A mutant of *Saccharomyces cerevisiae* defective for nuclear fusion

(yeast/heterokaryon/defective zygote)

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ABSTRACT   A mutant unable to fuse nuclei during mating has been isolated from standard wild-type *Saccharomyces cerevisiae*. Tetrad analysis of the mutation responsible for this defect (*kar*1-1) shows that it segregates as a single Mendelian factor. The defect in *kar*1-1 appears to be nuclear limited. Cytological and genetic evidence shows that in this mutant the events associated with zygote formation are normal until the point of nuclear fusion. The consequence of this defect is the formation of a multinucleate zygote which in subsequent divisions can segregate heterokaryons and haploid heteroplasmons.

The sexual cycle in haploid strains of *Saccharomyces cerevisiae* occurs by an orderly progression of several sequential events (Fig. 1): cell fusion to form zygotes, nuclear fusion to form diploids, and meiosis to form haploid organisms. In standard laboratory strains of *S. cerevisiae*, nuclear fusion follows immediately after cell fusion with no intervening cell or nuclear division. Once the nuclei have fused the zygote gives rise to diploid vegetative cells by mitotic budding. Several laboratories (1–4) have described instances where nuclear fusion seems to fail. This aberration occurs at low frequency and gives rise to "heteroplasmons," strains which contain the cytoplasmic components of both parents and the nuclear genotype of one parent.

In this report, we describe a mutation (*kar*1-1) which causes a defect in nuclear fusion. The existence of such a mutation suggests that nuclear fusion is an autonomous morphogenetic process, dissectable by genetic analysis. The *kar*1-1 mutation alters the usual sequence of morphogenetic events involved in the sexual cycle of yeast in several ways. Zygotes from a *kar*1-1 X wild-type cross form diploids at a low frequency; the majority of the zygotes from this cross segregate heteroplasmons or become heterokaryons. The production of heteroplasmons and heterokaryons by strains carrying *kar*1-1 extends the life cycle of *Saccharomyces* and provides new tools for its genetic analysis.

MATERIALS AND METHODS

Yeast Strains and Genetic Methods. The yeast strains used are JC1 (*a his4 ade2 can1 nys*^{R} [*rho*$^-$]) and GF4836-8C (*a leu1 thr1* [*rho*$^+$]) where [*rho*$^+$] stands for the presence of the cytoplasmic determinant for respiratory ability. The [*rho*$^-$] strains lack mitochondrial function and are unable to grow on nonfermentable carbon sources like glycerol and ethanol. The genetic methods and nomenclature are those described in the Cold Spring Harbor yeast course manual (5), unless otherwise stated.

Culture Media. *Complete medium:* yeast extract 1%, peptone 2%, and glucose 2%. *Minimal medium:* yeast nitrogen base without amino acids (Difco) 0.7%; glucose 2%. *Selective medium:* minimal medium supplemented with histidine 0.3 mM
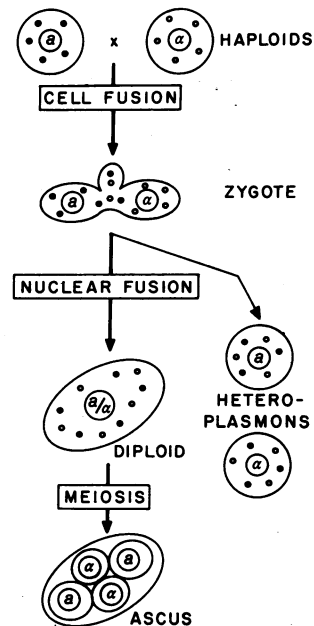
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FIG. 1. The sexual cycle in *Saccharomyces cerevisiae*. The two alleles of the mating type locus are *a* and *α*. The large circles represent cells and the small ones which encircle the mating type genotypes represent nuclei. The black and white dots represent mitochondria from one or the other of the two haploid parents. The arrow going from the zygote to the heteroplasmons is thinner as an indication of the low frequency of this event in wild type.

and adenine 0.15 mM, and with 3% glycerol plus 0.1% glucose instead of 2% glucose as carbon source. This medium was buffered with 0.1 M citrate-phosphate buffer at pH 6.5, added as a 10-fold concentrated solution after sterilization. Canavanine sulfate (60 mg/liter) was added after sterilization, and nystatin (2 mg/liter of Squibb Mycostatin) when the medium had cooled to approximately 50°.

To supplement auxotrophic requirements, we added histidine 0.3 mM, adenine 0.5 mM, leucine 2 mM, or threonine 1 mM to minimal medium. All the media were solidified with 2% agar.

Mutagenesis. Ethyl methanesulfonate mutagenesis was carried out as described by Fink (6).

Selection of Strains Defective for Nuclear Fusion. The standard cross used to select strains defective for nuclear fusion was JC1 X GF4836-8C. In this cross, the nucleus as well as the cytoplasm of each strain is marked with easily scored genetic traits. These two strains were mixed to permit mating and then plated on the selective medium. On this medium, the GF4836-8C parental haploid is selected against because of its nutritional requirements and drug sensitivities. The JC1 parent does not give significant growth on this medium (even though there is 0.1% glucose) because it is [*rho*$^-$]. Moreover, JC1 like other [*rho*$^-$] strains fails to revert to [*rho*$^+$]. Diploids fail to

Table 1. Genotype of zygotic clones from wild × wild and mutant × wild crosses

Cross			Genotype of the zygotic clones			
α *his4 ade2* *can1 nys*ᴿ	×	a *leu1 thr1*	Wild-type diploids	α *his4 ade2* *can1 nys*ᴿ	a *leu1* *thr1*	Mixed
JC1		GF4836-8C	29	1	0	0
P24		GF4836-8C	3	9	14	9
P24 [*rho⁻*]		GF4836-8C	1	4	9	6

Zygotes were micromanipulated from each cross, and the genotype of the viable zygotic clones determined as described in the *text*.

grow because they are heterozygous for the two recessive resistance markers *can1* and *nys*ᴿ, and therefore sensitive to both canavanine and nystatin. It is then possible to select for heteroplasmons with the nuclear genotype of JC1 and the mitochondrial genotype of GF4836-8C.

To find mutants, we adopted the following experimental protocol. Strain JC1 was treated with ethyl methanesulfonate and allowed to grow for 2–3 generations to allow the expression of potential mutations. A mating mixture was then prepared by mixing 10⁸ cells per ml of JC1 with 10⁹ cell per ml of GF4836-8C in water. Aliquots (0.2 ml) of the mixture were spread on the surface of complete medium plates, and the cells allowed to mate for 20–50 hr at 30°. The cells from each mating plate were then resuspended in 1 ml of water, diluted, and 0.2 ml of the 1:10 dilutions spread on the surface of selective medium plates. After 5–15 days at 30°, the colonies able to grow on the selective plates were isolated and tested. Most of the colonies were α His⁻ Ade⁻ Canᴿ Nysᴿ [*RHO⁺*] (heteroplasmons with the JC1 nucleus and the GF4836-8C mitochondria). These heteroplasmons were considered putative mutants defective for nuclear fusion and were characterized further.

Staining of Nuclei. Fixed cells were stained with Giemsa according to the procedure of Robinow as modified by Hartwell (7). Fresh preparations of cells were stained with mithramycin according to the procedure of Slater (8). Mithramycin was a gift from Nathan Belcher of Pfizer Inc., Groton, Conn.

RESULTS

Selection of mutants defective for nuclear fusion

Strain JC1 was mutagenized with ethyl methanesulfonate, crossed with strain GF4836-8C, and plated on selective medium to isolate heteroplasmons harboring the JC1 nucleus and the GF4836-8C mitochondria, according to the protocol described in *Materials and Methods*.

The heteroplasmons isolated by this procedure were tested for the presence of a mutation preventing nuclear fusion by determining their ability in subsequent matings to generate heteroplasmons at a frequency higher than the parental strain JC1. Every heteroplasmon was converted to [*rho⁻*] by ethidium bromide treatment (5) and crossed to strain GF4836-8C in an experiment formally identical to the cross in which these heteroplasmons were selected; a control cross JC1 × GF4836-8C was included in every experiment. Several heteroplasmons gave rise to α His⁻ Ade⁻ Canᴿ Nysᴿ [*rho⁺*] segregants at a frequency at least 10 times higher than that of the control cross. One of them, strain JCX1 P24 (abbreviated P24), was particularly promising because it gave heteroplasmons at 1000-fold higher frequency than the control cross.

Mating behavior of P24

Crosses of the mutant strain P24 show that heteroplasmons are formed as a direct consequence of mating and not by any other

events. Both P24 and its petite derivative P24 [*rho⁻*] were crossed by strain GF4836-8C. The frequency of formation of zygotes (approximately 20%), as observed under the microscope, was as high as in control JC1 × GF4836-8C crosses, and the morphology of the zygotes appeared normal. Typical zygotes, with a clearly visible, central zygotic bud, were isolated by micromanipulation and allowed to form colonies on complete medium. After 3 days at 30°, these zygotic colonies were resuspended in water, diluted, and plated on complete medium, and the phenotypes of the resulting colonies determined.

The results are shown in Table 1. In the control (wild × wild) cross all but one of the zygotic clones are wild-type diploids. By contrast, most of the clones derived from mutant × wild-type zygotes have one or the other of the two parental genotypes. Others are a mixture of parental genotypes and wild-type diploids. No recombinant types were observed. A minority of the clones derived from mutant × wild-type zygotes are wild-type diploids. These rare diploids, as will be shown later, sporulate normally and give the expected meiotic segregation of markers. In this experiment, there was no selection for heteroplasmons, and no demand was made for cytoplasmic mixing. Nevertheless, the cells with the P24 nucleus isolated from the cross P24 [*rho⁻*] × GF4836-8C were always [*rho⁺*] which shows that they were heteroplasmons arising from true zygotes.

The above facts suggest that in crosses involving the mutant strain P24 cell fusion is normal, but nuclear fusion fails with a high frequency. The failure of nuclear fusion results in the segregation of heteroplasmons harboring one or the other of the haploid parental nuclei. This segregation event seems to take place very soon after mating, because most of the zygotic clones, rather than containing equal numbers of both heteroplasmons, are homogeneous populations of one or the other type.

Comparison of the zygotic cell cycle in mutant and wild-type crosses

More direct evidence supporting the interpretation that mutant P24 is defective in nuclear fusion was obtained through cytological analysis. P24 and, as a control, JC1 were each mated to GF4836-8C. After 5 and 13 hr of mating at 30°, cells were collected, their nuclei stained with mithramycin and observed under the fluorescence microscope. Typical results obtained in this analysis are shown in Fig. 2.

The sequence of events taking place in wild × wild control zygotes is the one described by Hartwell (9) for wild-type *Saccharomyces* strains. The first zygotic bud does not start to be formed until nuclear fusion has been completed (A1). Bud formation is a morphological landmark for the initiation of the first diploid mitotic cycle, which culminates with the division of the first diploid nucleus in the bridge between zygote and daughter bud (A2), and the separation of the first diploid bud (A3). In mutant × wild crosses the zygotic cell cycle is profoundly altered. Even though nuclear fusion does not take place, the first zygotic bud is formed (B1). The two haploid, unfused
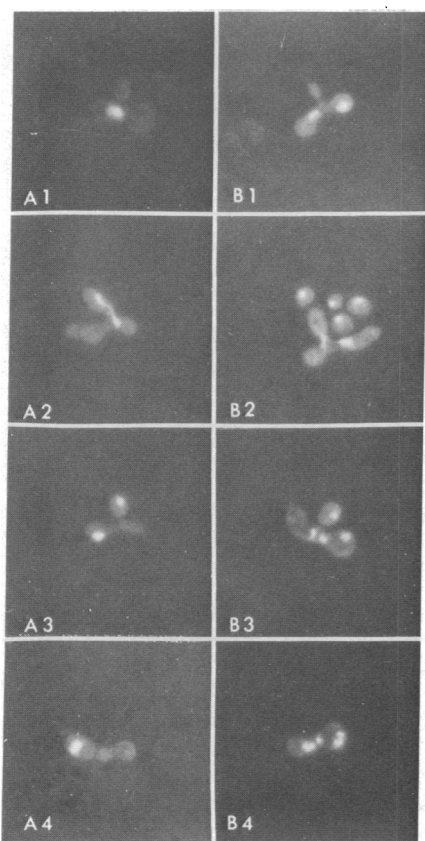
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FIG. 2.   Zygotes from wild × wild (A1 to A4) and mutant × wild (B1 to B4) crosses. JC1 × GF4836-8C (wild × wild) and P24 × GF4836-8C (mutant × wild) mating mixtures were incubated at 30° for different periods of time, and stained with mithramycin according to the procedure described in *Materials and Methods*. A1 to A3 and B1 to B3 were sampled after 5 hr of mating; A4 and B4, after 15 hr. ×1120.

nuclei undergo an apparently normal mitotic cycle which culminates usually in synchronous nuclear divisions (B2). The distribution of daughter nuclei between the zygote and the bud is not always coordinated: two daughter nuclei can migrate to the bud which results in the formation of a dikaryon. More frequently we have observed that only one of them migrates (B3), with the formation of a monokaryotic bud and trikaryotic zygote. As successive mitotic cycles take place, the difference between wild and mutant zygotes is more and more apparent. Whereas wild × wild zygotes from old mating mixtures are invariably mononucleate (A4), those from mutant × wild zygotes are polynucleate (B4). We have observed as many as 10 to 14 nuclei per zygote. We conclude from these results that mutant P24 is defective for nuclear fusion.

Meiotic analysis of *kar*1

Most of the zygotes formed when P24 is mated form heteroplasmons, but a few of them form prototrophic diploid clones (Table 1) which undergo meiosis when placed on sporulation medium. One of these diploids was sporulated and 36 four-spored tetrads were analyzed (Table 2). The segregation of known markers was typically 2:2. It would have been cumbersome to score the 36 tetrads for the nuclear-fusion-defective phenotype of P24 by looking for enhanced frequency of heteroplasmon formation. To simplify the initial analysis of tetrads, we took advantage of the fact that the *kar*1-1 mutation causes a weak response in the standard complementation test (6). This

Table 2.   Meiotic analysis of a diploid from a mutant × wild-type cross

Marker	Tetrad segregations		
	2:2	3:1	1:3
+:his⁻	35	0	1
+:ade⁻	36	0	0
+:leu⁻	36	0	0
+:thr⁻	33	2	1
+:can R	34	2	0
+:nys R	36	0	0
a:α	36	0	0
Strong:weak*	23	0	0

The cross was P24 (α *his4 ade2 can1 nys*R *kar*1-1) × GF4836-8C (a *leu*1 *thr*1). The numbers in the table refer to the number of tetrads showing a particular segregation pattern. Seventy-two tetrads were dissected; 36 of them had four viable spores, and spore viability was 83%.

* Strong:weak refers to the complementation response. The weak complementation phenotype was scored only for the 23 asci in which all four spores were auxotrophic.

difference from wild type presumably results from the inability of strains carrying the *kar*1-1 mutation to form large numbers of stable diploids which are required for vigorous growth in the complementation test. All tetrads analyzed by the complementation test segregated two weak complementers:two strong complementers (Table 2).

To confirm the correspondence between the weak complementation phenotype and enhanced frequency of heteroplasmon formation, we crossed 16 spores from four complete tetrads with wild-type haploid strains of opposite mating type, and analyzed the zygotic clones for heteroplasmons. The weak-complementing spores always formed heteroplasmons at high frequency, whereas the strong-complementing spores formed only wild-type diploids. As an additional confirmation, the four spores of a tetrad were intercrossed in all the possible combinations and the composition of the zygotic clones analyzed. The results can be seen in Fig. 3, where a 2:2 segregation is also found.

The above results show that the mutation harbored in P24 behaves as a single, Mendelian gene responsible for both the weak complementation and high frequency of heteroplasmon formation. We have named this gene *KAR*1 and the mutation in P24, *kar*1-1. *kar*1-1 is a leaky mutation, since a small fraction (5–10%) of the binucleated zygotes give rise to true diploids, which makes meiotic manipulation possible. The mutation is dominant in the binucleate zygote containing a *kar*1-1 nucleus and a *KAR*1⁺ nucleus, but is recessive in binucleate zygotes containing two diploid *KAR*1⁺/*kar*1-1 nuclei, so we denote it by small letters.

We imagined that if strains carrying *kar*1-1 were truly defective in nuclear fusion then it should be possible to select for heterokaryons arising from a *kar*1-1 × *kar*1-1 cross.

Construction of *kar*1 heterokaryons

The construction of heterokaryons was attempted by crossing *kar*1-1 strains containing complementary auxotrophic markers and plating the mating mixture on media where only diploids or complementing heterokaryons could grow. In many crosses two colony types resulted: those with a large, regular shape, and those with a small, irregular shape. The small colonies were subcloned at least three times on minimal medium by streaking and single colony isolation. Upon subcloning, these small
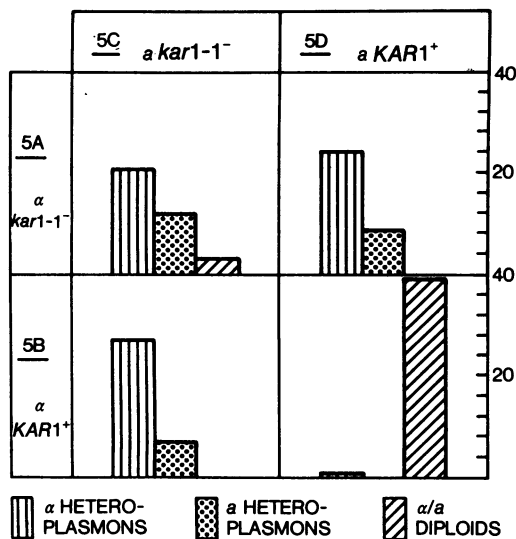
FIG. 3. The phenotype of zygotic clones from intercrosses between the spores of a tetrad resulting from the cross of *kar*1-1 × *KAR*1⁺ shown in Table 2. The four spores from tetrad 5 (5A, 5B, 5C, 5D) were intercrossed in all the possible *a* × *α* combinations. Forty zygotes were micromanipulated from each cross, and the phenotype of the viable zygotic clones determined. The terms *a* and *α* heteroplasmon mean that the clone derived from the zygote has the nuclear genotype of the *a* or *α* parent, respectively.

colonies gave rise again to two classes, small and large. The larger colonies were thought to represent prototrophic diploids or polyploids formed by random nuclear fusions within the heterokaryons; the small colonies were thought to represent heterokaryons. If this hypothesis were correct, the small colonies should segregate both parental genotypes when grown on nonselective medium. To test this prediction, we inoculated small colonies representing the initial isolates on liquid complete medium, grown until stationary phase, and then diluted and plated on complete medium so that isolated colonies could be obtained. Several clones grown in this way segregated a mixed population, some individuals showing one parental phenotype and some the other. In addition, there were a few prototrophic segregants. None of the segregants showed a reassortment of parental markers.

Additional important evidence in support of the heterokaryotic nature of the small clones arising from a *kar*1-1 × *kar*1-1 cross was obtained by visualizing the nuclei of these cells with both the Giemsa and the mithramycin stains according to the
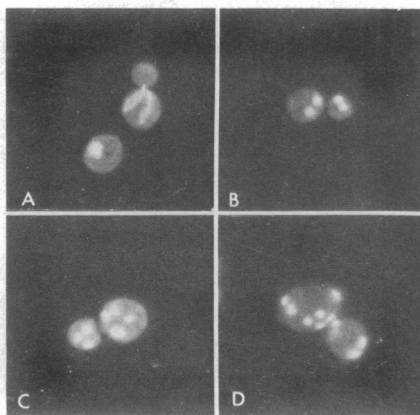

FIG. 4. *kar*1-1 × *kar*1-1 heterokaryons stained with mithramycin. In A, two nuclei are dividing synchronously. ×1120.

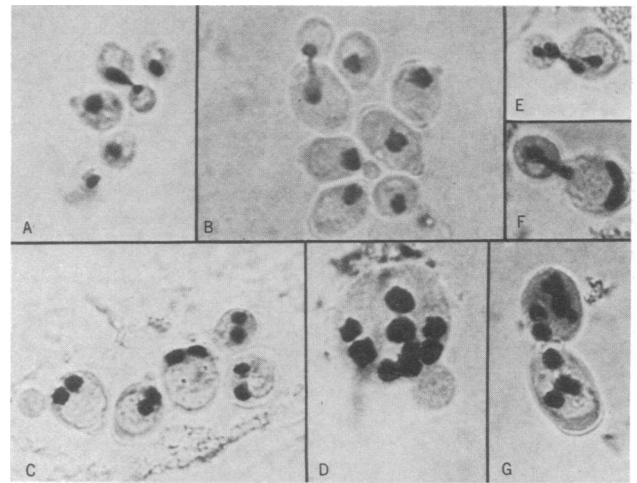FIG. 5. *kar*1-1 × *kar*1-1 heterokaryons stained with Giemsa. (A) haploid *kar*1-1 cells. (B) diploid *kar*1-1 × *kar*1-1 cells. (C-G) *kar*1-1 × *kar*1-1 heterokaryons. In (E) two nuclei are dividing synchronously in the bridge between mother and daughter cells. In (F) two nuclei are dividing synchronously, but only one is in the division bridge. In (G) only two of the four nuclei in a tetrakaryon are dividing synchronously. ×1080.

procedures mentioned in *Materials and Methods*. Some examples of the results are shown in Figs. 4 and 5. Most of the cells had from two to six nuclei. Controls of *kar*1-1 haploid or *kar*1-1/*kar*1-1 diploid cells, when stained in the same way, had only a single nucleus per cell. We conclude that the slow-growing colonies derived from these crosses are heterokaryons because (*i*) they are multinucleate and (*ii*) they segregate haploid cells manifesting the two parental genotypes.

DISCUSSION

We have designed a general procedure for the selection of mutations which during mating allow transfer of cytoplasm without concomitant nuclear fusion. Because both a mutant and a non-mutant nucleus are present in the same cell, the scheme will select only dominant or "nuclear limited" mutations. The *kar*1-1 mutation obtained by this selection fulfills these expectations. Crosses involving *kar*1-1 lead to a number of developmental outcomes which are rare or absent in standard yeast crosses. In fact, it is possible to isolate and propagate three distinct cell types from zygotes of *Kar*1⁺ × *kar*1-1 crosses: heteroplasmons, heterokaryons, and true diploids. In wild-type strains, zygote morphogenesis involves cell fusion, nuclear fusion, bud formation, and nuclear division. The existence of the *kar*1-1 mutation shows that nuclear fusion is not a necessary consequence of cell fusion. Furthermore, our results suggest that bud formation and nuclear division can proceed in the absence of nuclear fusion.

The unique aspect of the *kar*1-1 defect is that *KAR*1⁺ nuclei cannot provide the function missing in *kar*1-1 nuclei. Preliminary observations suggest that this recessive behavior of *KAR*1⁺ does not result from a general inhibition of nuclear fusion in *kar*1-1, *KAR*1⁺ zygotes. For example, some of the *KAR*1⁺ heteroplasmons are diploid or polyploid suggesting that the *KAR*1⁺ nucleus was able to fuse with a sister *KAR*1⁺ nucleus in the zygote, a result unlikely if the presence of the *kar*1-1 nucleus precluded nuclear fusion within the cell. Moreover, the fact that *KAR*1⁺/*kar*1-1 diploid nuclei fuse normally suggests that *kar*1-1 does not inhibit *KAR*1⁺ function. It is more likely that the *kar*1-1 mutation causes a nuclear-limited defect. The outstanding ultrastructural feature of zygote formation in yeast

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is the fusion of the two spindle plaques (10). It is an intriguing possibility that kar1-1 might affect the structure and function of the spindle plaques. However, these speculations should take into account the fact that kar1-1 strains show no other obvious mitotic or meiotic abnormalities.

Heterokaryons are produced as a consequence of the defect in strains carrying kar1-1. We assume that several nuclei can exist in a yeast cell only when there is a defect in nuclear fusion since normal cells fail to form heterokaryons at a detectable frequency. The cellular morphology of heterokaryons is not notably different from normal vegetative cells, although the heterokaryotic cells may be larger than haploids or diploids, their size apparently related to the number of nuclei in the cell. Some synchronization of nuclear division is present in heterokaryons, but as can be seen in Fig. 5G, this appears to be localized. Often two, three, or more adjacent nuclei can be seen dividing in one portion of the cell while another nucleus lies quiescent in a different locale, a condition suggestive of diffusible inducers of nuclear division. This mixture of synchrony and asynchrony is probably responsible for the variation in the number of nuclei per heterokaryon. The number of nuclei per heterokaryotic cell fluctuates considerably; for newly formed heterokaryons it approaches two, but in older ones it can be as high as eight to ten. Diploids emerge from heterokaryons grown on selective medium, but they appear less frequently from these multinucleate vegetative cells than they do from the binucleate zygote of a kar1-1 × kar1-1 cross.

The kar1-1 defect in nuclear fusion provides new tools for the genetic analysis of yeast: heteroplasmons and heterokaryons. Heteroplasmons can be isolated from crosses homozygous or heterozygous for kar1-1 with frequencies greater than 95%. These heteroplasmons allow an investigation of the consequences of cytoplasmic mixing without nuclear fusion. This alternative to the usual life cycle provides a potentially powerful approach to problems of cytoplasmic inheritance. For example, using heteroplasmons produced by the kar1-1 crosses we have shown that the yeast killer trait (11) is transmitted through the cytoplasm (unpublished results). Heterokaryons are of potential use for identifying nuclear limited traits—genes whose products fail to diffuse out of the nucleus. Mutations in genes controlling spindle plaques, nuclear membranes, chromosome structure, and RNA processing could be expressed in heterokaryons but not in diploids. There has already been a report of a regulatory gene whose product appears unable to affect the other nuclei in a *Neurospora* heterokaryon (12). In fact, the $KAR1^+$ gene product is an example of a nuclear limited trait. It will be interesting to contrast the expression of mutations of the cell division cycle (especially those which affect the nuclear division cycle) in heterokaryons as compared with diploids.

1. Fowell, R. F. (1951) "Hybridization of yeasts by Lindegren's technique," *J. Inst. Brew. London* 180.
2. Wright, R. E. & Lederberg, J. (1957) "Extranuclear inheritance in yeast heterocaryons," *Proc. Natl. Acad. Sci. USA* 43, 919–923.
3. Zakharov, J. A., Yurchenko, L. V. & Yarovot, B. F. (1969) "Cytoduction: The autonomous transfer of cytoplasmic hereditary factors during pairing of yeast cells," *Genetika* 5, 136–141.
4. Aigle, M. & Lacroute, F. (1975) "Genetical aspects of (URE3), a non-mitochondrial, cytoplasmically inherited mutation in yeast," *Mol. Gen. Genet.* 36, 327–335.
5. Sherman, F., Fink, G. R. & Lawrence, C. W. (1974) *Methods in Yeast Genetics* (Cold Spring Harbor Laboratory for Quantitative Biology, Cold Spring Harbor, N.Y.).
6. Fink, G. R. (1970) "The biochemical genetics of yeast," in *Methods in Enzymology*, eds. Tabor, H. & Tabor, C. W. (Academic Press, New York and London), Vol. XVIIA, pp. 59–78.
7. Hartwell, L. H., Culotti, J. & Reid, B. (1970) "Genetic control of the cell division cycle in yeast," *Proc. Natl. Acad. Sci. USA* 66, 352–359.
8. Slater, M. L. (1976) "A rapid nuclear staining method for *Saccharomyces cerevisiae*," *J. Bacteriol.* 126, 1339–1341.
9. Hartwell, L. H. (1973) "Synchronization of haploid yeast cell cycle, a prelude to conjugation," *Exp. Cell Res.* 76, 111–117.
10. Byers, B. & Goetsch, L. (1975) "Behavior of spindles and spindle plaques in the cell cycle and conjugation of S. *cerevisiae*," *J. Bacteriol.* 124, 511–523.
11. Fink, G. R. & Styles, C. (1972) "Curing of a killer factor in *Saccharomyces cerevisiae*," *Proc. Natl. Acad. Sci. USA* 69, 2846–2849.
12. Burton, E. G. & Metzenberg, R. L. (1972) "Novel mutation causing derepression of several enzymes of sulfur metabolism in *Neurospora crassa*," *J. Bacteriol.* 109, 140–151.