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# *Saccharomyces cerevisiae* Nuclear Fusion Requires Prior Activation by Alpha Factor

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**We have developed a protocol for efficient fusion of spheroplasts of the same mating type. Nuclear fusion in this whole-cell system is also efficient and closely parallels nuclear fusion in heterosexual mating of intact cells. In the spheroplast fusion system, nuclear fusion is dependent on both the *KAR1* gene and prior exposure to alpha factor. The major products of nuclear fusion in the spheroplast fusion assay were true diploids that were homozygous at the mating-type locus. An additional 10% of the products were cells of ploidy greater than diploid. The dependence of nuclear fusion on alpha factor treatment could not be replaced by synchronization in G1 by mutations in *CDC28* and *CDC35* or by prior arrest in stationary phase. These data suggest that nuclear fusion is not a constitutive function of the nucleus, but rather is specifically induced by mating hormone.**

In the yeast *Saccharomyces cerevisiae*, the nuclear envelope remains intact throughout mitosis, meiosis, and conjugation (1). Consequently, formation of the diploid nucleus during conjugation (karyogamy) requires fusion of the nuclear membranes of the two haploid nuclei. Observation of conjugation by electron microscopy (2, 3) indicates that nuclear fusion (karyogamy) is mediated by extranuclear microtubules emanating from the spindle plaques embedded within the nuclear envelope. These microtubules span the gap between the two haploid nuclei, which then move together and fuse in the region of the spindle plaques. As a result, the newly formed diploid nucleus includes a novel zygotic spindle plaque. During conjugation, nuclear fusion occurs with high fidelity; usually more than 95% of the zygotes produce buds having strictly diploid nuclei.

Several mutations have been identified that disrupt nuclear fusion without affecting cell fusion (4, 5, 7, 9, 21; J. H. Thomas, doctoral thesis, Massachusetts Institute of Technology, 1984). The first mutation to be associated with a defect in nuclear fusion is *kar1-1* (4, 9). In crosses with *kar1-1*, nuclear fusion fails in approximately 90 to 95% of all matings. This defect is manifest to the same extent whether *kar1-1* is mated with the wild type or with *kar1-1* strains. If both the cytoplasm and nuclei of the parents are appropriately marked, it can be shown that the buds from these zygotes contain one or more haploid nuclei and cytoplasmic contributions from both parents. These recombinant progeny are called cytoductants. Subsequent buds show no higher frequency of diploid nuclei than do the first buds (18), indicating that the nuclei within the zygote do not subsequently fuse. These features of nuclear fusion suggest that the process is not solely a passive fusion of the two membranes, but is mediated by specific gene products.

In the normal sequence of events in conjugation, cell fusion is an obligatory prelude to nuclear fusion. Therefore, it is difficult to separate the functions required for cell contact and fusion from the functions required for nuclear fusion. Yeast cells must be activated so that they can leave the mitotic cycle and undergo the developmental events required for cell fusion. Activation leads to arrest in the G1 portion of the cell cycle, agglutination with cells of the opposite mating type, morphological distortion (shmooing), and selective removal of a portion of the cell wall to permit cytoplasmic fusion. A similar activation of the nucleus could be required for nuclear fusion. Alternatively, nuclear fusion could be constitutive, occurring spontaneously once the two nuclei are present in the same cell. In this report we use a spheroplast fusion assay to fuse cells of the same mating type. Our results show that nuclei, like cells, require activation by conjugation-specific signals in order to fuse.

## MATERIALS AND METHODS

**Strains and media.** The *S. cerevisiae* strains used in this study are described in Table 1. Strains MY346 and MY348 were transformed with a plasmid carrying the wildtype *KAR1* gene. The isolation of this plasmid will be described elsewhere.

Yeast strains were propagated nonselectively in liquid YPD medium (2% peptone, 1% yeast extract, and 2% glucose) or on the surface of agar plates (YPD with 2% agar) essentially as described in Sherman et al. (27). Selective medium was YNB (yeast nitrogen base [Difco Laboratories], 0.67%) supplemented with glucose to 2% and any essential

TABLE 1. Strain list

| *S. cerevisiae* strain | Relevant genotype or phenotype |
|---|---|
| MY573 | *MATa his4-34 ura3-52* |
| MY577 | *MATa leu1 leu2 ura3-52* |
| MY578 | *MATa kar1-1 his4-34 ura3-52* |
| MY575 | *MATa kar1-1 leu1 leu2 ura3-52* |
| MY248 | *MATa his4-34 ura3-52* Ery$^r$ |
| MY346 | *MATa leu1 leu2 kar1-1 ura3-52* (pMR6) |
| MY348 | *MATa his4-34 kar1-1 ura3-52* (pMR6) |
| MY415 | *MATa leu2-3 leu2-112 cdc28-1 ura3-52* |
| MY416 | *MATa leu2-3 leu2-112 ura3-52* |
| MY418 | *MATa his4-34 ura3-52* |
| MY419 | *MATa his4-34 cdc28-1 ura3-52* |
| L455 | *MATα/α lys1-1/lys1-1* |
| L94 | *MATα lys1-1* |
| 67-1D | *MATa his4-34 ura3-52* |
| 67-3C | *MATa his4-34 leu2-3 leu2-112* |
| 67-11B | *MATa his4-34 cdc35-1 ura3-52* |
| 67-12D | *MATa his4-34 cdc35-1 leu2-3 leu2-112* |

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nutritional requirement where appropriate as described in Sherman et al. (27). YNB medium was supplemented routinely with a mixture of all amino acids plus adenine and uracil (except for those specific supplements for which selection against auxotrophy was desired).

Spheroplasts were regenerated in a layer of top agar (regeneration agar) composed of YNB medium with 1 M sorbitol and 3% agar as described by Hinnen et al. (15) except that no YPD medium was added. The regeneration agar also normally contained a mixture of all amino acids save histidine and leucine, except when the regeneration frequency was measured, in which case all amino acids were added.

Erythromycin resistance was assessed on YPGE medium (2% peptone, 1% yeast extract, 2% glycerol, 2% ethanol, and 2% agar) to which 4.0 mg of erythromycin per ml was added.

**Spheroplast fusion.** Spheroplast fusion was performed essentially as described by van Solingen and van der Plaat (29). Strains were grown to a density of $1 \times 10^7$ to $2 \times 10^7$ cells per ml in YPD medium at 30°C. Prior to alpha factor treatment, the cells were centrifuged briefly and suspended in 2 volumes of YPD medium that had been adjusted to pH 4.0 with hydrochloric acid. Synthetic alpha factor (Sigma Chemical Co.) dissolved in methanol was added to a final concentration of 5 μM, and the cells were incubated with shaking until more than 90% of the cells had arrested (2 h at 30°C) and begun to undergo the characteristic morphological change associated with mating factor arrest (shmooing). In experiments with temperature shifts, cultures were grown at 24°C and then incubated at 34°C for 3 h, by which time the majority of the *cdc* mutant cells had arrested with characteristic morphology. Half of the culture was then incubated with alpha factor for 2 h at 34°C, and half was incubated without alpha factor at 34°C for 2 h. Approximately $10^9$ cells were harvested by centrifugation and suspended in 10 ml of 1 M sorbitol. Glusulase (Endo Labs) was added to 2% (vol/vol), and the cells were incubated with gentle shaking at 30°C for 2 h until spheroplasts formed. The culture was assumed to have been adequately converted to spheroplasts when more than 95% of the cells lysed after dilution into 0.1% sodium dodecyl sulfate. An additional indication that cells had been converted to spheroplasts was provided by measurement of the difference in the viable titer of a culture that had been diluted in $H_2O$ before and after Glusulase treatment. Viability after dilution in $H_2O$ was routinely reduced about 100-fold by treatment with Glusulase. Cells were centrifuged and washed in 10 ml of 1 M sorbitol supplemented with 10 mM Tris hydrochloride (pH 7.5) and 10 mM $CaCl_2$ (STC). Spheroplasts were centrifuged again and suspended at $2 \times 10^9$ cells per ml in STC. The actual fusion was carried out by mixing together 0.1 ml of each parent strain and then adding 2 ml of a solution of 40% polyethylene glycol 4000 (Baker)–10 mM Tris hydrochloride (pH 7.5)–10 mM $CaCl_2$. The cells were incubated in this solution at room temperature for 15 to 20 min, centrifuged for 5 min at 2,000 rpm, and suspended in 1 ml of STC. Spheroplasts were diluted in STC, and 0.2-ml samples were added to 10 ml of regeneration agar and plated onto YNB agar. Plates were then incubated at 24 or 30°C for up to 1 week. For each experiment, the frequency of regeneration of each parental strain was measured by plating the spheroplasts in regeneration agar containing all the required growth supplements. The frequency of regeneration was routinely between 1 and 10%. The frequency of prototroph formation is expressed as number of prototrophs per regenerant, where the regeneration frequency used is that of the strain with the lower regeneration frequency.

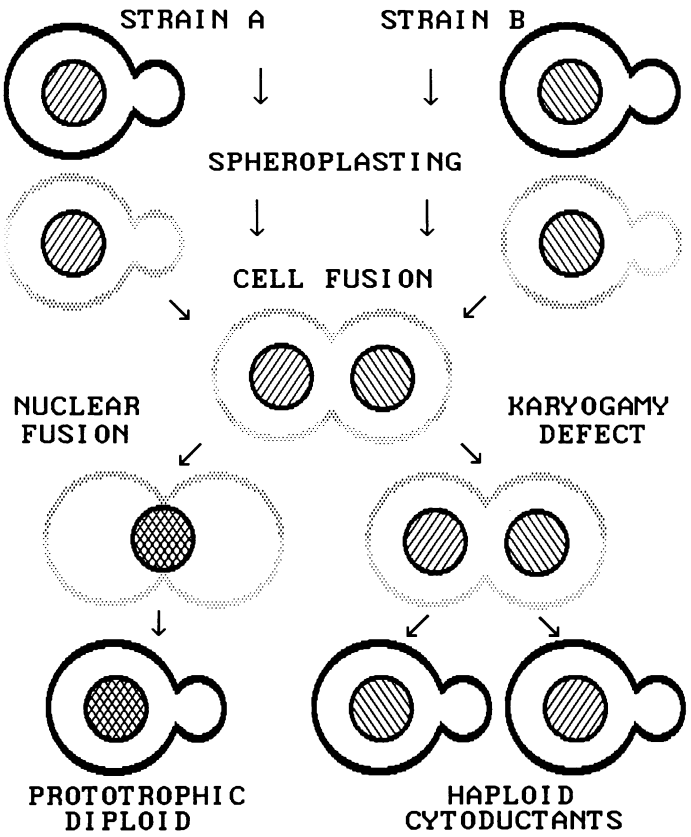


FIG. 1. Spheroplast fusion assay. See text for details.

## RESULTS

**Spheroplast fusion assay.** Cell fusion can have two different outcomes. If the haploid nuclei fuse, the resulting nucleus will be diploid and, if appropriately marked genetically, prototrophic. Growth of the prototrophic diploid will lead to the formation of a macroscopic colony on selective minimal medium. If nuclear fusion fails, the fused cells will be transiently prototrophic heterokaryons that can divide on the minimal medium until the individual nuclei segregate into different daughter cells. The progeny of the transient heterokaryons should be a mixture of cells of both haploid nuclear genotypes containing cytoplasmic elements from both parents.

Our standard spheroplast fusion assay is depicted in Fig. 1. Each of the strains to be fused has a different set of nonreverting auxotrophic mutations. The nonreverting mutations include a deletion mutation in the *HIS4* gene (10), a Ty1 insertion mutation in the *URA3* gene (26), a pair of mutations in the *LEU1* and *LEU2* genes, and a double mutation in the *LEU2* gene (15). Both strains are the same mating type, *MAT***a**, to prevent mating. The cells are treated separately with Glusulase to remove their cell walls. Cell fusion is promoted by mixing the spheroplasts together and treating them with $CaCl_2$ and polyethylene glycol (PEG). The fusion mixture and appropriate dilutions are then added to regeneration agar and plated onto rich medium to measure the frequency of regeneration and minimal medium to select cells that have become prototrophic.

TABLE 2. Effect of alpha factor treatment on prototroph formation

| Strain A | Phenotype | Strain B | Phenotype | Spheroplasting | Alpha factor | No. of prototrophs[a] |
|---|---|---|---|---|---|---|
| MY573 | Kar+ | MY577 | Kar+ | + | − | 1.0 |
| MY573 | Kar+ | MY577 | Kar+ | + | + | 1,500 |
| MY573 | Kar+ | MY577 | Kar+ | − | + | 0.01 |
| MY573[a] | Kar+ | MY577[b] | Kar+ | − | + | <0.01 |
| MY573 | Kar+ | | | + | + | <0.1 |
| | | MY577 | Kar+ | + | + | <0.1 |

[a] Expressed as number of prototrophs per 100,000 regenerating cells, where the frequency for the more poorly regenerating parent is used in the denominator.

[b] Cells were treated with 0.1 M lithium acetate prior to mixing instead of with Glusulase.

In practice, the diploids can be easily distinguished on the basis of colony size; the diploids form large colonies and the heterokaryons form very small colonies. In the selective medium, the transient heterokaryons give rise to small colonies whose size is determined by the number of generations prior to segregation of the nuclei and the length of time that the cells remain phenotypically prototrophic (19). In some experiments, prior to the formation of spheroplasts, the cells were prearrested by treatment with the peptide mating hormone alpha factor by holding temperature-sensitive cell division cycle (*CDC*) mutants at their nonpermissive temperature or by a combination of both alpha factor treatment and temperature arrest.

**Alpha factor-arrested cells.** When mitotically growing cells were converted to spheroplasts and mixed in our fusion protocol, the frequency of nuclear fusion, as assayed by prototroph formation, was quite low (Table 2). About 1 prototroph was formed per 100,000 of the cells able to regenerate. Regeneration was typically between 1 and 10%. In contrast, when cultures were first treated with the mating hormone alpha factor, the frequency of prototroph formation was increased over 1,000-fold. In some experiments, as many as 10% of the regenerating cells were prototrophic. The prototrophs fused their nuclei, as shown by the stability of the new phenotype after many generations of growth on nonselective medium and subsequent genetic analysis (next section). Control experiments in which cultures were not mixed showed that alpha factor treatment did not simply increase the reversion frequency. When cells were treated with the mating hormone and mixed in regeneration agar without prior conversion to spheroplasts, prototroph formation was very low. Therefore, prototroph formation is dependent on both alpha factor arrest and spheroplast formation. This combination of treatments bypasses a normal requirement for mating, that intact cells be of opposite mating type for fusion to occur. In our assay, lithium acetate treatment (16), which, like spheroplast formation, makes cells competent for DNA-mediated transformation, did not make intact cells competent for fusion.

**Prototrophs are true diploids.** The prototrophs produced by fusion of alpha factor-treated spheroplasts were analyzed to determine their ploidy, because genetic events other than nuclear fusion could give rise to prototrophy. One event which has been reported to produce prototrophs without nuclear fusion is chromosome transfer from one nucleus to another (6, 20). The prototrophs resulting from this type of chromosome transfer are basically haploid (with one or more extra chromosomes), whereas true nuclear fusants should be diploid. Haploids resulting from chromosome transfer can be distinguished from diploids by mating the strains in question to a haploid and a diploid (homozygous at the mating type locus) strain and subsequently analyzing the viability of the spores produced. If the prototroph is a diploid, mating to the haploid will produce a triploid and mating to the diploid will produce a tetraploid. If the prototroph is a haploid, mating to the haploid will produce a diploid and mating to the diploid will produce a triploid. In *S. cerevisiae*, both diploids and tetraploids give spores whose viability approaches 100%, whereas triploids give spores whose viability is below 10%.

Fifty-one prototrophic colonies were picked and mated to diploid strain L455 (MATα/MATα *lys1-1*/*lys1-1*) and haploid strain L94 (MATα *lys1-1*). Twelve prototrophic strains were derived from $KAR^+$ by $KAR^+$ fusions, 12 were from $KAR^+$ by *kar1-1* fusions, and 27 were from *kar1-1* by *kar1-1* fusions. Forty-six of the strains gave good spore viability when mated with the α/α tester strain but poor spore viability when mated with the α tester strain. The remaining five strains gave good spore viability when mated with either of the two tester strains and are probably of higher ploidy than diploid. None of the strains tested were haploid. Thus, the principal prototrophic product of spheroplast fusion is a diploid nucleus arising from nuclear fusion. Prototroph formation will be referred to throughout the remainder of this paper as nuclear fusion. Occasionally, spheroplast fusion produced cells of higher ploidy, possibly by fusion among more than two parents. Conjugation without the spheroplast protocol gives rise to cells with more than two parents at a frequency of less than 1 in 10,000 matings (25).

**Cell cycle-synchronized cells.** The large stimulation in nuclear fusion in cells treated with alpha factor is consistent with two different models: (i) nuclear fusion is activated by mating or (ii) nuclear fusion requires that the mating pairs be synchronized at the same point of the cell cycle. To address this issue, we synchronized cells at G1 using conditions other than alpha factor treatment and determined whether G1 arrest per se was sufficient to promote nuclear fusion.

G1 arrest was achieved with temperature-sensitive mutations in the *CDC28* gene. At the nonpermissive temperature, cells with a temperature-sensitive mutation in *CDC28* arrest in the cell cycle at a point thought to be identical to that at which cells treated with alpha factor arrest (14, 23). Cells arrested at the *cdc28* block retain mating competence at the nonpermissive temperature (14, 23, 24). During conjugation at the nonpermissive temperature, the *cdc28* mutation measurably increases the frequency of cytoductant formation, although the frequency of nuclear fusion is not appreciably diminished (7). Our assay is insensitive to small changes in the frequency of nuclear fusion which can result in large increases in the frequency of cytoductant formation.

The effect of the *cdc28* mutation on nuclear fusion in the spheroplast matings was tested in a variety of ways. We first performed a control fusion in which spheroplasts were prepared from *cdc28* cells preincubated at both high and low temperatures and then treated with alpha factor at high and low temperatures. This experiment determined whether the *cdc28* mutation had any deleterious effect on nuclear fusion in our spheroplast fusion assay. The results of this experiment (Table 3) show that the temperature sensitivity of the *cdc28* mutation had no deleterious effect on nuclear fusion. It should be noted that the *cdc28* mutant strains fused nuclei with lower efficiency than wild-type strains even at 24°C (Table 3, compare line 7 with line 3). Preincubation of the mutant strains at 34°C (line 8) resulted in a slight reduction in nuclear fusion, but even wild-type cells showed reduced fusion at 34°C than at 24°C (lines 3 and 4). Furthermore, the relative level of reduction in the *cdc28* fusions at 34°C was

comparable to that found in the wild type. This suggests that the lowered efficiency of nuclear fusion at all temperatures in the strains containing *cdc28* is due to differences in the genetic background rather than to the *cdc28* mutation per se.

The stimulation of nuclear fusion by alpha factor treatment of *cdc28* strains suggests that the *cdc28* mutation does not block nuclear fusion in the spheroplast fusion assay. To determine whether synchronization with the *cdc28* mutation was equivalent to alpha factor treatment, we followed nuclear fusion between two *cdc28* mutant strains at 34°C in the absence of alpha factor. Cell cycle arrest at 34°C failed to stimulate fusion in the absence of alpha factor (Table 3, compare line 6 with lines 5 and 8), suggesting that synchronization by *cdc28* cannot replace activation by alpha factor.

A second method for testing the effect of the *cdc28* mutation involved temperature arrest of only one of the parents. In our standard spheroplast assay, treatment of only one of the parents with alpha factor was sufficient to stimulate nuclear fusion. In fact, nuclear fusion after stimulation of one parent was reduced only 10-fold compared with that after treatment of both parents (Table 3, line 9). Therefore, it was important to test whether the arrest of one *cdc28* parent would, like alpha factor treatment of one parent, stimulate nuclear fusion. In these experiments, wild-type cells were used as the other parent. In one series of experiments, the wild-type parent was pretreated with alpha factor. As can be seen in Table 3, nuclear fusion was not stimulated when strains carrying the *cdc28* mutation were arrested at the nonpermissive temperature (compare line 12 with line 11). When the wild-type parent was not pretreated with alpha factor, a very modest increase in nuclear fusion was observed when the *cdc28* parent had been held at the high temperature (compare line 14 with lines 13 and 15). However, this increase was close to the limit of the sensitivity of the assay and is not comparable with the large increase observed when fusion was stimulated by alpha factor.

Two other means of synchronizing cells in the G1 phase of the cell cycle were used to determine whether cell cycle arrest or synchronization is sufficient to induce nuclear fusion. In one experiment, *cdc35* was used to synchronize cells. The *cdc35* mutation arrests cells just prior to the *cdc28*-dependent step. In another experiment, synchronization was achieved by allowing wild-type cells to grow to stationary phase. Cells in stationary phase are thought to be in a G1-like state (22). Experiments with *cdc35* show that cells containing this mutation are not activated for nuclear fusion in the spheroplast fusion assay. As can be seen in Table 4, incubation at the nonpermissive temperature did not stimulate nuclear fusion (compare line 5 with line 3). In contrast to the observation with the *cdc28* mutations, the *cdc35* strains gave only low levels of nuclear fusion at the permissive temperature. When the temperature-arrested cells were subsequently treated with alpha factor at the high temperature, the level of nuclear fusion was substantially reduced. This result is consistent with a *cdc35* arrest point prior to the *cdc28* and alpha factor arrest points or a requirement for *CDC35* to obtain the alpha factor response.

Similar experiments with stationary-phase cells suggest that such cells are not activated for nuclear fusion in the absence of treatment with alpha factor. Table 5 shows the results of the spheroplast fusion assay with stationary-phase cells. Spheroplasts prepared from stationary-phase cells (more than 90% of cells unbudded) were no more efficient in the production of prototrophs than were spheroplasts prepared from logarithmically growing cultures (Table 5, compare line 5 with line 1). When the stationary-phase cells were diluted directly into medium with alpha factor, they became competent for nuclear fusion (line 6). The lower level of activation observed for the stationary-phase cells probably reflects the long lag time required for stationary-phase cells to emerge from arrest and become responsive to alpha factor (only 50% of the stationary-phase cells formed shmoos in the same time that over 90% of logarithmically growing cells had

TABLE 3. *cdc28*-arrested cells are not activated for nuclear fusion

| Line no. | Strain A[a] | Temp (°C)[b] | Alpha factor | Strain B[c] | Temp (°C) | Alpha factor | No. of prototrophs[d] |
|---|---|---|---|---|---|---|---|
| 1 | *CDC*$^+$ | 24 | − | *CDC*$^+$ | 24 | − | 1.5 |
| 2 | *CDC*$^+$ | 34 | − | *CDC*$^+$ | 34 | − | <0.2 |
| 3 | *CDC*$^+$ | 24 | + | *CDC*$^+$ | 24 | + | 7,800 |
| 4 | *CDC*$^+$ | 34 | + | *CDC*$^+$ | 34 | + | 1,600 |
| 5 | *cdc28* | 24 | − | *cdc28* | 24 | − | 0.6 |
| 6 | *cdc28* | 34 | − | *cdc28* | 34 | − | 0.4 |
| 7 | *cdc28* | 24 | + | *cdc28* | 24 | + | 280 |
| 8 | *cdc28* | 34 | + | *cdc28* | 34 | + | 120 |
| 9 | *CDC*$^+$ | 24 | − | *CDC*$^+$ | 24 | + | 1,400 |
| 10 | *CDC*$^+$ | 34 | − | *CDC*$^+$ | 24 | + | 1,000 |
| 11 | *cdc28* | 24 | − | *CDC*$^+$ | 24 | + | 120 |
| 12 | *cdc28* | 34 | − | *CDC*$^+$ | 24 | + | 76 |
| 13 | *cdc28* | 24 | − | *CDC*$^+$ | 24 | − | <0.2 |
| 14 | *cdc28* | 34 | − | *CDC*$^+$ | 24 | − | 7.5 |
| 15 | *CDC*$^+$ | 34 | − | *CDC*$^+$ | 24 | − | 1.0 |

[a] *CDC*$^+$, MY416; *cdc28*, MY415.
[b] Temperature refers to the temperature of incubation prior to spheroplast fusion.
[c] *CDC*$^+$, MY418; *cdc28*, MY419.
[d] See Table 2, footnote *b*.

TABLE 4. *cdc35*-arrested cells are not activated for nuclear fusion[a]

| Line no. | Strain A[b] | Temp (°C) | Alpha factor | Strain B[c] | Temp (°C) | Apha factor | No. of prototrophs |
|---|---|---|---|---|---|---|---|
| 1 | *CDC*$^+$ | 34 | − | *CDC*$^+$ | 34 | − | 0.2 |
| 2 | *CDC*$^+$ | 34 | + | *CDC*$^+$ | 34 | + | 4,800 |
| 3 | *cdc35* | 24 | − | *cdc35* | 24 | − | <0.1 |
| 4 | *cdc35* | 24 | + | *cdc35* | 24 | + | 7 |
| 5 | *cdc35* | 34 | − | *cdc35* | 34 | − | <0.1 |
| 6 | *cdc35* | 34 | + | *cdc35* | 34 | + | 0.3 |

[a] See Table 2, footnote *b*, and Table 3, footnote *b*.
[b] *CDC*$^+$, 67-1D; *cdc35*, 67-12D.
[c] *CDC*$^+$, 67-3C; *cdc35*, 67-11B.

TABLE 5. Stationary-phase cells are not activated for nuclear fusion[a]

| Line no. | Strain A | Alpha factor | Strain B | Alpha factor | No. of prototrophs[b] |
|---|---|---|---|---|---|
| 1 | Log | − | Log | − | 0.1 |
| 2 | Log | + | Log | + | 5,500 |
| 3 | Log | + | Log | − | 480 |
| 4 | Log | − | Log | + | 730 |
| 5 | Sat | − | Sat | − | <0.1 |
| 6 | Sat | + | Sat | + | 350 |
| 7 | Log | + | Sat | − | 48 |
| 8 | Sat | − | Log | + | 3 |
| 9 | Log | + | Sat | + | 4,600 |
| 10 | Sat | + | Log | + | 1,200 |

[a] Strains used: Strain A, 67-1D; Strain B, 67-3C. Sat, Saturated cultures that had been grown for 2 days in YPD liquid medium at 30°C. Log, Logarithmically growing cultures.
[b] See Table 2, footnote *b*.

TABLE 6. *KAR1* is required for nuclear fusion during spheroplast fusion[a]

| Line no. | Strain A | Strain B | No. of prototrophs |
|---|---|---|---|
| 1 | *KAR*$^+$ | *KAR*$^+$ | 1,200 |
| 2 | *KAR*$^+$ | *kar1-1* | 120 |
| 3 | *KAR*$^+$ | *kar1-1*(YCp50-*KAR1*) | 890 |
| 4 | *kar1-1* | *KAR*$^+$ | 120 |
| 5 | *kar1-1*(YCp50-*KAR1*) | *KAR*$^+$ | 800 |
| 6 | *kar1-1* | *kar1-1* | 120 |

[a] Strain A: *KAR*$^+$, MY573; *kar1-1*, MY578; *kar1-1*(YCp50-*KAR1*), MY348. Strain B: *KAR*$^+$, MY577; *kar1-1*, MY575; *kar1-1*(YCp50-*KAR1*), MY346. All strains were treated with alpha factor prior to spheroplast fusion. Prototroph frequency is expressed as in Table 2, footnote *b*, except that the frequency is corrected for the percentage of cells containing the plasmid (80%).

formed shmoos). Moreover, treatment of one of the stationary-phase parents with alpha factor gave the same frequency of nuclear fusion as treatment of both stationary-phase parents with alpha factor (Table 5, compare lines 7 and 8 with lines 6, 9, and 10). Thus, stationary-phase cells are not activated for nuclear fusion.

***KAR1* is required for nuclear fusion.** One criterion for determining whether the spheroplast fusion assay measures a nuclear fusion process akin to that which occurs during normal heterosexual mating is whether mutations that disrupt nuclear fusion when intact cells are mated also disrupt nuclear fusion in the spheroplast assay. When one of the parent strains contained the *kar1-1* mutation, nuclear fusion was reduced to about 10% of the value obtained for experiments in which both parents were *KAR1*$^+$ (Table 6, compare lines 2, 4, and 6 with line 1). This reduction by *kar1-1* in the spheroplast assay was the same relative decrease as was observed for diploid formation with intact cells. Significantly, when the *kar1-1* parent was transformed with a plasmid carrying the wild-type *KAR1* gene, the high levels of nuclear fusion typical of *KAR1* × *KAR1* fusions were restored (lines 3 and 5), indicating that the reduction was the result of the *kar1-1* mutation. The *kar1-1* defect was unilateral in the spheroplast assay just as it is during the mating of intact cells. Thus, the *KAR1* gene product is required for nuclear fusion in both activated spheroplasts and intact cells.

**Formation of unstable heterokaryons.** In the absence of alpha factor treatment, more than 99% of the colonies that appeared on the selective plate were very small (microcolonies). These microcolonies were composed of small clusters of about 100 or more cells and were readily visible under a dissecting microscope. The microcolonies were not formed if the complementing spheroplasts were not first mixed together during the fusion procedure. The microcolonies were reduced to only 5% of the total when the cells were pretreated with alpha factor, while the frequency of the large prototrophic colonies (resulting from nuclear fusion) was increased more than 1,000-fold. When *kar1-1* spheroplasts were pretreated with alpha factor, the frequency of microcolonies increased 10- to 20-fold relative to isogenic *KAR1* spheroplasts, whereas the frequency of large prototrophic colonies was reduced about 10- to 20-fold.

The results of these studies made it likely that the microcolonies arose from the unstable heterokaryons resulting from fusion of cells without concomitant nuclear fusion. Such fusions would produce heterokaryons containing the separate nuclei from each parent and a mixed cytoplasm. To test this hypothesis, spheroplast fusion was performed on cells in which one of the parents contained mitochondria that had been marked genetically with an erythromycin resistance mutation. Sixty-one microcolonies were picked from the surface of the selective regeneration agar and purified nonselectively. The genotype of one randomly selected clone from each microcolony was determined. Twenty-eight of the clones had the parental genotype, whereas 33 of the clones were recombinant; the recombinant clones had the nuclear genotype of one parent and the mitochondrial genotype of the other parent. Thus, the microcolonies picked showed essentially random segregation of the mitochondria with respect to the nuclei. These data show that the microcolonies arise from heterokaryons that contain the unfused nucleus in a mixed cytoplasm.

In the spheroplast fusion assay, after treatment with alpha factor, the total number of CFU (counting both microcolonies and large prototrophic colonies) was the same regardless of whether the strains were *KAR1* or *kar1-1*. This result is important because it shows that *kar1-1* affects only the frequency of nuclear fusion and not the frequency with which spheroplasts fuse. Svoboda (28) measured the frequency of spheroplast fusion in a protocol similar to ours (but without alpha factor treatment) to be between 1 and 10% of the input spheroplasts. This frequency is identical to the frequency of prototroph formation we obtained after alpha factor treatment. These results indicate that alpha factor treatment affects the frequency of nuclear fusion and not the frequency of spheroplast fusion.

## DISCUSSION

Our analysis of nuclear fusion during spheroplast fusion indicates that the nuclei of mitotically growing cells are not ordinarily competent to fuse. Nuclei gain the ability to fuse after cells have been arrested by treatment with the mating hormone alpha factor. The level of nuclear fusion after treatment with alpha factor was at least 1,000-fold higher than that found when untreated spheroplasts were used. After treatment with alpha factor, as many as 10% of the regenerated spheroplasts had fused nuclei. The requirement for activation by alpha factor explains the observation of previous workers that untreated spheroplasts can be made to fuse at high frequency, whereas nuclear fusion occurs at low frequency (12, 28, 29). In the absence of alpha factor, spheroplasts fused to form unstable heterokaryons. The two nuclei constituting these heterokaryons failed to fuse in subsequent divisions.

Alpha factor-induced spheroplast fusion mimics many of the properties of normal mating. Fusion is dependent on entry into the mating pathway, and the major products of the reaction are diploids. The spheroplast fusion system, like the mating of intact cells of opposite mating type, is dependent on the *KAR1* gene to the same extent. Moreover, when spheroplasts fuse and karyogamy fails, the products of the fusion reaction are unstable heterokaryons, just as they are in *kar1-1* crosses. The spheroplast fusion system is a good way to transfer mitochondria when cells are not treated with mating hormone (12), and it is an efficient way to produce **a**/**a** diploids when mating hormone is used. The spheroplast fusion system should therefore provide an efficient method for mating sterile mutants (11) that have defects in the pathway of cell fusion but still respond to mating hormones.

The fact that activated nuclei fused regardless of their mating type indicates that during mating, sexual identity is solely a cell surface phenomenon and not a nuclear phenomenon; that is, once the barrier to cell fusion has been overcome, activated homosexual nuclei fuse with an efficiency similar to that found in heterosexual crosses. This

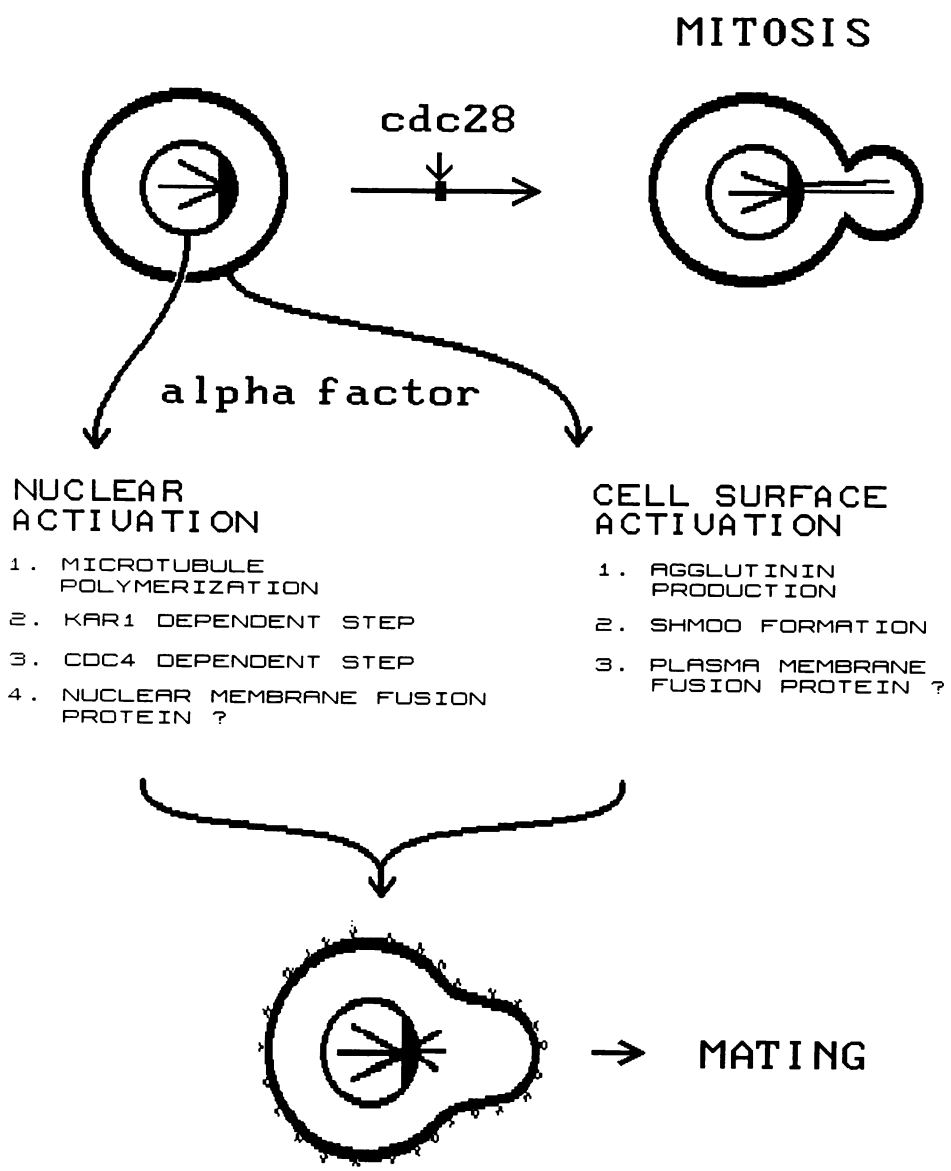


FIG. 2. Mating pathway diverges from the mitotic cell cycle. Haploid cells in the G1 portion of the cell cycle can enter either of two different developmental pathways. In the absence of mating hormone the cell can enter the mitotic cell cycle; in the presence of the appropriate mating hormone the cell can enter the mating pathway. The mating pathway is depicted as having two branches, one specific for preparing the cell for fusion and the other specifically preparing the nucleus for fusion. The pathways are separated because of the existence of mutations (*kar1*, *cdc4*, and *tub2*) that block nuclear fusion but not cell fusion during mating. Some of the functions assumed to be part of the different pathways are conjectural, such as the existence of specific fusion proteins for nuclear and plasma membrane fusion. Cells arrested by the *cdc28* mutation are in a portion of the cell cycle at which they can enter the mating pathway but are not in the pathway. Cells that have been arrested with alpha factor would reenter the cell cycle via the *cdc28*-dependent step. This configuration accounts for physiological differences between *cdc28*-arrested cells and alpha factor-arrested cells but allows for the equivalence of the two arrests indicated by reciprocal shift experiments (14).

result can be understood by supposing that the nuclei of both mating types become activated for nuclear fusion in a parallel fashion during mating. The alternative model, that nuclei become activated in a complementary fashion, so that different mating types are required for efficient fusion, is not supported by our data. The fact that all of the known mutations that prevent nuclear fusion do so regardless of the mating type of the parent also supports the homologous model.

Induction of nuclear fusion is not simply a consequence of synchronization of two nuclei in the cell cycle, nor is it solely a result of arrest in the G1 portion of the cell cycle. Cells which had been synchronized by growth to saturation or by preincubation of *cdc28* or *cdc35* mutants at high temperature were not activated for nuclear fusion. This result is of particular importance with respect to *cdc28*, whose point of arrest has been shown to be identical to the point of arrest of alpha factor. We suggest therefore that *cdc28* arrest and alpha factor arrest may not represent the same point of the cell cycle or equivalent physiological states. This point of view is supported by the observation that although *cdc28*-arrested cells resembles shmoos morphologically, they are

not induced for agglutination or other mating-specific functions. The apparent equivalence of the *cdc28* and alpha factor arrests as revealed by reciprocal shift experiments (14) can be explained by supposing that the *CDC28* step represents a branch point in the cell cycle. From this branch point, cells may either continue on into the normal mitotic cycle or, if the cell has been exposed to mating hormones, enter into a mating-specific pathway. A model depicting the relationship between the mating pathway and the cell cycle is shown in Fig. 2.

Harashima et al. (13) reported that spheroplast fusion accompanied by nuclear fusion is an obligate event during spheroplast transformation. We have not observed this phenomenon in our system (data not shown). The transformants we obtained by the spheroplast transformation procedure (15) showed no evidence of nuclear fusion. Transformation was not stimulated by pretreatment with mating hormone, and nuclear fusion was not stimulated by the presence of DNA in our spheroplast fusion assay. Our assay only detects fusion between genetically different spheroplasts and does not detect diploidization associated with self-fusion or spontaneous endomitosis. Differences in strains or subtle differences in transformation procedures may be responsible for the difference between our results and those of Harashima et al. (13).

The finding that nuclear fusion must be induced during mating explains some of the properties associated with the *kar1-1* mutation. This mutation is recessive, yet the wild-type gene product fails to complement the mutation in zygotes. The inability of the *kar1-1* nucleus to fuse persists from the time of the initial mating through subsequent mitotic divisions of the zygote. Nevertheless, Dutcher and Hartwell (8) showed that the wild-type gene product is accessible to the mutant nucleus by demonstrating that *kar1-1* cytoductants become transient $Kar^+$ phenocopies. Therefore, any model of nuclear fusion must explain both the initial failure of nuclear fusion and the persistence of the failure.

One explanation for the requirement that only one parent be mutant for fusion to fail is that *KAR1* acts prior to the point at which cells fuse during conjugation (4, 7–9). Wild-type function present after cell fusion would then be necessarily irrelevant for nuclear fusion in that cell cycle. The spheroplast fusion assay bypasses conjugation and permits cells to fuse prior to the point at which they fuse during normal mating. Therefore, the assay provides information about the timing of the functions induced for nuclear fusion. In spheroplast fusion, as in the mating of intact cells, the *kar1-1* mutation blocks nuclear fusion if only one parent is mutant, implying that the *KAR1* gene product must act prior to or during alpha factor treatment and certainly prior to the point at which cell fusion occurs. Our observation that nuclear fusion occurs only in response to alpha factor could explain the absence of nuclear fusion during subsequent divisions in the presence of the wildtype *KAR1* gene product. Regardless of the success or failure of nuclear fusion, the zygote is phenotypically identical to an **a**/α cell (capable of sporulation [17] and unable to mate). Our model suggests that nuclear fusion does not occur in subsequent mitotic cycles because an **a**/α heterokaryon, like an **a**/α diploid, cannot enter the mating pathway, a prerequisite for activation of the nucleus for nuclear fusion.

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