# *kem* Mutations Affect Nuclear Fusion in *Saccharomyces cerevisiae*

**Jinmi Kim,*,[1] Per O. Ljungdahl[†] and Gerald R. Fink*,[†]**

**Massachusetts Institute of Technology and [†]Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142*

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ABSTRACT

We have identified mutations in three genes of *Saccharomyces cerevisiae*, *KEM1*, *KEM2* and *KEM3*, that enhance the nuclear fusion defect of *kar1-1* yeast during conjugation. The *KEM1* and *KEM3* genes are located on the left arm of chromosome *VII*. *Kem* mutations reduce nuclear fusion whether the *kem* and the *kar1-1* mutations are in the same or in different parents (*i.e.*, in both *kem kar1-1* × wild-type and *kem* × *kar1-1* crosses). *kem1* × *kem1* crosses show a defect in nuclear fusion, but *kem1* × wild-type crosses do not. Mutant *kem1* strains are hypersensitive to benomyl, lose chromosomes at a rate 10–20-fold higher than *KEM*$^+$ strains, and lose viability upon nitrogen starvation. In addition, *kem1/kem1* diploids are unable to sporulate. Cells containing a *kem1* null allele grow very poorly, have an elongated rod-shape and are defective in spindle pole body duplication and/or separation. The *KEM1* gene, which is expressed as a 5.5-kb mRNA transcript, contains a 4.6-kb open reading frame encoding a 175-kD protein.

CONJUGATION in the yeast *Saccharomyces cerevisiae* occurs when cells of opposite mating type are mixed. Cells agglutinate, the cell walls separating the cells degrade, and the plasma membranes fuse to form a single cell. Nuclear fusion occurs immediately upon cell fusion resulting in the formation of a diploid zygote. The sequential events leading to nuclear fusion in newly formed zygotes have been characterized by electron microscopy (BYERS and GOETSCH 1974, 1975). Movement of the two nuclei toward one another appears to be mediated by extranuclear microtubules emanating from each spindle pole body. Nuclear fusion appears to initiate at the sites of the spindle pole bodies on the nuclear envelopes.

Mutational analysis has identified several genes that are required for efficient nuclear fusion. These include *KAR1*, *KAR2*, *KAR3* (CONDE and FINK 1976; FINK and CONDE 1976; POLAINA and CONDE 1982; ROSE, MISRA and VOGEL 1989), *TUB2* (THOMAS 1984; HUFFAKER, THOMAS and BOTSTEIN 1988), *CDC4*,*CDC37* (DUTCHER and HARTWELL 1982, 1983), and *BIK1* (BERLIN, STYLES and FINK 1990). The intracellular location of several of these gene products has been established, and it is clear that nuclear fusion requires the coordination of specific processes occurring in the endoplasmic reticulum, cytoplasm and nucleus. The ability of cells to properly coordinate these processes depends upon prior potentiation by mating pheromone (ROSE, PRICE and FINK 1986; CURRAN and CARTER 1986).

Several studies demonstrate a critical role for microtubules and spindle pole body in nuclear fusion. Functional cytoplasmic microtubules are required for nuclear fusion. Benomyl, a drug which induces depolymerization of microtubules, inhibits nuclear fusion (DELGANO and CONDE 1984). Cold-sensitive mutations in the β-tubulin gene *TUB2* (THOMAS 1984; HUFFAKER, THOMAS and BOTSTEIN 1988) block nuclear fusion. The *BIK1* gene product is a microtubule associated protein (BERLIN, STYLES and FINK 1990). *KAR3* encodes a kinesin homolog that associates with cytoplasmic microtubules (MELUH and ROSE 1990).

The *KAR1* gene product is thought to be a component of a multimeric complex, most likely the spindle pole body, because either under or over production of KAR1 results in an abnormally enlarged but unduplicated spindle pole body (ROSE and FINK 1987). *kar1* mutations result in the aberrant proliferation of both intra- and extranuclear microtubules. Overproduction of *KAR1* leads to cell cycle arrest and a spindle plaque morphology similar to that observed in arrested *cdc31* mutants (BYERS 1981). Quantitative mating studies with *kar1-1* strains have shown that the frequency of diploid formation during mating is reduced by 85–90% as compared with *KAR1* × *KAR1* crosses (CONDE and FINK 1976). Only 10–15% of Kar$^-$ zygotes from either *KAR1* × *kar1-1* or *kar1-1* × *kar1-1* crosses fuse nuclei to form fully functional diploids (*i.e.*, the *kar1-1* mutant defect is unilateral).

We isolated new mutations, called *kem* (*K*ar$^-$ *e*nhancing *m*utations), that reduce or abolish the residual nuclear fusion of the *kar1-1* mutation. These mutations define at least three genes, *KEM1*, *KEM2* and *KEM3*. Mutations in *KEM1* affect several cellular func-

The nucleotide sequence reported in this paper has been submitted to the GenBank™/EMBL Data Bank with the accession number X54717.

[1] Current address: Department of Microbiology, Chungnam National University, Daejeon 302-764, Korea.

tions in addition to nuclear fusion, leading to reduced chromosome stability and defects in spindle pole body duplication and/or separation. Furthermore, mutant *kem1* yeast strains lose viability under conditions of nitrogen starvation and homozygous diploids are unable to sporulate.

## MATERIALS AND METHODS

**Strains:** The yeast strains used in this study are listed in Table 1. Isogenic *KEM1* and *kem1-1* strains (JK204 and JK205) were derived from *kem1-1* strain JK336 by the two step gene replacement method described by BOEKE *et al.* (1987). Strain JK336 was transformed with integration plasmid pJI61, and Ura$^-$ Kem$^+$ (JK204) and Ura$^-$ kem$^-$ (JK205) strains were obtained by counterselection on medium containing 5-fluoroorotic acid. *KEM1* and *kem1-5* strains (JK190 and JK191) were derived from *kem1-5* strain JK335 by the same method. Though each pair of *KEM1* and *kem1* strains was made isogenic by transformation, the original *kem1-1* and *kem1-5* strains (JK336 and JK335) are not isogenic. Isogenic *KEM1* and *kem1* null (*kem1Δ2::URA3*, *kem1Δ3::LEU2*) strains were derived by replacing the wild-type *KEM1* segment on the chromosome with a disrupted copy of *kem1* by gene replacement (ROTHSTEIN 1983). A linear *Bam*HI-*Xho*I DNA fragment of pJI112 was transformed into *KEM1* strain JK251 to construct *kem1Δ2::URA3* strain JK245. Similarly, a linear *Bam*HI-*Xho*I DNA fragment of pJI113 was transformed into *KEM1* strain JK251 to construct *kem1Δ3::LEU2* strain JK246. [$\rho^0$] segregants were obtained by growing [$\rho^+$] strains in synthetic dextrose (SD) medium plus required amino acids containing 10 μg/ml ethidium bromide (SHERMAN, FINK and LAWRENCE 1979). Only nonsuppressive [$\rho^0$] strains were used. *Escherichia coli* strains HB101 (BOYER and ROULLAND-DUSSOIX 1969) and JM109 (YANISCH-PERON, VIERA and MESSING 1985) were used for plasmid progagation and phagemid production.

**Media and genetic analysis:** Yeast media, culture conditions and tetrad analysis were as described by SHERMAN, FINK and LAWRENCE (1979). Genetic map distances were calculated according to MORTIMER and SCHILD (1981). Cycloheximide medium is YPG containing 3% glycerol as the sole carbon source and 3 μg/ml cycloheximide. Benomyl medium is YEPD containing either 10 or 15 μg/ml benomyl. This medium was made by slowly adding a stock solution of benomyl (10 mg/ml in dimethyl sulfoxide, stored at −20°) to warm YEPD medium with vigorous swirling to prevent precipitation. Benomyl was a generous gift from E. I. Du Pont deMours and Co., Inc. Canavanine medium is synthetic complete (SC) without arginine to which 60 μg/ml canavanine sulfate has been added. Nitrogen starvation medium contained 0.17% Difco yeast nitrogen base (without amino acids and ammonium sulfate) and 2% glucose. Sporulation medium contained 1% potassium acetate with or without 0.1% glucose. Bacterial media were made as described by DAVIS, BOTSTEIN and ROTH (1980).

**Transformation and DNA manipulation techniques:** Yeast transformation was carried out by the lithium acetate method (ITO *et al.* 1983) using 50 μg of sonicated calf thymus DNA per transformation as carrier. Yeast transformants were selected by plating cells on appropriate selective media. *Escherichia coli* transformations were performed by either the procedure of MANDEL and HIGA (1970) or the method of HANAHAN (1985). Plasmid DNA from *E. coli* was obtained by the boiling lysis method (HOLMES and QUIGLEY 1981). Plasmids from yeast were isolated and passaged through *E. coli* as described by HOFFMAN and WINSTON (1987). Yeast DNA was prepared as described by BOEKE *et al.* (1985). Restriction endonuclease analysis and agarose gel electrophoresis were carried out as described in MANIATIS, FRITSCH and SAMBROOK (1982).

**Isolation of mutants:** Two schemes for isolating mutants were used. In scheme 1, strain JK77 (Kar$^+$) cells were

**TABLE 1**

**Yeast strains used**

| Strain[a] | Genotype |
|---|---|
| JK77 | *MAT***a** *ura3 leu1 ade2 can1 cyh2* |
| JK78 | *MAT***a** *ura3 ade2 can1 cyh2 kar1-1* |
| JK130 | *MATα ura3-52 his4-29 kem3-1 kar1-1* |
| JK132 | *MATα ura3-52 his4-29 kar1-1* |
| JK136 | *MATα ura3-52 his4-29 kem3-1* |
| JK137 | *MATα ura3-52 his4-29* |
| JK156 | *MATα his4-29 kem1-1* |
| JK158 | *MATα his4-29 ade2 can1 kem1-1 kar1-1* |
| JK190 | *MATα ura3-52 his4-29 cyh2* |
| JK191 | *MATα ura3-52 his4-29 cyh2 kem1-5* |
| JK196 | *MATα ura3-52 his4-29 cyh2* [$\rho^0$] |
| JK197 | *MATα ura3-52 his4-29 cyh2 kem1-5* [$\rho^0$] |
| JK200 | *MAT***a**/*MATα ura3-52/ura3-52 trp1-1/trp1-1 lys2-801/lys2-801* |
| JK204 | *MATα ura3-52 his4-29* |
| JK205 | *MATα ura3-52 his4-29 kem1-1* |
| JK217 | *MAT***a** *trp1-1 ade2 leu2-3,112 cyh2* [$\rho^0$] |
| JK218 | *MAT***a** *trp1-1 ade2 leu2-3,112 cyh2 kar1-1* [$\rho^0$] |
| JK219 | *MATα trp1-1 ade2 cyh2* [$\rho^0$] |
| JK245 | *MATα ura3-52 his4-34 leu2-3,112 kem1Δ2::URA3* |
| JK246 | *MATα ura3-52 his4-34 leu2-3, 112 kem1Δ3::LEU2* |
| JK251 | *MATα ura3-52 his4-34 leu2-3,112* |
| JK278 | *MATα ura3-52 his4-34 leu2-3,112* (pJI98) |
| JK280 | *MATα ura3-52 his4-34 leu2-3,112 kem1Δ3::LEU2* (YEp24) |
| JK282 | *MATα ura3-52 his4-34 leu2-3,112 kem1Δ3::LEU2* (pJI98) |
| JK301 | *MATα ura3-52 his4-29 ade2 cry1 kem2-1* |
| JK306 | *MATα ura3-52 lys5 kem1-1* |
| JK311 | *MAT***a** *his4-29 cyh2* |
| JK313 | *MATα his4-29 cyh2 kem1-5* [$\rho^0$] |
| JK314 | *MAT***a** *leu1 ade2 kem1-5* |
| JK315 | *MAT***a** *leu2-3,112 his1 can1* |
| JK316 | *MAT***a** *leu2-3,112 his1 can1 kem1-5* |
| JK326 | *MAT***a**/*MATα ura3-52/+ trp1-1/+ +/leu2-3,112 +/his1 +/can1* |
| JK327 | *MAT***a**/*MATα ura3-52/+ trp1-1/+ +/leu2-3,112 +/his1 +/can1 +/kem1-1* |
| JK328 | *MAT***a**/*MATα ura3-52/+ trp1-1/+ +/leu2-3,112 +/his1 +/can1 +/kem1-5* |
| JK329 | *MAT***a**/*MATα ura3-52/+ trp1-1/+ leu2-3,112/leu2-3,112 +/his1 +/can1 kem1-1/kem1-1* |
| JK330 | *MAT***a**/*MATα +/ura3-52 +/his4-29 +/cyh2 leu2-3,112/+ his1/+ can1/+ kem1-5/kem1-5* |
| JK331 | *MAT***a**/*MATα ura3-52/+ trp1-1/+ leu2-3,112/leu2-3,112 +/his1 +/can1 kem1-1/kem1-5* |
| JK335 | *MATα ura3-52 his4-29 cyh2 kem1-5* |
| JK336 | *MATα ura3-52 his4-29 leu1-1 kem1-1* |
| JK340 | *MATα his4-29 cyh2 kem1-1* |
| 6947-2B | *MATα leu2-3 lys1-1 met3* |
| 7523-6A | *MATα leu2-3 lys1-1 kar1-1* |
| 5916-6a | *MAT***a** *his4-29* |
| F760[b] | *MAT***a** *ura3-52 trp1-1 lys2-801* |

[a] Strains designated JK were constructed for this study.
[b] Obtained from D. BOTSTEIN.

mutagenized with ethyl methanesulfonate (EMS) as described by FINK (1970). Cells were diluted and spread on YEPD plates such that after incubation at 30° for 3 days 200 colonies formed per plate. Colonies were mated to a lawn of strain 6947-2B (*KAR1*) and to a lawn of strain 7523-6A (*kar1-1*). Mating plates were incubated at 30° for 4 h, and then diploids were selected by replica-plating these mating plates onto minimal SD medium. Colonies which formed diploids at the wild-type level in *KAR1* crosses but not in *kar1-1* crosses were considered putative mutants and characterized further.

In scheme 2, strain JK78 (*kar1-1*) cells were mutagenized and spread on YEPD plates as described in scheme 1, but plates were incubated at 24°. Colonies were mated to a lawn of strain 6947-2B (*KAR1*) at two different temperatures, 24° and 34°. After incubation, mating plates were replica-plated onto minimal SD media and diploids were allowed to grow at 24°. Colonies unable to form diploids at 34° were picked and rescreened by microscopic examination to eliminate steriles. Colonies capable of forming zygotes were considered putative *kem* mutants.

**Assays of cytoduction, chromosome loss and mitotic recombination:** Cytoduction assays (DUTCHER 1982) were performed using yeast strains with the desired Kem and Kar genotypes in either of the following genetic backgrounds; *MATα* Cyh$^s$[$\rho^+$] or *Mata* Cyh$^r$[$\rho^0$]. The appropriate strains were then crossed, and Cyh$^r$[$\rho^+$] cytoductants were selected on medium containing a nonfermentable carbon source and cycloheximide. The presumed cytoductants were considered to be haploid if they had the nutritional requirements of the parental *MAT***a** strain and could mate with *Matα* tester strains. Only haploid cells were counted as cytoductants. The frequency of diploid formation was monitored by assaying the complementation of auxotrophic markers present in the haploid parents.

The frequency of chromosome loss and mitotic recombination on chromosome *V* was measured by the method of HARTWELL and SMITH (1985). In this method chromosome *V* is marked on one arm with the *can1* mutation, which confers recessive resistance to canavanine, and on the other by the auxotrophic marker *his1*. A strain is constructed which is heterozygous for *can1* and *his1*, and therefore, phenotypically Can$^s$His$^+$. Loss of chromosome *V* results in Can$^r$His$^-$ cells, whereas mitotic recombination on one arm results in Can$^r$His$^+$ cells. Three clones of each diploid strain from SD media were streaked on SC medium and incubated at 30°. Six to eight colonies (about $10^6$ cells per colony) were assayed from each diploid strain. Individual colonies were cut from the plate on a block of agar, resuspended in 1 ml of 0.85% saline, and sonicated to disrupt clumps of cells. Appropriate dilutions were plated onto SC medium to determine the total number of viable cells and onto canavanine medium to select for canavanine resistance cells. The canavanine resistant colonies arising on canavanine medium were replica-plated onto histidine dropout medium (SC without histidine) to obtain the frequency of His$^-$ (chromosome loss) and His$^+$ (mitotic recombination and chromosome loss) colonies.

**Cloning of *KEM1*:** The *KEM1* gene was isolated by its ability to complement the benomyl sensitivity and nuclear fusion defect of a *kem1* strain. Strain JK336 (*ura3-52, his4-29, leu1, kem1-1*) was transformed with DNA from a plasmid yeast genomic library constructed in the YCp50 vector (ROSE *et al.* 1987). Ura$^+$ transformants were selected at 30° then replica-plated onto benomyl medium and incubated overnight at 26°. Two of 16,000 transformants were identified as being benomyl-resistant. Both benomyl-resistant transformants formed diploids when crossed with a *kar1-1* strain. Plasmids pJI43 and pJI44 were recovered from these benomyl-resistant strains. Each plasmid complemented both *kem1-1* and *kem1-5* alleles. Restriction endonuclease analysis of pJI43 and pJI44 identified a 9.2-kb overlapping yeast DNA fragment.

**Plasmid constructions:** Plasmid pJI82 was constructed by inserting the 5.8-kb *Pvu*II-HindIII fragment of *KEM1* into *Bam*HI and *Hin*dIII digested YCp50 (JOHNSTON and DAVIS 1984); the *Bam*HI site was regenerated. Plasmid pJI74 contains the 7.5-kb *Pvu*II-*Nru*I fragment of *KEM1* inserted into *Bam*HI and *Nru*I digested YCp50; the *Bam*HI site was regenerated. A high copy vector containing *KEM1*, pJI98, was constructed by inserting the 8.2-kb *Bam*HI-*Xho*I fragment into *Bam*HI and *Sal*I digested YEp24 (BOTSTEIN *et al.* 1979). The integration plasmid, pJI61, was constructed by inserting the 9.1-kb *Bam*HI-*Nru*I fragment of *KEM1* into *Bam*HI and *Nru*I digested YIp5 (BOTSTEIN *et al.* 1979). A *URA3* marked gene disruption plasmid pJI90 (*kem1Δ1::URA3*) was constructed by replacing the internal *KEM1* 1.6-kb *Pvu*II-*Bst*EII fragment in plasmid pJI74 with a 1.1-kb *Hin*dIII fragment containing the *URA3* gene. Disruption plasmid pJI112 (*kem1Δ2::URA3*) was constructed by replacing the internal *KEM1* 3.3 kb *Sna*BI fragment in plasmid pJI74 with a 1.1-kb *Hin*dIII fragment containing the *URA3* gene. Plasmid pJI113, containing a *LEU2* marked gene disruption of *KEM1* (*kem1Δ3::LEU2*), was constructed by inserting a *Sal*I-*Xho*I fragment containing the *LEU2* gene between the *Sna*BI sites of plasmid pJI74.

**DNA sequencing:** The 5.8-kb *Bam*HI-*Hin*dIII fragment from plasmid pJI82, which corresponds to the 5.8-kb *Pvu*II-*Hin*dIII genomic DNA fragment (Fig. 4), was subcloned in both directions into the *Sma*I site of pUC118 (VIERA and MESSING 1987). Nested deletions of the insert fragment were generated by digestion with *Exo*III as described by HENIKOFF (1984) except that *Exo*VII was substituted for S1 nuclease. Single stranded phagemid DNA was prepared as described by VIERA and MESSING (1987) and sequenced by the dideoxy chain termination method of SANGER, NICKLEN and COULSEN (1977).

**Preparation and analysis of RNA:** Total nucleic acid was prepared by the method of ELDER, LOH and DAVIS (1983). Formaldehyde denatured RNAs were fractionated by electrophoresis through a 1.2% agarose gel and transferred to nitrocellulose as described by MANIATIS, FRITSCH and SAMBROOK (1982). RNA blots were washed and hybridized with labeled probes (FEINBERG and VOGELSTEIN 1983) under conditions of high stringency following protocol b as described by DAVIS, BOTSTEIN and ROTH (1980). Band sizes were determined by comparison to a RNA ladder from Bethesda Research Laboratories (Life Technologies, Inc., Gaithersburg, MD), 25S and 18S rRNA bands. S1 mapping was carried out with uniformly labeled single stranded cDNA under conditions described by WEAVER and WEISSMAN (1979).

**Protein manipulations:** Total yeast protein was obtained by trichloroacetic acid precipitation as described by OHASHI *et al.* (1982). Total and insoluble *E. coli* proteins were extracted according to KOERNER *et al.* (1990). Protein extracts were fractionated by electrophoresis through gels containing a 7–12.5% linear gradient of acrylamide stabilized within a 3–12% sucrose gradient as described by LAEMMLI (1970), except that SDS was omitted from gel and lower electrode buffer. Fractionated proteins were electrophoretically transferred to nitrocellulose sheets by the method of TOWBIN, STAEHELIN and GORDON (1979). Immunoblots were blocked for 4 hr with a solution containing 5% instant nonfat milk, 0.2% Tween 20 in PBS (137 mM NaCl, 3 mM KCl, 7 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$) and

rinsed with 0.2% Tween 20 in PBS. Rinsed blots were incubated 4 hr with antiserum diluted 1:500 in 0.2% Tween 20 in PBS. The blots were then washed five times with blocking solution containing 0.1% Triton X-100, 0.02% SDS and 1 mM EDTA. Immunoreactive bands were visualized after incubation with protein A-gold and subsequent silver enhancement (Bio-Rad Laboratories).

**Preparation of antibody to the *KEM1* gene product:** An inframe gene fusion between the *E. coli trpE* gene and *KEM1* was made by ligating the 3′ *Cla*I-*Hin*dIII fragment of *KEM1* into the *Cla*I site of pATH10 (KOERNER *et al.* 1990). The resulting plasmid encoded a hybrid protein containing the first 324 amino acids of the TrpE protein and 18 polylinker amino acids fused to the carboxy-terminal 408 amino acids of the Kem1 protein. Induction of the *trpE* operon with indoleacrylic acid (SPINDLER, ROSSER and BERK 1984) resulted in the overexpression of a 79-kD fusion protein, a size in accord with the predicted molecular mass of the hybrid fusion protein based on known sequence data. The trpE-KEM1 fusion protein was recovered in the insoluble protein fraction as described by KOERNER *et al.* (1990) and partially purified by preparative electrophoresis (LAEMMLI 1970). Gel strips containing the fusion protein were forced through an 18 gauge needle and incubated overnight at 65° in 10 ml of 25 mM Tris, 190 mM glycine and 0.1% SDS. The fusion protein, which was quantitatively recovered in the supernatant and two subsequent washes of the gel matrix, was concentrated by ultrafiltration using a PM30 centricon filtration unit (Amicon Div., W. R. Grace & Co., Danvers, Massachusetts). Forty microgram of fusion protein was emulsified in synthetic adjuvant (RIBI Immunochem Research, Inc., Hamilton, Montana) and injected intraperitoneally into mice. After three weeks a similar injection was given as a boost and blood was collected six days later.

**Staining of nuclei with DAPI:** DAPI (4′,6′-diamino-2-phenylindole, Accurate Chemical and Scientific Corp., Westbury, New York) was used to reveal the position of the nucleus in newly formed zygotes. Cells were prepared as described by DUTCHER (1982). A sample of $1 \times 10^7$ cells were suspended in 1 ml of Carnoy fixative (3:1, methanol:glacial acetic acid) and fixed at room temperature for 30 min. The cells were washed twice with 0.85% saline, resuspended in 1 ml of 1 μg/ml DAPI solution and incubated at room temperature for 45 min. The cells were washed twice with 0.85% NaCl, sonicated briefly to disrupt clumps of cells and examined by fluorescence microscopy.

**Immunofluorescence:** Microtubules were visualized by indirect immunofluorescence microscopy using antitubulin antibodies as described by ADAMS and PRINGLE (1984) with some modifications. Cells ($1–5 \times 10^7$) were resuspended in 10 ml of 0.1 M potassium phosphate buffer, pH 6.5, and fixed at room temperature for 2 hr after the addition of 1 ml of 37% formaldehyde. Cells were washed twice with 0.1 M potassium phosphate buffer, pH 6.5, after which cells were washed and resuspended in 1 ml of buffer A (1.2 M sorbitol, 0.1 M potassium phosphate buffer, pH 6.5). Five microliters of β-mercaptoethanol and 30 μl of zymolyase (10 mg/ml, 60K activity, Kirin Brewing Co., Japan) were added to the cell suspension in order to digest cell walls. After 1.5-hr incubation at 30° with gentle shaking, cells were washed once and resuspended in 3 ml of buffer A. Fifteen microliters of cell suspension was applied to a well of a polylysine coated slide (8-well slides, Flow Laboratories, Inc., McLean, Virginia). After 20 min, the wells were gently aspirated and the slides were immersed in cold methanol (−20°) for 6 min and placed immediately into cold acetone (−20°) for 30 sec. The cells were washed with buffer B (10 mg/ml bovine serum albumin, 1.2 M sorbitol, 0.1 M potassium phosphate, pH 6.5) and 15 μl of a 1:40 dilution of YOL1/34 (rat antitubulin antibody, Accurate Chemical and Scientific Corp.) in buffer B was added to each well. The slides were then incubated in a moist chamber at room temperature for 1 hr. Cells were washed four times with buffer B and 15 μl of a 1:500 dilution of rhodamine-conjugated anti-rat antibodies (Boehringer Mannheim Biochemicals) in buffer B was added to each well. After incubation at room temperature in the dark for 1 hr, cells were washed four times with buffer B and 15 μl of a 1 mg/ml DAPI solution was added to stain nuclear DNA. After 5 min, cells were washed with buffer A and coverslips were mounted with *p*-phenylenediamine in 90% glycerol. Cells were examined by fluorescence microscopy.

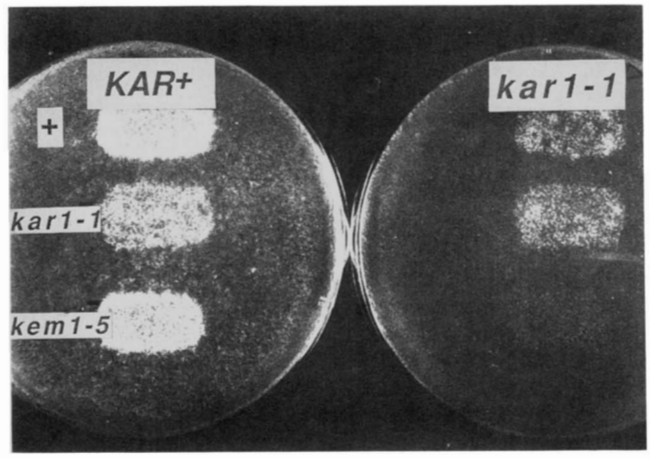



FIGURE 1.—The nuclear fusion defect in *kem* mutants as measured by a plate mating assay. Patches of strains grown on a YEPD plate were mated to a lawn of *KAR* or *kar1-1* cells. After incubation at 30° for 4 hr, plates were replica-plated onto YNB plates to select for diploids. From the top, the patches are *KEM1 KAR1* (JK137), *KEM1 kar1-1* (JK132), and *kem1-5 KAR1* (JK191). Strains on the plate on the left were mated with a *KAR1* lawn (6947-2B) and strains on the plate on the right were mated with a *kar1-1* lawn (7523-6A).

## RESULTS

**Isolation of *kem* mutants:** Mutations called *kem* (*K*ar⁻ *e*nhancing *m*utations) were isolated that lower the ability of *kar1-1* cells to form diploids. Since *KEM kar1-1* cells form diploids at a discernible frequency, mutants exhibiting a reduced ability to form diploids can be identified by a simple replica plate mating assay (see Figure 1). Two screens were used to identify *kem* mutants. In the first screen, Kar$^+$ cells were mutagenized and mutant isolates that lowered the frequency of diploid formation in crosses with *kar1-1* cells were identified. In the second, mutagenized *kar1-1* strains were screened and *kem kar1-1* double mutants which lowered the frequency of diploid formation in crosses with *KAR1* cells were obtained. Eight independent *kem* mutants were isolated in screens of 26,000 EMS mutagenized cells. Four of these strains were examined further because they exhibited a reduction in diploid formation that could be followed easily in subsequent crosses. The Kem⁻ phenotype associated

with these four strains segregated 2.2 in tetrads, indicating in each case that the Kem$^-$ phenotype resulted from a single nuclear mutation. In a *KAR1* background, all the Kem$^-$ mutants are defective in diploid formation in crosses with *kar1-1*, even though one of the mutants (*kem3*) was isolated in a *kar1-1* background (scheme 2). Strains carrying *kem3-1* are temperature sensitive for growth at 37° on complete medium. The temperature sensitivity of *kem3-1* cosegregates with the defect in diploid formation (examined at 34°) suggesting that *KEM3* encodes a vital function.

***kem* mutations define three genes:** Functional tests were assessed by constructing diploids that were capable of mating; *KEM/kem* diploids for dominance tests and *kemx/kemy* diploids for complementation tests. These strains were derived from diploids heterozygous for the *cry1* allele (*cry1* is recessive and tightly linked to the *MAT* locus). Diploids homozygous for the mating locus were selected on cryptopleurine plates (YEPD containing 1 μg/ml cryptopleurine). Once these strains were constructed, the Kem phenotype of each of these diploids was determined; failure to give a mating response with a *kar1-1* strain was diagnostic of the Kem$^-$ phenotype. The four *kem* mutations analyzed are recessive. *kem1-1* and *kem1-5* mutations failed to complement, whereas *kem2* and *kem3* complemented each other as well as *kem1-1* and *kem1-5* mutations. These results suggest that there are at least three *KEM* complementation groups and that *kem1-1* and *kem1-5* are functionally identical. Subsequent tetrad analysis indicated that the *KEM1*, *KEM2* and *KEM3* complementation groups represent three distinct genetic loci.

**Mapping of *KEM1* and *KEM3*:** Both *KEM1* and *KEM3* were initially mapped by hybridization to whole yeast chromosomes separated by pulse field electrophoresis (CARLE and OLSON 1985). Full length chromosomes, isolated from yeast strains with fragmented chromosomes *VII* (VOLLRATH *et al.* 1988) were electrophoretically separated, transferred to a nitrocellulose filter, and hybridized with radioactively labeled probes specific to either *KEM1* or *KEM3* (see subsequent section for cloning of *KEM1* and see KIM (1988) for cloning of *KEM3*). Both probes hybridized to DNA sequences located on the left arm of chromosome *VII*.

To determine the precise chromosomal location of *KEM1* and *KEM3*, we carried out crosses involving known markers on the left arm of chromosome *VII* and analyzed the resulting tetrads. Mapping data presented in Table 2 establish the gene order and map distances (centimorgans) as follows: *CEN VII-met13*-(16 cM)-*kem3*-(22 cM)-*lys5*-(16 cM)-*kem1*.

***kem* mutations reduce nuclear fusion:** To verify that decreased diploid formation in *kem* × *kar1-1* crosses results from enhancement of the Kar$^-$ defect rather than from a defect in zygote formation or zygote viability, we assayed failure of nuclear fusion directly. Failure to complete nuclear fusion would result in the formation of cytoductants, haploid progeny which contain the nuclear genotype of one parent and the cytoplasmic components of both parents. The ratio of cytoductants to diploids is an indication of the efficiency of nuclear fusion during mating. We measured the cytoductants as cyh$^r$[$\rho^+$] cells in a cross of cyh$^s$[$\rho^+$] × cyh$^r$[$\rho^0$] cells. Table 3 summarizes the results of quantitative cytoduction experiments with a set of *kem* strains. The cytoductant to diploid ratio in crosses with *kar1-1* strains was found to be between 5 and 6 (Table 3; lines 1 and 2), consistent with previously reported values (DUTCHER 1982, DUTCHER and HARTWELL 1982). In crosses of *kem* × *kar1-1* or *kem kar1-1* × *KAR*, the cytoductant to diploid ratio increased substantially (Table 3; lines 3–5 and 6–7, respectively). The *kem1* and *kem3* mutations lead to elevated cytoduction frequencies whether or not they are in the same nucleus as *kar1-1* (Table 3; compare lines 3 and 6, lines 5 and 7). In crosses to a wild-type strain the *kem* mutants do not show a significant reduction in nuclear fusion. These results confirm that *kem* mutations reduce the residual diploid formation of the *kar1-1* mutation primarily by reducing nuclear fusion.

Further evidence that *kem* mutations reduce nuclear fusion was obtained by direct microscopic observation. Mutant *kem* × *kar1-1* or *kem kar1-1* × *KAR* crosses were incubated for 3–4 hr after which cells were fixed and stained with DAPI. Zygote formation (cell-cell fusion) in mutant crosses occurred at rates observed in wild-type crosses (data not shown). The number of zygotes with a single nucleus and the number of zygotes with two unfused nuclei were counted (Table 4). In *kem* × *kar1-1* or *kem kar1-1* × *KAR* crosses, fewer than 1% of the zygotes contain nuclei that have fused (Table 4, lines 3–6). Fifteen percent of zygotes resulting from *KEM kar1-1* crosses have visibly fused nuclei (Table 4, lines 1 and 2). The results obtained by the direct visualization of nuclear fusion supports the conclusions obtained from mating tests (Figure 1) and

**TABLE 2**

**Genetic mapping of *kem1* and *kem3***

| Gene pair | Segregation pattern[a] (number of tetrads) PD | NPD | TT | Map distance (cM) |
|---|---|---|---|---|
| *kem1-lys5* | 60 | 0 | 27 | 16 |
| *lys5-kem3* | 112 | 2 | 67 | 22 |
| *kem1-kem3* | 30 | 7 | 36 | 53 |
| *kem3-met13* | 32 | 0 | 15 | 16 |

[a] PD, parental ditype; NPD, nonparental ditype; TT, tetratype.

**TABLE 3**

**The ratio of cytoductant to diploid formation in crosses of *kem* and *kar1-1* mutants**

| | *MAT***a** *cyh*$^r$ [$\rho^0$] parent | |
|---|---|---|
| *MATα CYH*$^r$ [$\rho^+$] parent | *KAR* (JK217) | *kar1-1* (JK218) |
| 1. *KEM KAR* (JK137) | 0.001 (0.0006/0.6)[a] | 5.0 (0.10/0.02) |
| 2. *kar1-1* (JK132) | 5.0 (0.15/0.03) | 6.3 (0.19/0.03) |
| 3. *kem1-1* (JK156) | 0.005 (0.0009/0.19) | 200 (0.10/0.0005) |
| 4. *kem2-1* (JK301) | 0.001 (0.0003/0.25) | 40 (0.10/0.0025) |
| 5. *kem3-1* (JK136) | 0.007 (0.002/0.28) | 26 (0.18/0.007) |
| 6. *kem1-1 kar1-1* (JK158) | 180 (0.09/0.0005) | 800 (0.08/0.00001) |
| 7. *kem3-1 kar1-1* (JK130) | 28 (0.11/0.004) | 32 (0.16/0.005) |

[a] Data in parentheses are frequencies of cytoductant and diploid formation. Cytoductant frequency = (Cyh$^r$ [$\rho^+$] colonies/total colonies). Diploid frequency = (prototrophic colonies/total colonies).

**TABLE 4**

**Percentage of zygotes with a single nucleus in crosses of *kem* and *kar1-1* mutants as measured by DAPI staining**

| | *MAT***a** [$\rho^0$] parent | |
|---|---|---|
| *MATα* [$\rho^+$] parent | *KAR1* (JK217) | *kar1-1* (JK218) |
| 1. *KEM KAR* (JK137) | 98 (197)[a] | 16 (118) |
| 2. *kar1-1* (JK132) | 15 (213) | 14 (79) |
| 3. *kem1-1* (JK156) | 90 (136) | 0 (205) |
| 4. *kem3-1* (JK136) | 100 (125) | 1 (139) |
| 5. *kem1-1 kar1-1* (JK158) | 0 (139) | 0 (62) |
| 6. *kem3-1 kar1-1* (JK130) | 0 (109) | 0 (48) |

[a] The number of zygotes examined are shown in parentheses.

measurement of the frequency of cytoduction (Table 3).

***kem1* has a bilateral nuclear fusion defect:** Nuclear fusion defects are defined as bilateral, if both mating partners of a cross must be mutant for nuclear fusion to fail, or unilateral, if only one partner must be mutant for nuclear fusion to fail. Strains carrying *kar1-1* show a unilateral defect (CONDE and FINK 1976), whereas β-tubulin mutants exhibit a bilateral fusion defect (HUFFAKER, THOMAS and BOTSTEIN 1988). Nuclear fusion occurs normally in *KAR kem1* × *KAR KEM1* crosses, and thus *kem1* mutations do not appear to have unilateral defects (see Tables 3 and 4).

In order to determine whether the *kem1* mutations exhibited mating defects independent of *kar1-1*, *KAR kem1* strains were intercrossed. Examination of the distribution of nuclear DNA in zygotes by fluorescence microscopy showed that nuclear fusion is defective in *kem1* × *kem1* crosses, whereas nuclear fusion is normal in crosses of *kem1* × *KEM1*. Zygotes from crosses of *kem1* × *KEM1* (Figure 2, panels C and D) appeared similar to those from the wild-type cross *KEM1* × *KEM1* (Figure 2, panels A and B). Matings in these crosses resulted in a zygote with a single bright DAPI staining region. However, in *kem1* × *kem1* crosses approximately 20–30% of zygotes appeared to have unfused nuclei (Figure 2, panels E–H). These results suggest that *kem1* has a bilateral nuclear fusion defect.

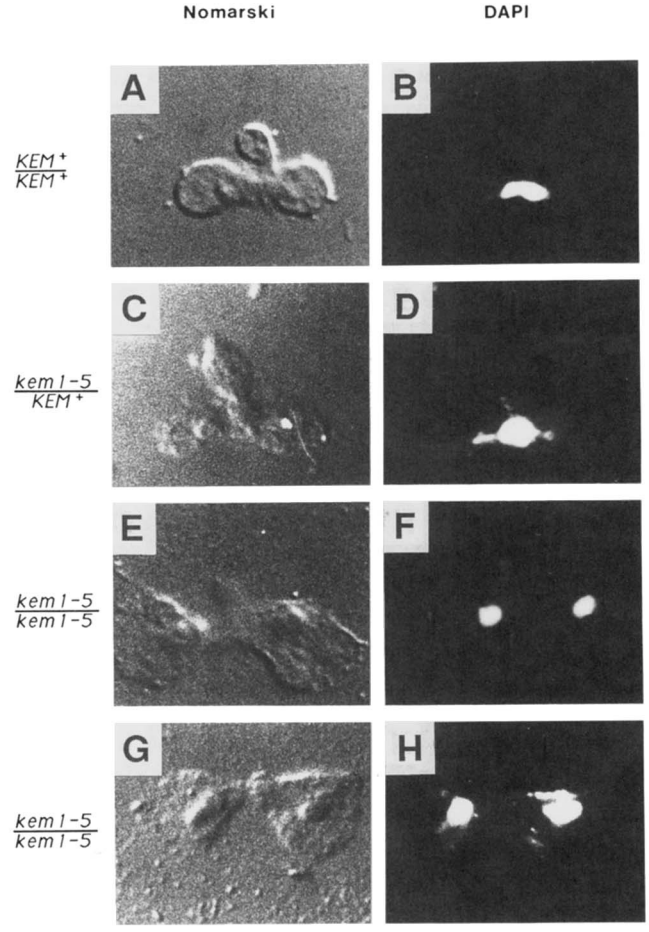



FIGURE 2.—Photomicrographs of zygotes from *KEM1* × *KEM1* and *kem1* × *kem1* crosses. Each row of photomicrographs represents the same zygote observed by Nomarski optics and DAPI staining. Zygotes are from the following crosses: *KEM1* × *KEM1* (JK219 × JK311) panels A and B; *kem1-5* × *KEM1* (JK313 × JK311) panels C and D; *kem1-5* × *kem1-5* (JK313 × JK314) panels E–H.

The ratio of cytoductant to diploid formation was measured in crosses of *KEM1* × *KEM1*, *kem1-5* × *KEM1*, and *kem1-5* × *kem1-5* (Table 5). In a *kem1-5* × *kem1-5* cross the cytoductant to diploid ratio is increased 20-fold as compared to the ratio observed in

**TABLE 5**

**The ratio of cytoductant to diploid formation in crosses of *kem1-5* mutants**

| Cross | Relevant genotype | Cytoductant frequency[a] | Diploid frequency[b] | Cytoductant/ diploid |
|---|---|---|---|---|
| JK196 × JK315 | *KEM1* × *KEM1* | 0.0008 | 0.45 | 0.002 |
| JK196 × JK316 | *KEM1* × *kem1-5* | 0.0009 | 0.28 | 0.003 |
| JK197 × JK315 | *kem1-5* × *KEM1* | 0.001 | 0.31 | 0.005 |
| JK197 × JK316 | *kem1-5* × *kem1-5* | 0.02 | 0.18 | 0.11 |

JK196 and JK197 are isogenic strains; JK315 and JK316 are derived from sister spores.
[a] Cyh$^r$ [$\rho^+$] colonies/total colonies.
[b] Prototrophic colonies/total colonies.

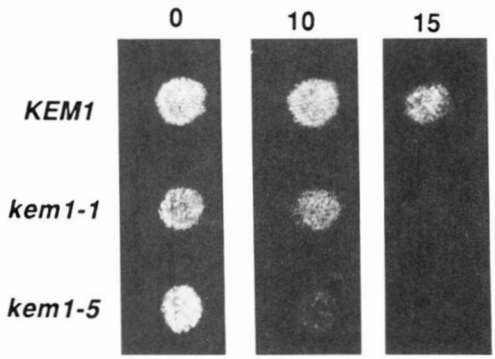



FIGURE 3.—Benomyl sensitivity of *kem1* strains. Growth of *KEM1* (JK204), *kem1-1* (JK205), and *kem1-5* (JK191) yeast strains on media containing 0, 10, and 15 μg/ml of benomyl. The plates were incubated for 2 days at 30° and photographed.

**TABLE 6**

**Frequency of chromosome loss and mitotic recombination in *kem1/kem1* diploids**

| Diploid strain | Chromosome loss[a] | Mitotic recombination[b] |
|---|---|---|
| *KEM1/KEM1* (JK326) | 1.2 | 2.30 |
| *KEM1/kem1-1* (JK327) | 1.1 | 0.93 |
| *KEM1/kem1-5* (JK328) | 3.2 | 1.61 |
| *kem1-1/kem1-1* (JK329) | 22.9 | 1.18 |
| *kem1-5/kem1-5* (JK330) | 16.4 | 1.87 |
| *kem1-1/kem1-5* (JK331) | 27.7 | 1.51 |

Numbers listed are averages of 6–8 independent experiments.
[a] (Number of Can$^r$ His$^-$ cells)/(total number of diploid cells) × $10^5$.
[b] (Number of Can$^r$ His$^+$ cells)/(total number of diploid cells) × $10^4$.

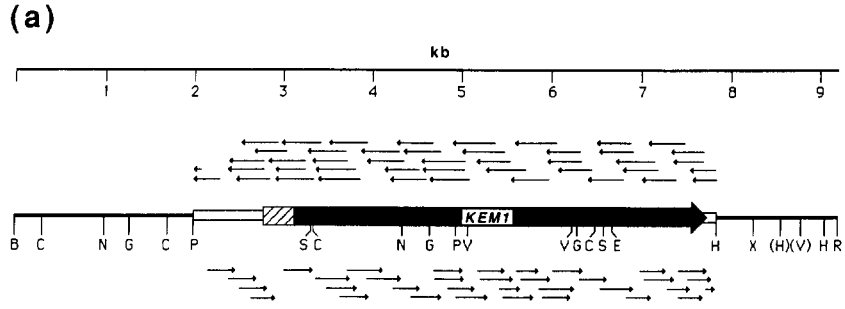
a *kem1-5* × *KEM1* cross. These results are consistent with our cytological observations (Figure 2) and provide quantitative genetic evidence that *kem1* has a bilateral nuclear fusion defect. It is important to emphasize that *kem1* mutations have no apparent effect on the frequency of cell fusion.

***KEM1* mutants are benomyl sensitive and lose chromosomes:** The growth of *kem1* mutants was compared with that of an isogenic *KEM1* wild-type strain at various concentrations of benomyl to determine whether there was any differential sensitivity. Benomyl inhibits the growth of *kem1* mutants at concentrations (10 μg/ml) that do not affect the growth of the wild-type strain (Figure 3). The benomyl hypersensitive phenotype and the *kem1* mutation cosegregate 2:2 in tetrads from a *kem1* × *KEM1* cross (results not shown).

Benomyl sensitivity has been associated with mutations that increase the frequency of chromosome loss (HUFFAKER, HOYT and BOTSTEIN 1987; HUFFAKER, THOMAS and BOTSTEIN 1988; HOYT, STEARNS and BOTSTEIN 1990). We measured the frequency that mitotically growing diploid cells lose chromosome *V* (Table 6). Wild-type and heterozygous *kem1* diploids (*KEM1/KEM1*, *KEM1/kem1-1*, and *KEM1/kem1-5*) lost chromosome *V* at a frequency of 1–3 × $10^{-5}$. *kem1-1/kem1-1*, *kem1-1/kem1-5*, and *kem1-5/kem1-5* diploids lost chromosome *V* at a frequency of 20–30 × $10^{-5}$. These results indicate that there is a 10–20-fold increase in the frequency of chromosome loss in *kem1* homozygous diploids and that this phenotype is recessive. Mitotic recombination in either *KEM1/kem1* or *kem1/kem1* strains occurred at frequencies similar to that observed in *KEM1/KEM1* diploids.

**Cloning of *KEM1*:** We cloned the *KEM1* gene by complementation of the benomyl sensitivity of *kem1* strains. A *kem1* strain was transformed with a genomic yeast library and two benomyl resistant transformants were obtained. These transformants simultaneously gained benomyl resistance and the ability to form stable diploids when crossed with a *kar1-1* strain. Plasmids from these strains were recovered in *E. coli* and purified. These plasmids complemented both *kem1-1* and *kem1-5* alleles upon retransformation. The restriction map of the 9.2-kb overlapping DNA fragment is shown in Figure 4a.

To demonstrate that the cloned sequence contains the *KEM1* gene, we determined whether this DNA sequence could integrate along with the plasmid vector at the *KEM1* chromosomal locus by homologous recombination. The 9.2-kb *Bam*HI-*Nru*I fragment was subcloned into the integrating vector YIp5 (pJI61). This vector carries the *URA3* marker as well as the pBR322 sequence and can only give stable transformants by integration into the genome. Ura$^+$ transformants, obtained by transforming strain F760 (*KEM1 ura3-52*) with plasmid pJI61, were crossed to a strain of genotype *kem1-1 ura3-52* (JK336) and to a




(b)

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-1131 GATCCTGGTTTTGCACCAAACATGCCCTGGTTCTGCGCAGGGGCCGAAGAGGTGCTTGTATTACCGAATAAAGAAGTGCCTCCTGCAGGTTTAGAGCCAAATAGTCCAGCTTGCATTATT -1012
-1011 CATGCCACTACTACTATTATTGATATTATTAGTGTTATTATTACCAAACAAACCACACTCGTCTGTGCATTGTTGGAACCAAACAAACTGCCAGAATTACTGTTCGAATTAGCGCCAAAT -892
 -891 AACCCGCCCCCCAGTGTTCGCCGTATTGTTAGGTTTGTTACCGAAAAGGCCCCCTCCGGTGGTGGCGCTTCCTCCAAATGCATTTGTCTGAGATTGTTGTTGCTGCTGACCAAATGATGCA -772
 -771 CCTAATCCTCCTGTAGATCCGGCTGGTTTAGCGCCAAATAGTCCACCTGTACTTGGGCCGGTGTTTTGTCCAGTTTGAGTCCCACCAAACGAAAACCCAGTATTCGCGTTTCCAGTGCTA -652
 -651 GCTCCGCTGGCCTGACCAAAGAGCCCACCTGCAGGTGTCGAAGATGCTTTATTTAATCCAAACATTGTGCCCACTACCCGTGAAAACCCTCTCAATTACTGTTTTTATCGTCTCATCACC -532
 -531 TGATCAGTAACTATTCTCACGATTAATGGTCTTTTTCAACTACATTTATTTGAGAAAGCTGTAATACATTCCGTGTTGAGTGATTTTCAACCTCCTTTGCTCGTCTTTTCGCCACCGC -412
 -411 AGAGCAAGTAACAACAGAGACAACACAGAAGAGGTTAGAAAGCATTTAAGCAGTTTTAACGACTACTCAAACGTCAAACTAAGTACTTACGACTTCCAATTACCGGTTGTAAAGTT -292
 -291 TATCTACGCAAATATAACAGTTGCAGCTTGCTTTTTTTTCAGCAGCTCTTCCGTTATTATAAAATCTGTTTGGCGTTCGCACAAGCGAACCGATTCTTTTATTATTTCTCTCTGCCTT -172
 -171 TTATTTCCGTTCCTGTTAATAGTTTATTTTCTAAAGGATACTGTCTTCTTCCCGTACTTATAATCGGTTCACAATAAGCAATTGACTAATCCTAGGACGATTCGTGTACTATAAGGAGAA  -52

  -51 AAAAAATCAACACTTGTAACAACAGCAGCAACAAATATATATCAGTACGGTATGGGTATTCCAAAATTTTTCAGGTACATCTCAGAAAGATGGCCCATGATTTTACAGCTTATTGAGGGA   69
                                                                                  M  G  I  P  K  F  F  R  Y  I  S  E  R  W  P  M  I  L  Q  L  E  G
   70 ACACAGATTCCTGAGTTTGATAACTTATACCTGGATATGAATTCGATTTTACATAATTGTACGCATGGTAACGACGATGATGTAACCAAGCGATTAACTGAAGAAGAGGTTTTTGCAAAA  189
       T  Q  I  P  E  F  D  N  L  Y  L  D  M  N  S  I  L  H  N  C  T  H  G  N  D  D  V  T  K  R  L  T  E  E  V  F  A  K
  190 ATCTGTACGTATATCGATCACCTTTTTCAAACAATCAAGCCCAAGAAGATTTTCTACATGGCTATTGATGGTGTGGCCCCTCGTGCCAAGATGAATCAACAAAGAGCTCGTAGATTCAGA  309
       I  C  T  Y  I  D  H  L  F  Q  T  I  K  P  K  K  I  F  Y  M  A  I  D  G  V  A  P  R  A  K  M  N  Q  Q  R  A  R  R  F  R
  310 ACCGCTATGGATGCAGAAAAAGCCTTGAAGAAGGCTATTGAGAATGGTGACGAGATTCCTAAGGGTGAGCCATTTGATTCGAATTCTATTACTCCAGGTACGGAGTTTATGGCCAAATTG  429
       T  A  M  D  A  E  K  A  L  K  K  A  I  E  N  G  D  E  I  P  K  G  E  P  F  D  S  N  S  I  T  P  G  T  E  F  M  A  K  L
  430 ACCAAAAACTTACAATATTTTATTCACGACAAGATTTCTAACGATTCCAAATGGAGGGAAGTGCAAATCATATTTTCTGGCCATGAAGTTCCAGGTGAAGGTGAACACAAGATCATGAAC  549
       T  K  N  L  Q  Y  F  I  H  D  K  I  S  N  D  S  K  W  R  E  V  Q  I  I  F  S  G  H  E  V  P  G  E  G  E  H  K  I  M  N
  550 TTTATAAGGCATTTAAAATCCCAAAAGGATTTCAACCAGAATACGAGACATTGTATTTACGGTCTTGACGCAGATTTGATTATGCTGGGTTTGTCTACTCATGGCCACATTTTGCGTTA  669
       F  I  R  H  L  K  S  Q  K  D  F  N  Q  N  T  R  H  C  I  Y  G  L  D  A  D  L  I  M  L  G  L  S  T  H  G  P  H  F  A  L
  670 TTGAGAGAAGAAGTGACATTTGGTAGAAGAAATAGTGAAAAAAAATCGCTTGAACATCAAAATTTCTACTTATTACATCTTTCTTTATTAAGAGAATACATGGAGTTGGAAATCAAAGAA  789
       L  R  E  E  V  T  F  G  R  R  N  S  E  K  K  S  L  E  H  Q  N  F  Y  L  L  H  L  S  L  L  R  E  Y  M  E  L  E  F  K  E
  790 ATTGCCGATGAAATGCAATTTGAATACAATTTGAATCATGTATTTGGATGATTTTATTCTTGTCATGGTTCGTCATTGGTAATGATTTCTTGCCCAATTTGCCAGATTTGCACCTTAACAAA  909
       I  A  D  E  M  Q  F  E  Y  N  F  E  R  I  L  D  D  F  I  L  V  M  F  V  I  G  N  D  F  L  P  N  L  P  D  L  H  L  N  K
  910 GGAGCATTTCCCGTTTTGTTACAAACGTTCAAAGAAGCTCTTTACATACTGATGGCTACATTAATGAACATGGTAAAATAAATTTAAAGAGATTAGGTGTCTGGTTAAATTATCTGTCT 1029
       G  A  F  P  V  L  L  Q  T  F  K  E  A  L  L  H  T  D  G  Y  I  N  E  H  G  K  I  N  L  K  R  L  G  V  W  L  N  Y  L  S
1030 CAATTTGAGTTATTAAAATTTCGAAAAGGATGATATAGACGTTGAGTGGTTCAACAAGCAATTAGAGAATATTTCTTTGGAGGGTGAAAGGAAAAGACAGAGGGTTGGTAAAAAATTACTG 1149
       Q  F  E  L  L  N  F  E  K  D  D  I  D  V  E  W  F  N  K  Q  L  E  N  I  S  L  E  G  E  R  K  R  Q  R  V  G  K  K  L  L
1150 GTAAAACAACAGAAGAAATTAATTGGAAGTATAAAACCATGGTTGATGGAGCAATTACAGGAAAAATTATCGCCTGATTTACCAGATGAAGAAATTCCAACTTTAGAGTTACCTAAGGAC 1269
       V  K  Q  Q  K  K  L  I  G  S  I  K  P  W  L  M  E  Q  L  Q  E  K  L  S  P  D  L  P  D  E  E  I  P  T  L  E  L  P  K  D
1270 TTAGACATGAAAGATCATTTAGAATTTTTAAAAGAATTCGCTTTTGATTTGGGTCTTTTTATAACGCATTCCAAATCCAAAGGTAGTTATTCGCTAAAAATGGATCTTGATTCTATTAAT 1389
       L  D  M  K  D  H  L  E  F  L  K  E  F  L  K  G  F  L  T  H  S  K  S  K  G  S  Y  S  L  K  M  D  L  D  S  I  N
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       P  D  E  T  E  E  E  F  Q  N  R  V  N  S  I  R  K  T  I  K  K  Y  Q  N  A  I  I  V  E  D  K  E  E  L  E  T  E  K  T  I
1510 TATAATGAAAGGTTTGAACGTTGGAAGCATGAGTATTATCACGACAAGTTAAAATTTACGACAGACAGTGAAGAAAAAGTGAGAGATCTTGCTAAAGACTACGTTGAAGGTTTACAATGG 1629
       Y  N  E  R  F  E  R  W  K  H  E  Y  Y  H  D  K  L  K  F  T  T  D  S  E  E  K  V  R  D  L  A  K  D  Y  V  E  G  L  Q  W
1630 GTTCTATATTATTATTATAGAGGATGTCCATCTTGGTCGTGGTACTATCCGCACCATTATGCACCAAGAATCTCCGACTTAGCCAAGGGTTTAGATCAAGACATTGAATTTGATTTGAGC 1749
       V  L  Y  Y  Y  Y  R  G  C  P  S  W  S  W  Y  Y  P  H  H  Y  A  P  R  I  S  D  L  A  K  G  L  D  Q  D  I  E  F  D  L  S
1750 AAACCATTTACTCCATTCCAGCAACTAATGGCAGTTTTACCGGAAAGGTCCAAAAATCTGATACCTCCGCCTTTAGGCCATTAATGTACGATGAACAGTCGCCAATCCACGATTTCTAT 1869
       K  P  F  T  P  F  Q  Q  L  M  A  V  L  P  E  R  S  K  N  L  I  P  P  A  F  R  P  L  M  Y  D  E  Q  S  P  I  H  D  F  Y
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       P  A  E  V  Q  L  D  K  N  G  K  T  A  D  W  E  A  V  V  L  I  S  F  V  D  E  K  R  L  I  E  A  M  Q  P  Y  L  R  K  L
1990 TCACCTGAAGAAAAAACGAGAAATCAATTTGGCAAAGACTTGATATATTCCTTTAATCCTCAAGTTGATAACCTTTATAAGAGTCCGTTGGGCGGCATTTTTCTGATATTGAACACAAT 2109
       S  P  E  E  K  T  R  N  Q  F  G  K  D  L  I  Y  S  F  N  P  Q  V  D  N  L  Y  K  S  P  L  G  G  I  F  S  D  I  E  H  N
2110 CATTGTGTCGAAAAAGAGTACATCACCATCCCATTGGACAGCTCCGAGATTCGGTATGGTTTATTACCTAATGCTAAACTCGGTGCCGAAATGCTGGCGGGTTTCCCCACGTTATTGTCT 2229
       H  C  V  E  K  E  Y  I  T  I  P  L  D  S  S  E  I  R  Y  G  L  L  P  N  A  K  L  G  A  E  M  L  A  G  F  P  T  L  L  S
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       L  P  F  T  S  S  L  E  Y  N  E  T  M  V  F  Q  Q  P  S  K  Q  Q  S  M  V  L  Q  I  T  D  I  Y  K  T  N  N  V  T  L  E
2350 GACTTTTCCAAGAGGCATTTAAACAAAGTGATTTATACAAGATGGCCATATTTAAGAGAATCCAAATTGGTCTCTTTAACGGATGGTAAGACTATCTATGAATATCAGGAGTCCAATGAT 2469
       D  F  S  K  R  H  L  N  K  V  I  Y  T  R  W  P  Y  L  R  E  S  K  L  V  S  L  T  D  G  K  T  I  Y  E  Y  Q  E  S  N  D
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       K  K  K  F  G  F  I  T  K  P  A  E  T  Q  D  K  K  L  F  N  S  L  K  N  S  M  L  R  M  Y  A  K  Q  K  A  V  K  I  G  P
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       M  E  A  I  A  T  V  F  P  V  T  G  L  V  R  D  S  D  G  G  Y  I  K  T  F  S  P  T  P  D  Y  Y  P  L  Q  L  V  V  E  S
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       V  V  N  E  D  E  R  Y  K  E  R  G  P  I  P  I  E  E  E  F  P  L  N  S  K  V  I  F  L  G  D  Y  A  Y  G  G  E  T  T  I
2830 GACGGTTACAGCAGTGACCGCAGACTAAAAATTACTGTAGAAAAGAAGTTTTTGGATAGTGAGCCCACCATCGGCAAAGAAAGGTTACAAATGGATCATCAAGCCGTTAAATATTATCCG 2949
       D  G  Y  S  S  D  R  R  L  K  I  T  V  E  K  K  F  L  D  S  E  P  T  I  G  K  E  R  L  Q  M  D  H  Q  A  V  K  Y  Y  P
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3070 GAAGCTAGACACCAAAAGGTTTTAGGTTACGCGAGGAGGAACCCTAGGGGCTGGGAATACTCAAATTTAACTCTAAATTTACTGAAAGAGTATAGACAAACTTTCCCAGATTTTTTTTTC 3189
       E  A  R  H  Q  K  V  L  G  Y  A  R  R  N  P  R  G  W  E  Y  S  N  L  T  L  N  L  L  K  E  Y  R  Q  T  F  P  D  F  F  F
3190 AGGTTGTCCAAGGTAGGTAATGATATCCCAGTTTTGGAAGATCTTTTCCCCGATACTTCCACTAAGGATGCCATGAATTTATTAGATGGTATCAAACAATGGCTAAAGTATGTCTCATCG 3309
       R  L  S  K  V  G  N  D  I  P  V  L  E  D  L  F  P  D  T  S  T  K  D  A  M  N  L  L  D  G  I  K  Q  W  L  K  Y  V  S  S
3310 AAGTTTATCGCGGTATCTTTGGAGTCTGACTCCTTAACTAAGACATCGATTGCTGCCGTGGAAGATCATATCATGAAATACGCAGCTAACATCGAAGGTCATGAAAGAAAACAGTTAGCC 3429
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3430 AAAGTTCCTCGTGAGGCTGTTTTGAATCCAAGATCATCATTTGCACTCTTACGTAGTCAAAAGTTCGATTTGGGTGACCGTGTTGTTTATATCCAAGATTCTGGTAAGGTACCAATTTTC 3549
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3550 TCAAAGGGTACAGTTGTTGGCTATACTACTCTCAGTTCATCATTATCAATTCAGGTCTTATTTGATCATGAAATCGTGGCCGGTAATAATTTTGGCGGCAGGTTGCGCACGAATAGAGGC 3669
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       N  S  T  E  C  Q  S  P  K  S  Q  S  N  A  A  D  R  D  N  K  K  D  E  S  T  *
4630 AGTATATCTGAGACCTATATAAAGAGGGGTATTTGTATTTAATCGTTGATCTTGTTAAGAAAAAGCT 4696
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FIGURE 4.—Restriction endonuclease map and DNA sequence of the *KEM1* gene. (a) Restriction endonuclease map of the 9.2-kb *Bam*HI-*Nru*I genomic DNA fragment containing the yeast *KEM1* gene. The 5.8-kb *Pvu*II-*Hin*dIII DNA fragment was sequenced (open boxed

strain of genotype *kem1-5 ura3-52* (JK337). Diploids from each cross were sporulated and 20 tetrads from each cross were dissected. In each tetrad, the two $Ura^+$ spores were benomyl-resistant and the two $Ura^-$ spores were benomyl sensitive. These results demonstrate that the cloned sequence complementing the *kem1* mutations had integrated at the *KEM1* locus by homologous recombination, and confirm that the cloned DNA fragment contained the authentic *KEM1* gene.

Portions of the isolated genomic fragment were subcloned into the YCp50 vector. The smallest fragment of DNA tested that maintained full complementing activity was a 5.8-kb *Pvu*II-*Hin*dIII fragment (pJI82). Construction of frameshift mutations at the *Nco*I site and a *Bst*EII site within this fragment destroyed complementing activity, as did deletion of the 1.6-kb *Bgl*II fragment. Therefore, the *Nco*I, *Bgl*II, and *Bst*EII sites are internal to the complementing gene.

**DNA and predicted protein sequences of *KEM1* gene:** The nucleotide sequence of the *KEM1* gene was determined by DNA sequence analysis of the 5.8-kb genomic *Pvu*II-*Hin*dIII fragment (Figure 4b). An open reading frame of 4,583 base pairs beginning with the initiation codon ATG was found. The location and directionality of the open reading frame corresponds to that predicted by insertional-mutagenesis (data not shown) and restriction endonuclease mapping data. The *KEM1* open reading frame is capable of encoding a protein comprised of 1528 amino acids with the predicted molecular mass of 175 kD and a pI = 7.19 (FINER-MOORE *et al.* 1989). KEM1 showed no significant homology with any proteins in the National Biomedical Research Foundation (NBRF) protein data bases and the GenBank nucleic acid data base using homology comparison programs FASTA and TFASTA (PEARSON and LIPMAN 1988).

***KEM1* mRNA and protein analysis:** RNA blot-hybridization analysis revealed a single RNA species in total RNA isolated from strains containing a functional *KEM1* gene (Figure 5). The observed 5.5-kb transcript is substantially longer than the identified 4.58-kb *KEM1* open reading frame. S1-nuclease protection experiments with two independent single stranded DNA probes each with complementary sequences 5′ to the *KEM1* open reading frame, one beginning within the open reading frame at nucleotide +148 and the other beginning at nucleotide −100, identified a region in which transcription of *KEM1* mRNA initiates (data not shown). This region, nucleotides −372 to −337, is approximately 90 nucleotides downstream from a putative TATA box (Figure 4b). The amount of *KEM1* RNA observed was proportional to the copy number of *KEM1* gene (compare lanes 1, 3 and 5 of Figure 5). RNA isolated from *kem1* deletion strains did not contain sequences that hybridized to the *KEM1* probe.

The observed molecular weight of the KEM1 protein correlates well with that predicted by DNA sequence analysis. The KEM1 protein can be overproduced and visualized upon SDS-polyacrylamide electrophoresis of total yeast protein. An intensely stained band of approximately 165-kD molecular mass was observed in strains containing the *KEM1* gene in high copy (JK278 and JK282). Immunoblot analysis, using antiserum raised against a trpE::KEM1 encoded fusion protein, detected the same 165-kD band in total yeast protein extracts from strains with one or multiple copies of *KEM1* (data not shown). The amount of protein observed was proportional to the amount of *KEM1* RNA (Figure 5). Overproduction of KEM1, at the levels we observed, did not have deleterious effects on growth.

**Disruption of *KEM1*:** Diploid yeast strain JK200 was transformed with a linear *Bam*HI-*Xho*I fragment derived from plasmid pJI90. $Ura^+$ diploid transformants were selected, sporulated and tetrads dissected. All four spores derived from this diploid were viable, however, two spore derived colonies from each tetrad grew extremely slowly. The bottom array in Figure 6 shows the 2:2 segregation of this slow growth phenotype. The *URA3* disruption marker and the slow growth phenotype co-segregated indicating that the slow growth phenotype is due to disruption of the *KEM1* gene. This result was confirmed by Southern blot analysis of DNA obtained from the slow growing spore derived colonies. Genomic DNA was prepared from strain JK200, a $Ura^+$ diploid transformant and each of the four spores from a single tetrad. The altered DNA pattern co-segregated with the slow growth phenotype (data not shown). Southern blot experiments with genomic yeast DNA at both low and high stringency indicate that the *KEM1* gene is present

region), the phagemid templates from which sequence data was obtained are diagramed above and below the map. The 5′ and 3′ flanking regions that have not been sequenced are depicted as thin solid lines. The 4.58-kb *KEM1* open reading frame is shown as a solid arrow, and the 5′ untranslated sequence present in *KEM1* mRNA is shown as the cross-hatched region. Restriction endonuclease sites are labelled as follows: B, *Bam*HI; C, *Cla*I; E, *Bst*EII; G, *Bgl*II; H, *Hin*dIII; N, *Nco*I; P, *Pvu*II; R, *Nru*II; S, *Sna*BI; V, *Eco*RV; X, *Xho*I. The positions of restriction sites enclosed within parentheses are approximated. (b) Nucleotide sequence of the *KEM1* gene and the deduced amino acid sequence. Nucleotide residues are numbered relative to the ATG (+1) that initiates the open reading frame. *KEM1* mRNA transcription starts within a region −372 to −337 bp (bold face nucleotides with reduced font size) 5′ from the initiation codon (data not shown). The identified region of the mRNA start site is approximately 90 nucleotides downstream from a putative TATA box (underlined bold face nucleotides with reduced font size).

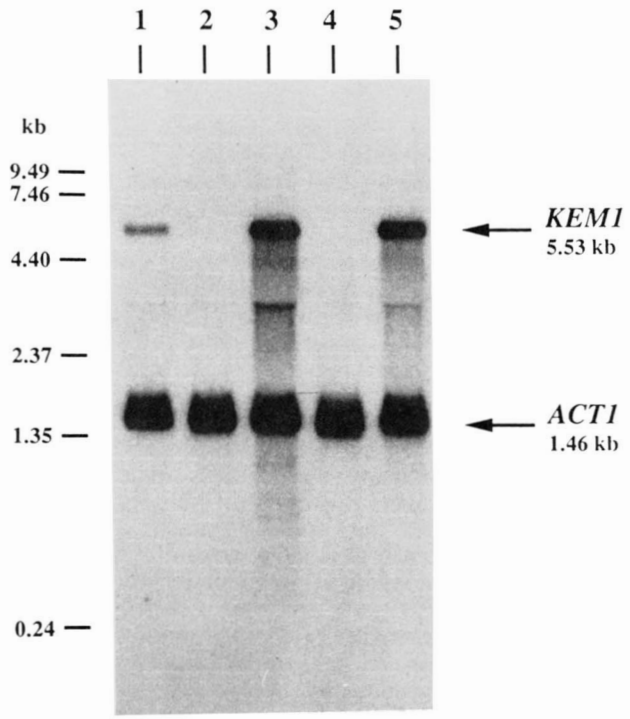


FIGURE 5.—Northern analysis of RNA isolated from exponentially growing yeast strains with zero, single and multiple copies of *KEM1*. Multiple copies of *KEM1* were introduced by transforming cells with the 2$\mu$ based plasmid pJI98. Five micrograms of total RNA were added in each lane. *KEM1* and *ACT1* transcripts were detected by hybridization to 1.28 kb *Eco*RV *KEM1* fragment and 1.65-kb *Bam*HI/*Hin*dIII *ACT1* fragment (GALLWITZ and SURES 1980; NG and ABELSON 1980), respectively. The 1.4-kb actin mRNA was used to standardize RNA loading. Lanes contain RNA prepared from isogenic strains as follows: lane 1, JK251 *KEM1* (single copy); lane 2, JK246 *kem1Δ3::LEU2* (zero copy); lane 3, JK278 *KEM1* (pJI98) (multiple copy); lane 4, JK280 *kem1Δ3::LEU2* (YEp24) (zero copy, vector control); lane 5, JK282 *kem1Δ3::LEU2* (pJI98) (multiple copy).

as a single copy in the haploid yeast genome (data not shown). Although the *KEM1* gene is not essential, the slow growth phenotype associated with the disruption allele suggests that the *KEM1* gene product is important for vegetative growth.

**A *kem1* null mutation affects spindle pole body duplication, cell viability upon nitrogen starvation, and sporulation:** Strains carrying a *kem1* null allele, *kem1Δ2::URA3*, exhibit altered cell morphology during mitotic cell growth. About 80% of *kem1Δ2::URA3* cells have an elongated rod shape and the size of these cells is roughly twice that of wild-type cells. In budding wild-type cells when the daughter bud is half the size of the mother cell, the spindle pole body appears duplicated and both spindle pole bodies appear connected by microtubules. When wild-type cells have progressed to the stage when mother and bud are of equal size, the nucleus is elongated and separated by long intranuclear microtubules (Figure 7a, A–F). In *kem1Δ2::URA3* strains, many large-budded cells contained a single focus of antitubulin staining (Figure

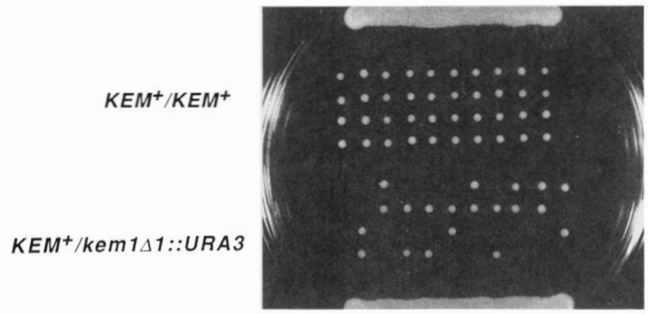


FIGURE 6.—Tetrad analysis of spores from control *KEM1/KEM1* (upper array) and *KEM1/kem1Δ1::URA3* (lower array) diploid strains. The upper array is tetrads from JK200 (*KEM1/KEM1*). The lower array of tetrads is from a Ura$^+$ transformant of diploid strain JK200 transformed with a linear *Bam*HI-*Xho*I DNA fragment derived from pJI90 (see MATERIALS AND METHODS). The resulting strain has a single intact and disrupted copy of the *KEM1* gene (*KEM1/kem1Δ1::URA3*). The plate was incubated for 2 days at 30° and photographed.

7b, G-I). This stained region presumably represents a single unduplicated spindle pole body or duplicated but unseparated spindle pole bodies. Ten percent of *kem1Δ2::URA3* cells contain two nuclei in one cell body (Figure 7b, J–O). We never observed two nuclei in one cell body in budding wild-type cells.

We examined and compared the ability of wild-type diploids and diploids homozygous for *kem1-1*, *kem1-5*, or *kem1Δ2::URA3* to sporulate. Diploid strains were transferred onto sporulation media, changes in cell morphology and nuclear DAPI-staining were monitored microscopically for a period of five days. A minimum of 200 cells from each diploid strain were examined. During the period of observation, 40–60% of wild-type diploid cells sporulated. Under the same conditions diploid strains homozygous for *kem1-1*, *kem1-5*, or *kem1Δ2::URA3* failed to sporulate. Diploids heterozygous for *kem1* sporulated as efficiently as wild-type strains, indicating that the sporulation defect is recessive. The sporulation phenotype cosegregated 2:2 with the *kem1* mutation.

*kem1* mutant cells (*kem1-1*, *kem1-5*, *kem1Δ2::URA3*) lose viability upon prolonged incubation on minimal media lacking nitrogen. Loss of viability was assayed qualitatively by replica plating colonies grown on nitrogen starvation medium for 4 days at 30° onto SC medium. The loss of viability phenotype cosegregated 2:2 with the *kem1* mutation. The response of *kem1* mutant cells to nitrogen starvation was also examined quantitatively by growing cells to log phase and then shifting them from YEPD rich medium to minimal media lacking nitrogen. Under this regime, wild-type cells complete their cell cycle, arrest uniformly as unbudded cells, and remain viable in this state for long periods of time. The initial response of the *kem1* mutants during the first 20 hr after the shift to medium without nitrogen was the same as that of wild-type cells; 90–95% of the cells arrested as unbudded

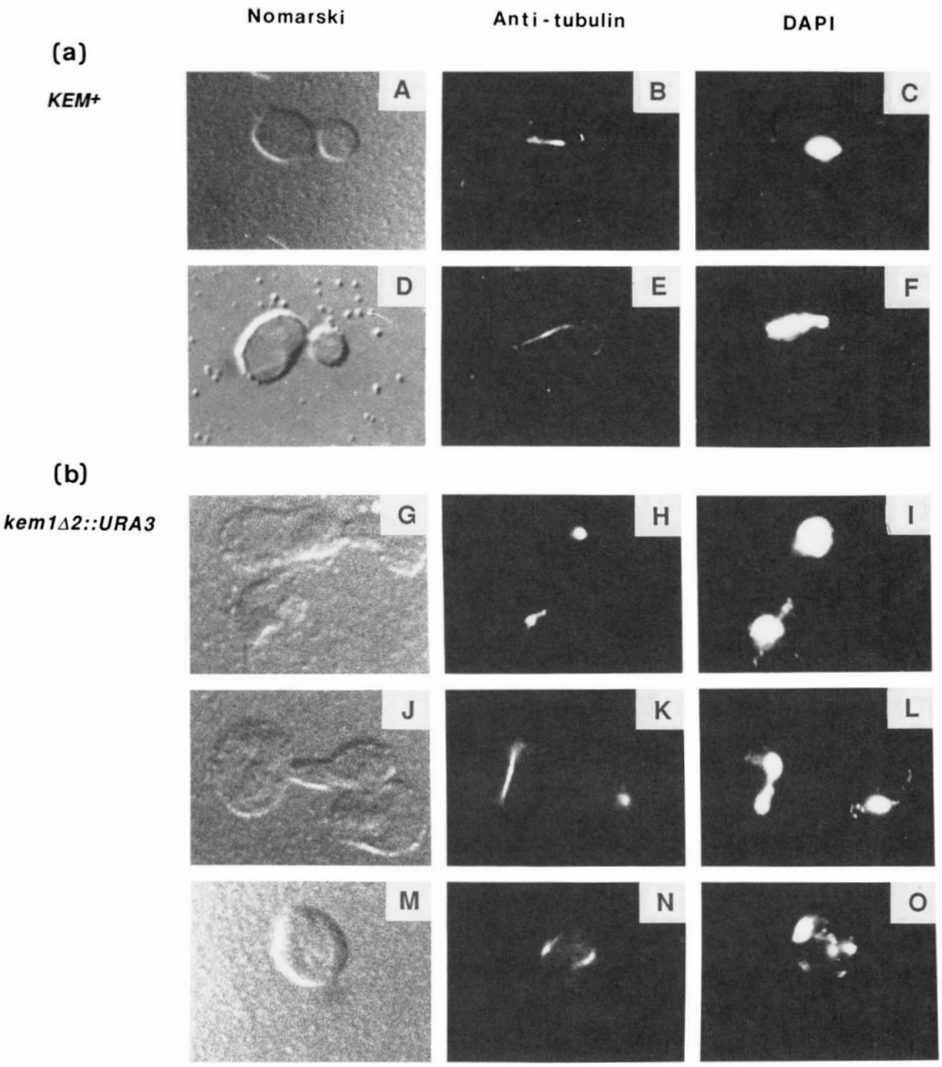


FIGURE 7.—Nuclear and microtubule staining of large-budded cells of *KEM1* and *kem1Δ2::URA3* cells. Exponentially growing cells were fixed, the microtubules were visualized by immunofluorescence using anti-tubulin antibodies, and the nuclear DNA was stained with DAPI. Each row of photomicrographs represents the same cell observed by Nomarski optics, anti-tubulin and DAPI staining, respectively. (a) Two cells (A–C and D–F) from strain JK251 (*KEM1*) are shown. (b) Three cells (G–I, J/L and M/O) from strain JK245 (*kem1Δ2::URA3*) are shown.

cells with no loss in viability. However, upon further incubation *kem1* mutants lost viability, whereas *KEM1* cells did not (Figure 8).

## DISCUSSION

New mutations that enhance the nuclear fusion defect in *kar1-1* crosses define at least three genes: *KEM1*, *2* and *3*. Some of these mutations have a dramatic effect on nuclear fusion. For example, the ability of *kar1-1* cells to form diploids is reduced more than 100-fold by mutations in the *KEM1* gene. One striking aspect of *kem* mutations is that they reduce nuclear fusion whether the *kem* and the *kar1-1* mutations are in the same or in different parents (Tables 3 and 4). This observation suggests that failure of nuclear fusion in *kem* × *kar1-1* crosses, but not in *kem* × *KAR1* crosses, must be a consequence of the unusual nature of the residual nuclear fusions that occur in a *kar1-1* cross. One explanation for the successful, albeit reduced, nuclear fusion in *kar1-1* strains is that the *kar1-1* allele is leaky. This leakiness could lead to the formation of an unstable, partially functional, spindle pole body. Perhaps during conjugation a few of the cells in the *kar1-1* population have spindle pole bodies with sufficient stability to complete nuclear fusion successfully. The partial spindle pole body stability in these competent *kar1-1* cells could require two intact copies of each *KEM* gene (one from each parent) to retain sufficient structural integrity.

Mutations in the *KEM1* gene have a number of diagnostic phenotypes in the absence of the *kar1-1* mutation: (1) *kem1* mutations cause a bilateral nuclear fusion defect, (2) *kem1* strains are hypersensitive to benomyl, a microtubule destabilizing reagent, (3) *kem1/kem1* diploids show a defect in mitotic chromosome stability, (4) *kem1* strains are sensitive to nitrogen starvation and (5) homozygous *kem1* diploids fail to sporulate. A null mutation of *KEM1* displays all these phenotypes and in addition leads to extremely slow growth, defects in spindle pole body duplication and/or separation, and aberrant nuclear division. Despite these defects *kem1* null mutants are alive.

Although we did not detect a genetic interaction between *kem1-1* and mutations in either β- or α-tubu-

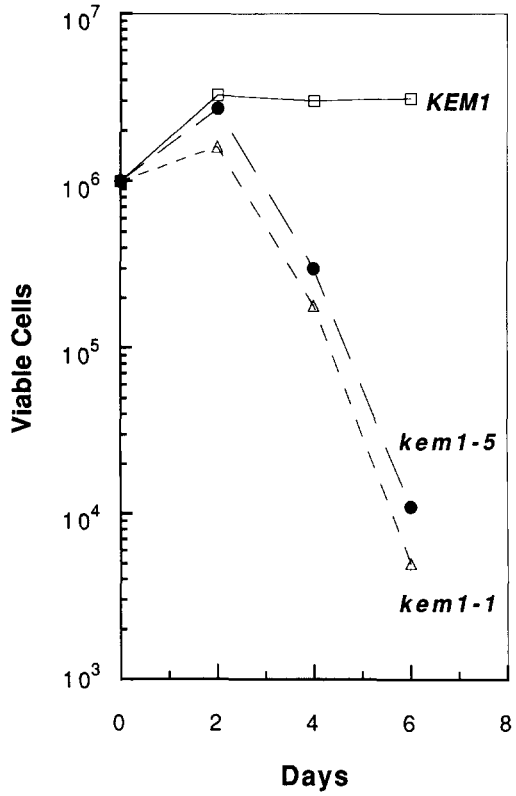


FIGURE 8.—Viability of *KEM1* and *kem1* mutant cells after nitrogen starvation. Exponentially growing cultures of *KEM1* (JK204), *kem1-1* (JK205), and *kem1-5* (JK191) were centrifuged, washed and resuspended at a cell density of $1 \times 10^6$ cells/ml in nitrogen starvation medium. After various incubation periods at 30°, samples were removed from cultures and aggregated cells were dispersed by sonication. Cell viability was determined by spreading appropriately diluted cell suspensions on YEPD plates, incubating plates 3–4 days at 30° and counting the number of colonies that formed.

lin genes (*tub2-104*, THOMAS, NEFF and BOTSTEIN 1985; *tub1-1*, STEARNS, HOYT and BOTSTEIN 1990, respectively) when double mutant strains were constructed, the *kem1* mutants are hypersensitive to the antimicrotubule drug benomyl (Figure 3). In *S. cerevisiae*, mutations conferring resistance to relatively high concentrations of benomyl occur almost exclusively in the β-tubulin gene *TUB2* (THOMAS, NEFF and BOTSTEIN 1985). Conversely, certain mutations in α-tubulin genes (cold-sensitive mutations in *TUB1* or a null allele of *TUB3*) result in hypersensitivity to benomyl (SCHATZ, SOLOMON and BOTSTEIN 1986; STEARNS, HOYT and BOTSTEIN 1990). Similarly, in *Aspergillus nidulans*, mutations in the β-tubulin gene were isolated by selecting for resistance to benomyl (SHEIRR-NEISS, LAI and MORRIS 1978) and hypersensitivity mutations were found in an α-tubulin gene (OAKLEY and MORRIS 1974). The benomyl-sensitive phenotype of *kem1* mutants implies that the *KEM1* gene may be required for normal microtubule function.

Many of the defects of *kem1* mutants could be explained by assuming that *KEM1* is a structural component required for proper assembly of the spindle. This model could explain the enhanced frequency of chromosome loss during mitotic cell division (Table 6) and the block in nuclear fusion (Figure 2 and Table 5). Alternatively, KEM1 may influence spindle function or assembly in an indirect way. The sporulation defect and increased sensitivity to nitrogen starvation of *kem1* mutants (Figure 8) closely resembles the phenotypes of mutants implicated in the RAS/adenylate cyclase pathway (*RAS2*$^{val19}$ and *bcy1*) (KATAOKA *et al.* 1984; UNO, MATSUMOTO and ISHIKAWA 1982; TODA *et al.* 1987) as well as that of the *ypt1* mutant (SEGEV and BOTSTEIN 1987). Studies with a cold-sensitive *ypt1* mutation (SEGEV and BOTSTEIN 1987) and galactose regulated *YPT1* (SCHMITT *et al.* 1986) have shown that a reduced concentration of functional YPT1 protein results in aberrant arrangements of microtubules. These observations support the notion that the function of microtubules can be influenced by intracellular signals concerning nutritional conditions. According to this view, the *KEM1* gene may specify a signaling or sensing function, relaying nutritional and/or developmental signals to the spindle pole body, which is required for proper microtubule function during conjugation and mitotic cell growth.

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