219 (4) into the *Hin*dIII site of pBR322 (5) or the large *Hin*dIII fragment of YRp7 (30), respectively (Fig. 1). Plasmid pLT1 (Fig. 1) was constructed by inserting a 2.2-kilobase (kb) *Sal*I-*Xho*I fragment bearing the yeast *LEU2* gene from YEp13 into the *Sal*I site of YRp7. pLT3 (Fig. 1) was constructed by deleting the smaller *Hin*dIII fragment from pLT1. pX (Fig. 1) is an 849-base pair miniplasmid spontaneously derived from the 1.4-kb yeast DNA fragment of YRp7, consisting of bases 54 to 902 of the 1.4-kb fragment according to Tschumper and Carbon (34) and containing *TRP1* and *ARS1* sequences (S. Harashima, Y. Shimada, and Y. Oshima, submitted for publication).

Media. Nutrient (YPAD) and minimal media for yeast, L broth as nutrient medium containing sodium ampicillin (Viccillin; 50 μg/ml; Meiji Seika Kaisha Ltd., Tokyo, Japan), and M9 medium used as minimal medium for *E. coli* were those described previously (32). Yeast cells were sporulated by smearing cells on an agar (2%) medium containing 0.5% anhydrous potassium acetate.

Genetic methods. Yeast sporulation was scored under a microscope by observing cells incubated on the sporulation medium at 30°C for 2 days after inoculation from YPAD. Cytoplasmic respiratory-deficient (Rho^-) clones of yeast were induced by cultivating the cells in YPAD medium containing 100 μg of ethidium bromide per ml.

Biochemical methods. Since all the plasmids used except for TRP1 RI circle and pX are able to replicate in *E. coli* hosts, the plasmid DNAs were prepared from *E. coli* JA221

TABLE 1. Principal microorganisms and plasmids

Organism or plasmid	Genotype[a] or characteristics or both	Source or reference
S. cerevisiae		
KM2C-43B	*ho MATα HMLα HMRa mar1-1[b] trp1 his1 ade6*	Klar et al. (22)
D13-1A	*ho MATa trp1 his3*	R. W. Davis
DS12-10B	*ho MATa trp1 leu2 his4 ade2*	T. Oshima
DKD-5D	*ho MATa trp1 leu2-3 leu2-112 his3*	Our stock
DK-13D	*ho MATα trp1 leu2-3 leu2-112 his3*	Our stock
C1457-3A	*ho MATα trp1 leu2 his4 thr4 arg4*	Our stock
K96	*ho mata-1 HMLa hmra-1 mar1-1[b] trp1-1 leu2 his4 met13 lys1-1 thr4*	A. J. S. Klar
SHY3 cir^{+}[c]	*ho MATa ste-VC9 ura3-52 trp1-289 leu2-3 leu2-112 his3-Δ1 ade1-101 can1-100* [*cir^{+}*]	Botstein et al. (6)
SHY3 cir^{0}	*ho MATa ste-VC9 ura3-52 trp1-289 leu2-3 leu2-112 his3-Δ1 ade1-101 can1-100* [*cir^{0}*]	Constructed from SHY3
ATG3	*ho MATa HMLα HMRa mar1-1trp1 leu2-3 leu2-112 his1 his3*	Our stock
NA87-11A cir^{+}	*ho MATα trp1 leu2-3 leu2-112 his3 pho3 pho5*	Our stock
NA87-11A cir^{0}	*ho MATα trp1 leu2-3 leu2-112 his3 pho3 pho5*	Our stock
E. coli		
JA221	F^{-} *leuB6 ΔtrpE5 lacY hsdR hsdM^{+} recA*	Clarke and Carbon (10)
Plasmid[d]		
YRp7	*TRP1*, *ARS1*, 5.8 kb	Struhl et al. (30)
pJDB219	*LEU2*, 2μ DNA replicon, 12.4 kb	Beggs (4)
YEp13	*LEU2*, 2μ DNA replicon, 10.7 kb	Broach et al. (9)
43A*	*LEU2*, 2μ DNA replicon, 16.7 kb	J. B. Hicks
pSH15*	*LEU2*, 2μ DNA replicon, 7.5 kb	Constructed
pSH19*	*LEU2*, 2μ DNA replicon, 8.3 kb	Constructed
pSH125	*TRP1*, *ARS1*, 12.6 kb	Harashima et al. (15)
pLT1*	*TRP1*, *LEU2*, *ARS1*, 8.0 kb	Constructed
pLT3*	*LEU2*, *ARS1*, 7.3 kb	Constructed
TRP1 RI circle	*TRP1*, *ARS1*, 1.4 kb	Zakian and Scott (38)
YCp19*	*TRP1*, *ARS1*, *CEN4*, 10.6 kb	R. W. Davis
pX*	*TRP1*, *ARS1*, 0.85 kb	S. Harashima, Y. Shimada, and Y. Oshima (submitted)
M13mp8::pX	*TRP1*, *ARS1*, 8.2 kb	Constructed

[a] The genetic symbols of *S. cerevisiae* are those described by Broach (8), and those for *E. coli* follow Bachmann (3) or the respective original authors listed.

[b] *mar1-1* is a mutant allele in the *SIR2* locus (8).

[c] The cir^{+} strains harbor 2μ DNA, and the cir^{0} strains were derived from the respective original strains by curing the plasmid by the method of Toh-e and Wickner (33).

[d] Gross structures of the plasmids with an asterisk are illustrated in Fig. 1.

cultivated in M9 medium at 37°C by CsCl-ethidium bromide equilibrium density gradient centrifugation at 36,000 rpm ($10^4 \times g$) in a RP65T rotor (Hitachi Ltd., Tokyo, Japan) for 36 h at 19°C by the method of Clewell and Helinsky (11). TRP1 RI circle (38) DNA was prepared by digestion of YRp7 by *Eco*RI and electrophoresis on polyacrylamide gel (7%), elution of the 1.4-kb *Eco*RI fragment from the gel, and circularizaton of the fragment by self-ligation with T4 ligase. pX DNA was prepared by digestion of hybrid phage M13mp8::pX with *Pst*I, electrophoresis on polyacrylamide gel, elution of the 0.85-kb *Pst*I fragment corresponding to pX from the gel, and circularization of the fragment by self-ligation.

Conditions for digestion of plasmid DNAs with various restriction endonucleases, agarose gel electrophoresis, and ligation of DNAs were described previously (32). Polyacrylamide gel (7%) electrophoresis was performed by the method of Maxam and Gilbert (24).

Yeast transformation and protoplast fusion. Transformation in yeast cells was performed as described by Beggs (4) with minor modification. Cells grown in 4 ml of YPAD medium by standing at 30°C overnight were washed once with 4 ml of 0.1 M sodium citrate buffer (pH 5.8) containing 1.2 M sorbitol and 0.01 M EDTA and suspended in the original volume of the same sodium citrate-sorbitol-EDTA buffer containing 0.1 mg of Zymolyase 60,000 (Kirin Brewery Co., Tokyo, Japan) per ml. The cell suspension was incubated at 30°C for 2 h unless otherwise noted. Protoplast formation was confirmed by placing one loopful of the cell suspension under a microscope and by observing cell lysis after dilution of the cell suspension with a drop of water. Protoplasts were washed with two 4-ml portions of 1.2 M sorbitol solution containing 10 mM $CaCl_2$ and resuspended by addition of 0.1 ml of the same solution to the cell pellet after centrifugation at $550 \times g$ for 5 min. To the protoplast suspension, 4 μl of DNA solution (250 μg/ml) in 10 mM Tris–1 mM EDTA buffer (pH 7.5) was added, and the mixture stood for 15 min at 25°C. Then, 2 ml of 20% polyethylene glycol 4,000 (Wako Pure Chemical Industries, Osaka, Japan) solution in 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM $CaCl_2$ was added. The final concentration of DNA was approximately 0.5 μg per ml of the transformation mixture. The reaction mixture was further incubated at 25°C for 15 min and protoplasts were harvested, resuspended in 0.3 to 0.5 ml of YPAD medium supplemented with 1.2 M sorbitol, and incubated at 30°C for 20 min. A 0.1-ml portion of the above protoplast suspension was put on the surface of an agar plate of selection medium containing 1.2 M sorbitol, and then 8 ml of minimal medium containing 1.2 M sorbitol and 3% agar (Difco Laboratories, Detroit, Mich.) at 46°C

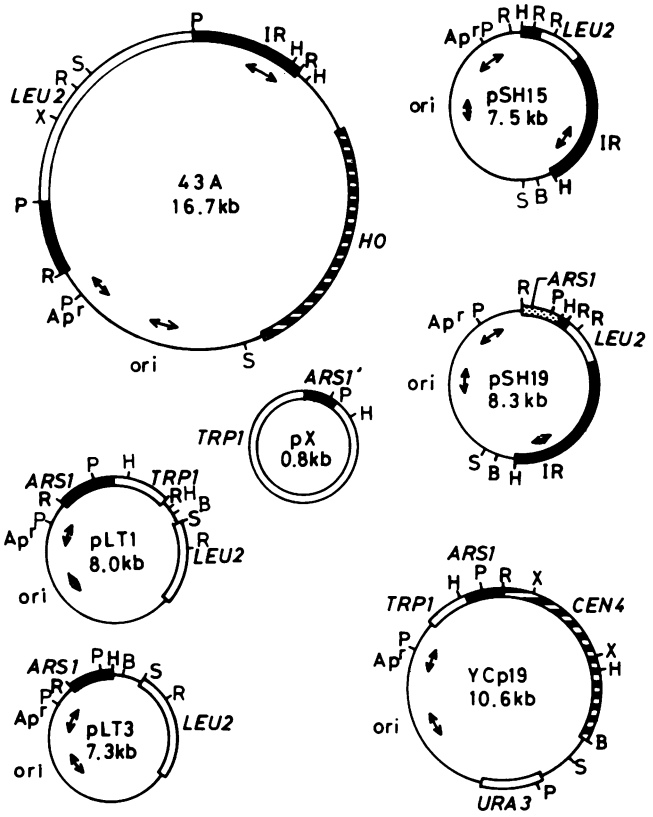

FIG. 1. Structure of novel plasmids used. All plasmids, except for pX, are drawn to the same scale. pX is illustrated on a scale five times greater than the other plasmids. A single thin line on the circle represents the DNA fragment derived from pBR322. A thick line represents the *ARS1* sequence in pX, pLT1, pLT3, and YCp19 or a 2μ DNA sequence in pSH15, pSH19, and 43A. The spotted region in pSH19 represents the *ARS1* region. Double thin lines represents a yeast DNA fragment containing the *LEU2*, *TRP1*, or *URA3* gene. The striped region in 43A represents a DNA fragment containing the *HO* gene and that in YCp19 is of *CEN4*. The region marked Ap^r encodes a gene for ampicillin resistance and that marked Tc^r corresponds to a gene for tetracycline resistance. IR indicates inverted repeated sequences of 2μ DNA. ori indicates the region containing the replication origin of the plasmid in *E. coli*. The restriction sites for *Eco*RI, *Bam*HI, *Hin*dIII, *Pst*I, *Sal*I, and *Xho*I are indicated by R, B, H, P, S, and X, respectively.

was poured onto the agar surface and mixed. When a mixture of protoplasts of two different strains was transformed, the protoplasts were prepared separately and mixed immediately before the addition of plasmid DNA solution. When a yeast strain harboring a plasmid was used as the host, the cells were cultivated in selective medium to avoid plasmid loss.

Estimation of the cell fusion frequency associated with transformation. Two methods were used to estimate the frequency of cell fusion during transformation. The first method employed a mixture of protoplasts of two strains, A and B, with X^- Y^+ Z^- [J^- K^+] and X^+ Y^- Z^- [J^+ K^-] genotypes. X^+ and Y^+ are mutually complementary nuclear genes dominant over their counter alleles, X^- and Y^-, respectively. Z^- is a recessive marker common to both strains, and J^+/J^- and K^+/K^- represent dominant and recessive alleles of cytoplasmic genes. A mixture of these protoplasts was transformed with a plasmid DNA bearing the dominant Z^+ marker. The Z^+ transformants scored were grouped into five classes according to their phenotypes: X^- Y^+ Z^+ J^- K^+ (class I; possible transformants of strain A), X^+ Y^- Z^+ J^+ K^- (class II; transformants of strain B), X^+ Y^+ Z^+ J^+ K^+ (class III; transformants with the fusant phenotype of strains A and B), X^- Y^+ Z^+ J^+ K^+ (class IV; transformants with the phenotype of cytoplasmic mixing of strains A and B and the nuclear marker of strain A), and X^+ Y^- Z^+ J^+ K^+ (class V; transformants with the phenotype of cytoplasmic mixing of strains A and B and the nuclear marker of strain B). Here we define "cytoplasmic mixing" as the process resulting in a fusant having the phenotype of both parents for the cytoplasmic genetic markers but the nuclear marker of only one parent and "nuclear fusion" as the process resulting in a fusant having the nuclear markers of both parents together with the phenotype of cytoplasmic mixing. Although the occurrence of nuclear fusion or cytoplasmic mixing by cell fusion during transformation can be detected by observing the class III, IV, and V transformants, the sum of these transformant classes does not represent the total frequency of cell fusion, because classes I and II of transformants also contain clones produced by cell fusion between two or more cells of an identical strain. In addition, the frequencies of these five transformant classes will be affected by the relative number of protoplasts of strains A and B. Thus, to evaluate the actual frequency of transformation-associated cell fusion, all of the five phenotypic classses of transformants should be taken into consideration. The procedures for calculation of the cell fusion frequencies based on these considerations are detailed in the Appendix. Here, we present only the principal equations.

The frequencies of cell fusion, designated as FCT values (frequency of cell fusion associated with transformation), were calculated from:

$$\mathrm{FCT} = \mathrm{FNF} + \mathrm{FCM} \tag{1}$$

where FNF and FCM values represent the frequency of nuclear fusion and frequency of cytoplasmic mixing associated with the transformation, respectively. These values are expressed by the following equations:

$$\mathrm{FNF} = \frac{\mathrm{fn}(N1 + N2) + N3}{N1 + N2 + N3 + N4 + N5} \times 100 \tag{2}$$

$$\mathrm{FCM} = \frac{\mathrm{fc}(N1 + N2) + N4 + N5}{N1 + N2 + N3 + N4 + N5} \times 100 \tag{3}$$

where $N1$, $N2$, $N3$, $N4$, and $N5$ are, respectively, the observed number of Z^+ transformants of classes I to V. The fn and fc values represent, respectively, the frequencies of nuclear fusion and cytoplasmic mixing among the transformants of classes I and II. For simplicity, we assumed that both the A and B strains had the same fn and fc values and neglected the possible occurrence of multiple cell fusion involving three or more cells. The fn and fc values ($0 \leqq \mathrm{fn} + \mathrm{fc} \leqq 1$) were determined for each experiment, to give minimal chi-square values, by comparison of the observed number of transformants of classes I to V with various assumed values calculated from the probability of the occurrence of an A to A, B to B, or A to B collision during the transformation process, based on a binominal distribution, $f = {}_2C_r R^r (1 - R)^{2-r}$ where R ($0 \leqq R \leqq 1$) is the fraction of protoplasts of strain A in the A plus B mixture.

Scored solely for nuclear phenotype, the Z^+ transformants fall into three classes: X^- Y^+ Z^+ (class A), X^+ Y^- Z^+ (class B), and X^+ Y^+ Z^+ (class AB). In this case, equations 2 and 3 were modified as follows.

$$FNF = \frac{fn(Na + Nb) + Nab}{Na + Nb + Nab} \times 100 \quad (4)$$

$$FCM = \frac{fc(Na + Nb)}{Na + Nb + Nab} \times 100 \quad (5)$$

where *N*a, *N*b, and *N*ab, are, respectively, the observed number of Z^+ transformants of class A, class B, and class AB. All calculations were performed with a personal computer (model MZ-80K2E; Sharp Electric Co., Ltd., Osaka, Japan).

In the second method for determination of the frequencies of cell fusion, we used a *sir* mutant, which allows expression of the silent mating-type information of the *HML* and *HMR* loci (14, 22, 27) as well as of the *MAT* locus (see reference 16 for the *HML* and *HMR* genes). A diploid cell with the *MAT***a**/*MAT***a** or *MAT*α/*MAT*α genotype at the mating-type locus would be able to sporulate if both the **a** and α information is encoded in the *HMR* and *HML* loci. Therefore, when a haploid strain carrying the *sir* mutation and with the *HML*α *HMR***a** or *HML***a** *HMR*α genotype is transformed, it should be possible to determine whether the transformants are haploid or diploid (or polyploid) by their ability to sporulate. However, since all diploid or polyploid cells do not necessarily sporulate, the values determined by this method might be underestimated.

RESULTS

Determination of cell-fusion frequency associated with transformation. To estimate the frequency of cell fusion associated with transformation (FCT value), two different protoplast suspensions were mixed in various combinations. The pairs of protoplasted strains always had identical mating types or were nonmating strains and were marked with auxotrophic markers of chromosomal genes complementary to each other and with a common recessive marker allelic to the dominant plasmid marker. To this mixture was added a yeast plasmid DNA. Transformants were selected primarily by the plasmid marker and scored for their nuclear phenotype. In all experiments, the three classes were found (Table 2). The frequencies of cell fusion (FCT) among the transformants were calculated by using equations 1, 4, and 5 and were found to have high values, i.e., 28 to 100%. If the cell fusion and transformation occurred independently, a much lower frequency of cell fusion would be expected, since both the transformation (2, 13) and cell fusion (21, 36) events are known to occur with a frequency of 10^{-5} (under these conditions, transformation and cell fusion frequencies were similar [10^{-4} to 10^{-6} per cell]). Thus, such high FCT values suggest that the yeast transformation is closely associated with cell fusion.

The FCT values, moreover, fell into two distinct classes that were apparently associated with the plasmids rather than the host strains (Table 2). Higher FCT values of 42 to 100% were observed with YRp7, pSH125, YCp19, pLT1, and pLT3, whereas lower values of 28 to 38% were observed with pSH15, pSH19, and pJDB219. Inspection of structural features of the plasmids suggested that the difference in the FCT values is related to the kind of replicon rather than to size or selective marker, because all the plasmids giving higher FCT value were YRp and YCp plasmids, which replicate by the *ARS1* sequence, whereas all the plasmids giving lower FCT values were YEp plasmids, whose replicon is 2μ DNA.

To confirm this hypothesis, transformations were performed with protoplasts prepared from haploid strains with the *sir* mutation. After transformation of KM2C-43B (*ho MAT*α *HML*α *HMR***a** *mar1-1*; phenotype is nonmater) with plasmid YRp7 and of ATG3 (*ho MAT***a** *HML*α *HMR***a** *mar1-1*; phenotype is nonmater) with YRp7, YCp19, TRP1 RI circle, pSH15, and pJDB219, the transformants were tested for sporulation. We observed that 26 to 95% of the transformants in each experiment were sporulation proficient (Table 3). (*mar1-1* is a mutant allele of the *SIR2* locus [8]. Although *mar1-1* haploids attempt to sporulate and form preasci, we did not score these preasci in the Spo^+ transformants.) This fact also indicates that the transformation is

TABLE 2. Frequency of occurrence of cell fusants in transformants

Plasmid	Replicon	Size (kb)	Host strain[a]		Marker for selection	No. of transformants				FCT (%)
						Total	Phenotypic class			
			A	B			A	B	AB	
YRp7	*ARS1*	5.8	1	2	Trp^+	438	193	84	161	75
			3	2	Trp^+	580	145	236	199	67
			1	4	Trp^+	328	234	37	57	42
			5	6	Trp^+	277	91	77	109	76
			3	7	Trp^+	197	15	128	54	56
pSH125	*ARS1*	12.6	3	2	Trp^+	647	155	237	255	76
YCp19	*ARS1*	10.6	1	2	Trp^+	329	70	51	208	100
			1	2	Leu^+	249	106	46	97	76
pLT1	*ARS1*	8.0	1	2	Trp^+	187	55	21	111	100
			1	2	Trp^+ Leu^+	252	77	27	148	100
pLT3	*ARS1*	7.3	1	2	Leu^+	115	52	14	49	83
pSH15	2μ DNA	7.5	1	2	Leu^+	318	158	116	44	31
pSH19	2μ DNA + *ARS1*[b]	8.3	1	2	Leu^+	315	230	53	32	28
pJDB219	2μ DNA	12.4	1	2	Leu^+	378	287	43	48	39
pX	*ARS1*	0.85	1	2	Trp^+	543	359	103	81	40
43A	2μ DNA	16.7	1	2	Leu^+	516	453	18	45	27
YEp13	2μ DNA	10.7	1	2	Leu^+	352	221	59	72	29

[a] The numbers correspond to the following strains: 1, DKD-5D; 2, DS12-10B; 3, D13-1A; 4, K96; 5, DK-13D; 6, C1457-3A; and 7, KM2C-43B. For their genotypes, see Table 1.

[b] pSH19 is considered to replicate with the 2μ DNA replicon but not with *ARS1* according to the characteristics of its mitotic stability, tetrad segregation, and the frequency of transmission in cytoduction (data not shown).

TABLE 3. Frequency of occurrence of sporulating clones in transformation of *sir* mutant

Plasmid	Replicon	Size (kb)	Host	Selection	Experiment no.[a]	No. of transformants			Frequency of Spo^+ transformation (%)
						Total	Spo^+	Spo^-	
YRp7	*ARS1*	5.8	ATG3	Trp^+	1	275	261	14	95
					2	319	209	110	66
YRp7	*ARS1*	5.8	KM2C-43B	Trp^+	1	190	170	20	90
					2	130	62	68	48
					3	83	51	32	61
					4	125	119	6	95
YCp19	*ARS1*	10.6	ATG3	Trp^+	1	285	130	155	46
					2	359	156	176	43
TRP1 RI circle	*ARS1*	1.4	ATG3	Trp^+		319	160	159	50
pSH15	2μ DNA	8.3	ATG3	Leu^+		320	84	238	26
pJDB219	2μ DNA	12.4	ATG3	Leu^+	1	319	99	220	31
					2	271	74	197	27

[a] All experiments were performed under the same conditions.

highly associated with cell fusion. It was also found that the higher frequencies (43 to 95%) of sporulation-proficient clones were associated with the YRp or YCp plasmid, whereas the lower frequencies (26 to 31%) were observed with YEp plasmids.

Cytoplasmic mixing associated with transformation. Since the genetic markers examined in the above experiments were nuclear genes, the cell-fusion frequencies estimated were based on nuclear fusion. In addition to transformants showing nuclear fusion, however, we also observed transformants judged to have haploid nuclei, which showed haploid cell size and shape and regular 2+:2− tetrad segregation of the nuclear markers when they were crossed with a haploid strain. To see whether the appearance of these haploid transformants was also associated with cell fusion, we tested the occurrence of cytoplasmic mixing associated with transformation by using two strains marked with cytoplasmic genetic markers.

Protoplasts from a [rho^-] derivative of strain DKD-5D harboring pSH15 (*LEU2*) were mixed with those of strain DS12-10B [rho^+] and transformed with YRp7 (*TRP1*) or YCp19 (*TRP1*), and Trp^+ transformants were scored for the phenotypes of their cytoplasmic markers and nuclear markers. In the same manner, transformations with pSH15 or pJDB219 were performed for a combination of two strains, DKD-5D [TRP1 RI circle, rho^-] and DS12-10B [rho^+]. The FCT values calculated from the above experiments indicated that some of the transformants had undergone cytoplasmic mixing but not nuclear fusion, and 96 to 100% of the Trp^+ or Leu^+ transformants were associated with either nuclear fusion or cytoplasmic mixing (Table 4). This indicates that the transformation of protoplasts of *S. cerevisiae* with plasmid DNA is directly associated with cell fusion.

Effect of plasmid stability on nuclear fusion. It is known that a YRp plasmid with an *ARS* replicon is generally less stably maintained in a host cell than a YEp plasmid with the replicon of 2μ DNA, for both the mitotic and meiotic divisions of the host cells. All the plasmids used in the above experiments were in accord with this rule. To investigate whether the plasmid stability or the kind of replicon affects the frequency of nuclear fusion associated with transformation, we used a stable mutant plasmid, pX, with the *ARS1* replicon and unstable plasmids with the 2μ DNA replicon, 43A and YEp13, to transform protoplast mixtures of two haploid strains, DS12-10B and DKD-5D (Table 2). Although a slightly lower FCT value was obtained with pX than with the other YRp plasmids (Tables 2 and 3), the results strongly suggest that the higher and lower frequencies of nuclear

TABLE 4. Frequency of occurrence of nuclear fusion and cytoplasmic mixing

Plasmid	Replicon	No. of transformants[a]						FNF (%)	FCM (%)	FCT (%)
		Total	Phenotypic class[b]							
			I	II	III	IV	V			
YRp7	*ARS1*	102	28.7	13.6	56	2.3	1.4	92	4	96
YCp19	*ARS1*	315	33.9	15.0	242	7.1	17.0	92	8	100
pSH15	2μ DNA	160	52.0	14.2	6	78.0	9.8	8	92	100
pJDB219	2μ DNA	138	35.2	11.2	22	60.8	8.8	26	74	100

[a] With plasmids YRp7 and YCp19, protoplast mixtures of strains DKD-5D [pSH15, rho^-] and DS12-10B [rho^+] were used. With plasmids pSH15 and pJDB219, protoplast mixtures of strains DKD-5D [TRP1 RI circle, rho^-] and DS12-10B [rho^+] were used.

[b] Phenotypic classes are described in the text. Since loss of the resident plasmid in strains DKD-5D [pSH15] and DKD-5D [TRP1 RI circle] or the rho^+ mitochondria in strain DS12-10 B [rho^+] was frequently observed during the protoplast regeneration and subsequent growth of cells in the colony formation under nonselective conditions, the observed numbers of transformants in each phenotypic class were corrected as $N1'$, $N2'$, $N4'$, and $N5'$ for the loss of plasmid or rho^+ mitochondria in each experiment by using the following equations: $N1' = N1 - j \cdot N1$; $N2' = N2 - k \cdot N5$; $N4' = N4 + j \cdot N1$; and $N5' = N2 + k \cdot N5$, where j and k are the coefficients of the loss of the cytoplasmic marker, J and K. The j value was calculated as the observed number of $X^+ Y^-$ and $X^+ Y^+$ transformants which had lost the J^+ cytoplasmic marker, in this case due to the presence of pSH15 (*LEU2*) or TRP1 RI circle (*TRP1*), divided by the total number of $X^+ Y^-$ and $X^+ Y^+$ transformants (which should originally have had the dominant cytoplasmic marker, J^+). The k value was similarly calculated as the observed number of $X^- Y^+$ and $X^+ Y^+$ transformants that had lost cytoplasmic marker K^+, in this case the Rho^+ phenotype, divided by the total number of $X^- Y^+$ and $X^+ Y^+$ transformants, which should originally have had the cytoplasmic marker K^+.

fusion depend on the kind of replicon but not on the stability of the plasmid.

To see the effect of resident 2μ DNA on nuclear fusion associated with transformation, cir^+ (cells harboring 2μ DNA) and cir^0 (cells cured of 2μ DNA) strains were used as hosts in transformation experiments with the pSH15 plasmid. The pSH15 plasmid has a deletion in the region of the *REP1* and *REP2* genes, which are known to exert some function in the stable maintenance of 2μ DNA in the host cell (7). Consequently, pSH15 should be unstable in a cir^0 host but stable in a cir^+ host because the *trans*-acting *REP1* and *REP2* gene products are supplied from the resident 2μ DNA in the cir^+ host but not in the cir^0 host. If the lower stability of a plasmid causes a higher frequency of nuclear fusion in the transformation, pSH15 should give a higher FCT value with the cir^0 host and a lower FCT value with the cir^+ host. Protoplast mixtures of two strains in combination, NA87-11A [cir^+] and SHY3 [cir^+] or NA87-11A [cir^0] and SHY3 [cir^0], were transformed with pSH15, and 38 and 224 Leu^+ transformants were scored from the cir^+ or cir^0 protoplast mixtures, respectively. The FCT values calculated from the results were 24% for the cir^+ protoplast mixture and 12% for the cir^0 mixture, which indicate that the FCT value does not depend on the plasmid stability.

Effect of protoplasting on frequency of nuclear fusion. To examine the effect of protoplasting of the recipient cells on the frequency of nuclear fusion, two strains, DKD-5D and DS12-10B, were protoplasted by Zymolyase treatment for various periods, and protoplasts were mixed and then transformed with YRp7. Samples taken at 15, 30, 60, 120, and 180 min from the reaction mixtures with Zymolyase gave FCT values of 84% (transformation frequency was 4×10^3 per μg of DNA), 55% (1×10^3), 75% (6×10^3), 84% (7×10^3), and 75% (7×10^3) for 250 to 300 Trp^+ transformants scored for each sample. These results clearly indicate that the FCT value is not affected by the degree of protoplasting.

Epistasis of the YEp plasmid over the YRp plasmid in relation to the frequency of nuclear fusion. Mixed transformations of a protoplast mixture of strains DKD-5D and DS12-10B was performed with two plasmids with different replicons, YRp7 (*TRP1*) and pSH15 (*LEU2*), and transformants showing the Trp^+ Leu^+ phenotype were selected. In two independent experiments in which, respectively, 316 and 109 Trp^+ Leu^+ transformants were scored, FCT values of 27 and 11% were obtained for the same combination. These FCT values are significantly lower than that obtained with the YRp plasmid alone (Tables 2 and 3) and comparable to values obtained with the YEp plasmids. When either Trp^+ or Leu^+ transformants were selected independently in the above transformation experiments with two plasmids, 181 (58%) of the 314 Trp^+ transformants showed the Leu^+ phenotype, and the remaining 131 clones (42%) were Leu^-; 196 (63%) clones of the primarily selected 311 Leu^+ transformants showed the Trp^+ phenotype, and the remaining 115 (37%) clones were Trp^- under these transformation conditions (1.0 μg of DNA, 0.5 μg each, per ml of the transformation mixture). The FCT values for those supposed cotransformants (Leu^+) and single transformants (Leu^-) in the primary selection of Trp^+ were 44 and 84%, respectively. The values in the Leu^+ primary selection were 14% for cotransformation and 27% for single transformation. This indicates that the higher frequency of nuclear fusion observed with the YRp plasmids is suppressed by the simultaneous presence of a YEp plasmid. Since the transformation frequency of the recipient protoplasts under these conditions was generally 10^{-4} to 10^{-6}, the frequency of double transformation observed is extraordinarily high and strongly suggests that a large fraction of transformants with either plasmid concomitantly acquired the other plasmid.

DISCUSSION

Transformation with plasmid DNA in protoplasted cells of *S. cerevisiae* is directly associated with cell fusion. The observations strongly suggest that the cell fusion is indispensable for DNA uptake. Completion of the transformation with the YRp and YCp plasmids appears to require nuclear fusion, whereas that with the YEp plasmids is more easily established by cytoplasmic mixing and appears not to require nuclear fusion.

Significantly high cotransformation occurred with the mixture of plasmids YRp and YEp, and lower FCT values were observed for the YRp plasmid than when it was used singly. Whether the high cotransformation frequency is the result of enhancement of the DNA incorporation, the more effective settlement of the YRp plasmid in the host cell, or any other reasons has not yet been solved. The high frequency of cotransformation suggests a possible method of selection for transformants with a plasmid lacking suitable selective markers.

Cell fusion might not be directly concerned with the transformation, because when cell fusants were primarily selected, the frequency of transformants among them was only 1% (data not shown). These observations strongly suggest that the cell fusion occurs independent of the transformation event, but the uptake of plasmid DNA depends on the cell fusion process.

We also examined the possibility of transformation during the sexual conjugation of two haploid clones with complementary mating types in YPAD medium containing 10 mM $CaCl_2$ and 20% polyethylene glycol 4,000. However, no transformants were detected (data not shown). Recently, Ito et al. (20) described an effective method for transformation of unprotoplasted cells of *S. cerevisiae* with plasmid DNA by treatment of the cells with various alkali cations. When we tested their method, all the transformants were found to be haploid cells (data not shown). Hence, the mechanism of DNA uptake with alkali cation treatment is different from that of protoplasted cells.

APPENDIX

Estimation of cell fusion frequency associated with transformation. Suppose that protoplasts of two strains, A (with the X^- Y^+ Z^- [J^+ K^-] genotype) and B (X^+ Y^- Z^- [J^- K^+]), are mixed and transformed with a plasmid DNA having the Z^+ marker, *X*, *Y*, and *Z* are nuclear genes, *J* and *K* are cytoplasmic genes, and + and − superscripts indicate the respective dominant and recessive alleles. Let *N*1, *N*2, *N*3, *N*4, *N*5, and *N* be, respectively, the observed number of transformants showing X^- Y^+ Z^+ J^+ K^- (class I phenotype), X^+ Y^- Z^+ J^- K^+ (class II), X^+ Y^+ Z^+ J^+ K^+ (class III), X^- Y^+ Z^+ J^+ K^+ (class IV), and X^+ Y^- Z^+ J^+ K^+ (class V) phenotypes and the total number of Z^+ clones. We assume that cytoplasmic fusion between two protoplasts results in cytoplasmic mixing with or without nuclear fusion. If the frequency of nuclear fusants (with cytoplasmic mixing) is designated as fn, that of cytoplasmic mixing (without nuclear fusion) as fc, and that of total cell fusants as f among the class I and II transformants, then the numbers of nuclear fusants, of fusants with cytoplasmic mixing, and of unfusants in classes I and II, designated, respectively, as *N*1n, *N*1c, *N*1u, *N*2n, *N*2c, and *N*2u, are given by the following equations:

$$f = fc + fn \quad (6)$$

$$N1c = N1 \times fc \quad (7)$$

$$N1n = N1 \times fn \quad (8)$$

$$N1u = N1(1 - fc - fn) \quad (9)$$

$$N2c = N2 \times fc \quad (10)$$

$$N2n = N2 \times fn \quad (11)$$

$$N2u = N2(1 - fc - fn) \quad (12)$$

Here we assumed that the frequencies of cytoplasmic mixing and nuclear fusion for strain A are equal to those of strain B, i.e., $N1c/N1 = N2c/N2$ and $N1n/N1 = N2n/N2$. Thus, the expected number of protoplasts which participated in nuclear fusion for strains A and B, designated as NpnA and NpnB, can be obtained from the following equations:

$$NpnA = 2 \times N1n + N3 \quad (13)$$

$$NpnB = 2 \times N2n + N3 \quad (14)$$

On the other hand, the expected number of protoplasts which took part in cytoplasmic mixing for strains A and B, designated as NpcA and NpcB, can be obtained by the following equations:

$$NpcA = 2 \times N1c + N4 + N5 \quad (15)$$

$$NpcB = 2 \times N2c + N4 + N5 \quad (16)$$

Thus, the total number of protoplasts which participated in cell fusion, regardless of cytoplasmic mixing and nuclear fusion, for the protoplast mixture of strains A and B, designated as NpA and NpB, can be calculated from the following equations:

$$NpA = NpnA + NpcA \quad (17)$$

$$NpB = NpnB + NpcB \quad (18)$$

When the protoplasts of strains A and B were mixed in proportions of R for A and $1 - R$ for B (where $1 \geqq R \geqq 0$), the theoretical probability of protoplast collision between A and A (i.e., $T1$), B and B ($T2$), and A and B ($T3$) during transformation can be obtained from an equation of binominal distribution:

$$T1, T2, \text{ or } T3 = {}_2C_rR^r(1 - R)^{2-r} \quad (19)$$

where $r = 0$ or 2 for $T1$ or $T2$ and $r = 1$ for $T3$, and $T1 + T2 + T3 = 1$. Then the frequencies of nuclear fusion, Fn, and cytoplasmic mixing, Fc, among total fusion events are calculated from the following equations:

$$Fn = \frac{fn}{fn - fc/2} \quad (20)$$

$$Fc = \frac{fc/2}{fn + fc/2} \quad (21)$$

Here it should be noted that a single event of cytoplasmic mixing gives rise to two cytoplasmic fusants, whereas one of nuclear fusion gives rise to one nuclear fusant. Thus, the theoretical number of nuclear fusants showing class I phenotype, $TN1n$, and class II phenotype, $TN2n$, or transformants with cytoplasmic mixing showing class I phenotype, $TN1c$, class II phenotype, $TN2c$, class III phenotype, $TN3n$, class IV phenotype, $TN4c$, and class V phenotype, $TN5c$, can be calculated from equations 22 to 28 with the equations $R = NpA/(NpA + NpB)$ and $1 - R = NpB/(NpA + NpB)$.

$$TN1n = T1 \times Fn \times [(NpA + NpB)/2] \quad (22)$$

$$TN1c = T1 \times Fc \times [(NpA + NpB)/2] \times 2 \quad (23)$$

$$TN2n = T2 \times Fn \times [(NpA + NpB)/2] \quad (24)$$

$$TN2c = T2 \times Fc \times [(NpA + NpB)/2] \times 2 \quad (25)$$

$$TN3n = T3 \times Fn \times [(NpA + NpB)/2] \quad (26)$$

$$TN4c = T3 \times Fc \times [(NpA + NpB)/2] \quad (27)$$

$$TN5c = T3 \times Fc \times [(NpA + NpB)/2] \quad (28)$$

Further, the theoretical number of protoplasts which did not participate in any fusion event, designated as $TN1u$ and $TN2u$, can be expressed by following equations:

$$TN1u = \frac{TN1n}{fn} \times (1 - fc - fn) = \frac{TN1c}{fc} \times (1 - fc - fn) \quad (29)$$

$$TN2u = \frac{TN2n}{fn} \times (1 - fc - fn) = \frac{TN2c}{fc} \times (1 - fc - fn) \quad (30)$$

Thus, the expected number of transformants showing the phenotype of classes I to V, $E1$, $E2$, $E3$, $E4$, and $E5$, respectively, is obtained by equations 31 to 35.

$$E1 = TN1n + TN1c + TN1u \quad (31)$$

$$E2 = TN2n + TN2c + TN2u \quad (32)$$

$$E3 = TN3n \quad (33)$$

$$E4 = TN4c \quad (34)$$

$$E5 = TN5c \quad (35)$$

These expected values, $E1$ to $E5$, can be calculated by putting various fc and fn values into equations 6 to 11. They are compared with the observed ones, $N1$ to $N5$, by chi-square statistics. Thus, we can test the fitness between observed values and calculated values with various assumed fn and fc values from 0 to 1 at intervals of 0.01 by equation 36.

$$\chi^2 = \frac{(E1 - N1)^2}{E1} + \frac{(E_2 - N2)^2}{E2} + \frac{(E3 - N3)^2}{E3} + \frac{(E4 - N4)^2}{E4} + \frac{(E5 - N5)^2}{E5} \quad (36)$$

The combination of assumed fc and fn values which gives a minimal chi-square value was determined for each individual experiment. Using the optimal fc and fn values, the frequencies of nuclear fusion (FNF) and cytoplasmic mixing (FCM) were calculated from equations 37 and 38.

$$FNF = \frac{fn(N1 + N2) + N3}{N} \times 100 \quad (37)$$

$$FCM = \frac{fc(N1 + N2) + N4 + N5}{N} \times 100 \quad (38)$$

When strains with only nuclear markers are used, this procedure can be modified as follows. When a protoplast mixture of two strains, A and B, having mutually complementary markers, $X^-\ Y^+\ Z^-$ and $X^+\ Y^-\ Z^-$, is transformed with a plasmid DNA bearing the Z^+ marker, transformants can be classified into three different phenotypic classes: $X^-\ Y^+\ Z^+$ (class A), $X^+\ Y^-\ Z^+$ (class B), and $X^+\ Y^+\ Z^+$ (class AB). In this case, equations 36, 37, and 38 should be replaced by the following equations:

$$\chi^2 = \frac{(Ea - Na)^2}{Ea} + \frac{(Eb - Nb)^2}{Eb} + \frac{(Eab - Nb)^2}{Eab} \quad (39)$$

$$FNF = \frac{fn(Na + Nb) + Nab}{N} \times 100 \quad (40)$$

$$FCM = \frac{fc(Na + Nb)}{N} \times 100 \quad (41)$$

where Na, Nb, and Nab are, respectively, the observed number of Z^+ transformants of class A, class B, and class AB, whereas Ea, Eb, and Eab are the numbers calculated by assuming various fn and fc

values. With the optimum fn and fc values, the FNF and FCM values can be calculated from equations 40 and 41. Then the frequency of cell fusion associated with transformation, FCT, is given by summation of the FNF and FCM values:

$$FCT = FNF + FCM \quad (42)$$

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