

Separate Domains of *KAR1* Mediate Distinct Functions in Mitosis and Nuclear Fusion

Elizabeth A. Vallen, Mark A. Hiller, Talma Y. Scherson, and Mark D. Rose

Department of Molecular Biology, Lewis Thomas Laboratory, Princeton University, Princeton, New Jersey 08544-1014

Abstract. The yeast *KAR1* gene is essential for mitotic growth and important for nuclear fusion. Mutations in *KAR1* prevent duplication of the spindle pole body (SPB), and affect functions associated with both the nuclear and cytoplasmic microtubules. The localization of hybrid *Karl-lacZ* proteins, described elsewhere (Vallen, E. A., T. Y. Scherson, T. Roberts, K. van Zee, and M. D. Rose. 1992. *Cell*. In press), suggest that the protein is associated with the SPB. In this paper, we report a deletion analysis demonstrating that the mitotic and karyogamy functions of *KAR1* are separate and independent, residing in discrete functional domains. One region, here shown to be essential for mitosis, coincided with a part of the protein that is both necessary and sufficient to target *Karl-lacZ* hybrid proteins to the SPB (Vallen, E. A., T. Y. Scherson, T. Roberts, K. van Zee, and M. D. Rose. 1992. *Cell*. In press). Complementation testing demon-

strated that deletions in this interval did not affect nuclear fusion. A second region, required only for karyogamy, was necessary for the localization of a *Kar3-lacZ* hybrid protein to the SPB. These data suggest a model for the roles of *Karlp* and *Kar3p*, a kinesin-like protein, in nuclear fusion. Finally, a third region of *KAR1* was found to be important for both mitosis and karyogamy. This domain included the hydrophobic carboxy terminus and is sufficient to target a *lacZ-Karl* hybrid protein to the nuclear envelope (Vallen E. A., T. Y. Scherson, T. Roberts, K. van Zee, and M. D. Rose. 1992. *Cell*. In press). Altogether, the essential mitotic regions of *KAR1* comprised 20% of the coding sequence. We propose a model for *Karlp* in which the protein is composed of several protein-binding domains tethered to the nuclear envelope via its hydrophobic tail.

IN eukaryotic cells, the microtubule organizing center plays a crucial role in nucleating the microtubules responsible for mitosis, meiosis, and the formation of the interphase cytoskeleton. In yeast, the microtubule organizing center is called the spindle pole body (SPB)¹ and is comprised of the spindle plaque (a disc-shaped structure embedded in the nuclear envelope) and associated osmophilic material. Mitosis, meiosis, and conjugation are associated with distinct and specific alterations in the morphology of the SPB (Byers and Goetsch, 1974, 1975; Byers, 1981).

As the nuclear envelope remains intact throughout mitosis, opposite faces of the spindle plaque are associated with the nuclear and cytoplasmic microtubules. Cytoplasmic microtubules are specifically required for nuclear movement and karyogamy (Delgado and Conde, 1984; Huffaker et al., 1988). In zygotes, the cytoplasmic microtubules span the gap between the two haploid parental nuclei. The nuclei then move together and nuclear fusion is initiated at or near the SPB to form the diploid nucleus (Byers and Goetsch, 1974, 1975).

Mutations in several genes (*KAR1*, *KAR2*, and *KAR3*) have

been isolated that cause severe defects in nuclear fusion (Conde and Fink, 1976; Fink and Conde, 1976; Polaina and Conde, 1982). Aside from their roles in karyogamy, all three genes have important or essential functions in various mitotic processes. *KAR1* is required for SPB duplication and various *kar1* mutations influence the length and morphology of cytoplasmic microtubules in mitotic and mating cells (Rose and Fink, 1987). Consistent with the genetic data, the localization of hybrid gene products containing parts of the *Karl* protein suggested that *Karlp* is associated with the SPB (Vallen et al., 1992). *KAR2* encodes the yeast homologue of mammalian BiP/GRP78, the HSP70 protein resident in the ER (Rose et al., 1989; Normington et al., 1989). *KAR2* has been implicated in the translocation and processing of secreted proteins (Vogel et al., 1990; Vogel, J., and M. Rose, unpublished observations). *KAR3* encodes a kinesin-like motor protein that has been implicated in antiparallel sliding of microtubules during both nuclear fusion and mitosis (Meluh and Rose, 1990). Major questions concerning the roles of the *KAR* genes in nuclear fusion are whether the mutant phenotypes arise as a consequence of prior defects in mitotic functions and whether their roles in mitosis and nuclear fusion are separate and distinct.

In this paper, we identify three distinct domains of *KAR1*

1. Abbreviations used in this paper: 5-FOA, 5-fluoro-orotic acid; SPB, spindle pole body.

that are required for mitotic viability and karyogamy. Remarkably, only two small regions, which together comprise 20% of the predicted protein, are uniquely important for viability. One region is essential for mitosis and may be involved in the association of the protein with the SPB. The second region is important for both viability and nuclear fusion. As the second region is composed of hydrophobic residues, it may form a membrane spanning domain. The third region is required only for nuclear fusion; deletion mutations have profound effects on the length of cytoplasmic microtubules and the localization of Kar3-lacZ hybrid proteins. Taken together, these data suggest a model for the structure of Karlp and for its role, together with Kar3p, in yeast nuclear fusion.

Materials and Methods

Strains and Microbial Techniques

Yeast strains used are listed in Table I. Yeast media and genetic techniques were essentially as described by Rose et al. (1990). Yeast transformations were performed by the lithium acetate procedure of Ito et al. (1983) using 50 μ g of sheared, denatured carrier DNA. Transformants were selected on synthetic complete media lacking uracil or leucine. Ura⁻ segregants of transformants were selected on 5-fluoro-orotic acid (5-FOA) medium as described by Boeke et al. (1984). Small scale plasmid DNA preparations were made by the boiling lysis method of Holmes and Quigley (1982). Yeast DNA for Southern blot analysis and PCR reactions was prepared by the method of Hoffman and Winston (1987).

Quantitative matings of yeast strains were performed essentially as described by Dutcher and Hartwell (1982). Approximately 5×10^6 exponentially growing cells of each parent were mixed together and concentrated on a 0.45- μ m pore size nitrocellulose filter. The mating mixtures were incubated for 5 h on yeast extract-peptone-dextrose (YPD) at 30°C and then diluted in sterile H₂O. Cells were plated on YPD, selective synthetic medium, and yeast extract-peptone-glycerol with cycloheximide (3 μ g/ml) to determine the titer of viable cells, diploids, and cytoductants, respectively.

For the semi-quantitative plate mating assay, a modification of the standard mating type test protocol was used (Rose et al., 1990). Fresh lawns of a wild type strain and fresh patches of strains to be tested were inoculated by replica plating the day before the mating. For mating, strains were replica plated together onto YPD plates and allowed to mate for 3 h at 30°C. Mating plates were then replica printed to synthetic medium to select for diploid cells. The frequency of diploid formation was compared to *KARI* and *karl-1* controls on the same plate. For the analysis of mutations on *CEN*-based vs. 2 μ m-based plasmids, we compared strains by testing them together on the same mating plate.

Plasmid Constructions

All enzymes were used according to the specifications of their suppliers. Linkers were purchased from Pharmacia Fine Chemicals (Piscataway, NJ), New England Biolabs (Beverly, MA), or synthesized at Princeton University. The construction of plasmids pMR95 (*karl-S72*), pMR97 (*karl-S88*), and pMR99 (*karl-S96*) has been previously described (Rose and Fink, 1987).

Two codon insertions into the TaqI and SspI enzyme recognition sites within the *KARI* gene were isolated by a technique described by Barany (1985a,b, 1988). DNA was linearized by restriction enzyme digestion in the presence of ethidium bromide, oligonucleotide linkers were added, and the plasmid was reclosed with DNA ligase. pCGTCGA (27-8546-01; Pharmacia Fine Chemicals) was inserted into TaqI sites to form *KARI-Q250*, *KARI-Q260*, and *KARI-Q270* (plasmids pMR1071, 1294, and 1295, respectively). CCGCGG (27-8430-01; Pharmacia Fine Chemicals) was inserted into SspI sites to form *KARI-P210*, *KARI-P220*, *karl-P230*, and *karl-P240* (plasmids pMR1420, 1422, 1424, and 1426). These insertions were isolated in plasmid pMR326, a pSB32 (gift of G. Fink, M.I.T., Cambridge, MA) derivative containing the *KARI* gene on a genomic HindIII-BamHI fragment of \sim 2.6 kb, *LEU2* and *CEN4*. Linker insertions were mapped by the acquisition of a novel restriction enzyme site. When phosphorylated linkers were used, multiple linker insertions were detected by the appearance of an additional restriction site created by the junction of two or more linkers.

To facilitate certain deletion constructions, 8-, 10-, and 12-bp linker insertions were isolated at two sites within *KARI*. For one set, pMR76 (*KARI* on YCp50) was linearized with SacI and blunted with T4 DNA polymerase. CGTCGACG, CCGTCGACCG, or CCGGTCGACCGG were ligated onto the blunt ends before reclosure to form *karl-C281*, *KARI-C282*, and *karl-C283* (pMR1449, 1451, and 1453), respectively. Similar sets of insertions were made into pMR1422 (*KARI-P220*) after cleavage with SacII and blunting with T4 DNA polymerase to form *KARI-P221*, *karl-P222*, and *karl-P223* (pMR1514, 1516, and 1518).

Duplications within the coding sequence of *KARI* were constructed from various linker insertions by ligating fragments containing a 5' portion of the gene on a BamHI-Sall or BamHI-XhoI fragment to fragments containing a 3' portion of the gene on a Sall-AatII or XhoI-AatII fragment. This resulted in the duplication of the region of the gene carried on both fragments. The fusion joints of these pairs of sequences were the linker insertions previously isolated (Rose and Fink, 1987). These duplications were then cloned into pSB32.

Deletion alleles of *KARI* were constructed on YCp50 in a manner similar to the duplications described above. Linker insertions previously described (Rose and Fink, 1987) as well as those described above were used. To construct $\Delta 27$, a Sall-StuI adaptor (TCGAGGCC) was inserted in the Sall site at the junction between the 5' and 3' fragments. To make $\Delta 29$, the plasmid containing the 5' and 3' fragments was digested with Sall, and the ends were filled in with the Klenow fragment of DNA polymerase I before reclosure of the plasmid. A complete deletion of the hydrophobic tail of *KARI* (*karl-P231*) was created by digesting *karl-P230* with SacII, blunting the ends with T4 DNA polymerase and inserting an Xba linker (CTCTAGAG) before religating. This resulted in a termination codon at position 413.

Analysis of Mutations

The phenotypes of the insertions were investigated either by the "spore viability test" (Rose and Fink, 1987) or by the "plasmid shuffle" method (Boeke et al., 1987). For spore viability, a diploid strain heterozygous for a deletion of *KARI* (MS136) was transformed with a centromere-based, *URA3* marked plasmid containing the mutant *KARI* gene. Transformants were sporulated and tetrads were dissected. Failure of the plasmid to suppress the chromosomal deletion resulted in only two viable spores per tetrad. Suppression of the chromosomal deletion allele resulted in two, three, or four viable spores from each tetrad.

We assayed for the presence of the plasmid by replica printing to media to select for the plasmid marker, usually *URA3*. In all cases, including those in which the plasmid could not suppress the chromosomal deletion of *KARI*, the plasmid was transmitted to a subset of the meiotic progeny. Therefore, the inviability associated with some of the deletions was not due to absence of the plasmid.

A caveat of the spore viability assay is that a mutation that only blocks spore germination cannot always be distinguished from a mutation that causes inviability. However, previous analysis has demonstrated that spores carrying a deletion of *KARI* are able to germinate and divide a few times (Rose and Fink, 1987). Although it is possible that *KARI* is not required for germination and the first few mitotic divisions, it is more likely that spores contain enough *KARI* mRNA or protein to proceed through several cell divisions. Regardless of the mechanism, these data suggest that the inviability caused by certain *KARI* mutations results from a mitotic, and not a germination, defect.

For plasmid shuffling, a haploid strain (MS397) was constructed that contained a deletion of *KARI* on the chromosome and a wild type *KARI* gene on a *URA3* marked centromeric plasmid (pMR76). For these experiments, the mutant *KARI* gene was carried on a *LEU2* marked plasmid, pMR326. Mutant derivatives of plasmid pMR326 were transformed into MS397 and transformants were subsequently grown on 5-FOA; Ura⁺ cells die but Ura⁻ cells continue to grow on this media. Functional *KARI* on pMR326 can suppress the chromosomal deletion so that cells can lose pMR76 by missegregation and become Ura3⁻ and 5-FOA^R. However, if the strain is dependent on the presence of the wild type *KARI* gene for viability, it will be 5-FOA^S because it cannot lose the *URA3*-based pMR76. Subsequently, strains containing mutant alleles of *KARI* generated either by plasmid shuffling or by the "spore viability test" were tested for heat and cold sensitive growth and karyogamy.

Certain mutations that failed to complement a chromosomal deletion of *KARI* had lower steady state levels of Kar1 protein as determined by Western blot analysis. To determine whether increased levels of these proteins would allow complementation, the deletion alleles were subcloned as a HindIII-EcoRI fragment onto the 2 μ m-based vector, CGS42 (J. Maio; Collaborative Genetics, Bedford, MA). These plasmids were then trans-

Table I. Yeast Strains

Strain	Genotype	Source*
MS10	<i>MATa ura3-52 leu2-3 leu2-112 ade2-101</i>	
MS52	<i>MATα ura3-52 leu2-3 leu2-112 trp1-Δ1</i>	
MS136	<i>MATa/α ura3-52/ura3-52 lys2-801/+ his4-539/+ kar1-102/+</i>	
MS147	<i>MATa trp1-Δ1 lys2-801 ade2-101 cyh^R [rho^o]</i>	
MS397	<i>MATα ura3-52 leu2-3 leu2-112 kar1-102</i> [pMR76: YCp50 <i>KAR1</i>]	
MS739	<i>MATα ura3-52 leu2-3 leu2-112 ade2-101 kar1-1</i>	
MS751	<i>MATa ura3-52 leu2-3 leu2-112 ade2-101</i> [pMR1300: YCp50 <i>KAR3-lacZ</i> hybrid]‡	
MS1113	<i>MATα ura3-52 leu2-3 leu2-112 trp1-Δ1 kar1-Δ15</i>	
MS1117	<i>MATα ura3-52 leu2-3 leu2-112 trp1-Δ1 kar1-Δ13</i>	
MS1274	<i>MATa ura3-52 trp1-Δ1 lys2-801 cyh^R kar1-Δ13</i>	
MS1461	<i>MATα ura3-52 leu2-3 leu2-112 trp1-Δ1 kar1-Δ13</i> [pMR1491: YCp50 <i>kar1-Δ17</i>]	
MS1463	<i>MATα ura3-52 leu2-3 leu2-112 trp1-Δ1 kar1-Δ13</i> [pMR1493: YCp50 <i>kar1-Δ18</i>]	
MS1465	<i>MATα ura3-52 leu2-3 leu2-112 trp1-Δ1 kar1-Δ13</i> [pMR1495: YCp50 <i>kar1-Δ19</i>]	
MS1467	<i>MATα ura3-52 leu2-3 leu2-112 trp1-Δ1 kar1-Δ13</i> [pMR1497: YCp50 <i>kar1-Δ20</i>]	
MS1473	<i>MATα ura3-52 leu2-3 leu2-112 trp1-Δ1 kar1-Δ13</i> [pMR76: YCp50 <i>KAR1</i>]	
MS1475	<i>MATα ura3-52 leu2-3 leu2-112 trp1-Δ1 kar1-Δ13</i> [YCp50]	
MS2059	<i>MATα ura3-52 leu2-3 leu2-112 trp1-Δ1 kar1-Δ13</i> [pMR95: YCp50 <i>kar1-S72</i>]	
MS2102	<i>MATa ura3-52 leu2-3 leu2-112 kar1-Δ13</i> [pMR1300: YCp50 <i>KAR3-lacZ</i> hybrid]‡	
MS2111	<i>MATα ura3-52 leu2-3 leu2-112 trp1-Δ1 kar1-Δ13</i> [pMR1738: pCGS42 <i>kar1-Δ17</i>]	
MS2113	<i>MATα ura3-52 leu2-3 leu2-112 trp1-Δ1 kar1-Δ13</i> [pMR1740: pCGS42 <i>kar1-Δ18</i>]	
MS2115	<i>MATα ura3-52 leu2-3 leu2-112 trp1-Δ1 kar1-Δ13</i> [pMR1742: pCGS42 <i>kar1-Δ19</i>]	
MS2117	<i>MATα ura3-52 leu2-3 leu2-112 trp1-Δ1 kar1-Δ13</i> [pMR1744: pCGS42 <i>kar1-Δ20</i>]	
MS2124	<i>MATα ura3-52 leu2-3 leu2-112 trp1-Δ1 kar1-Δ13</i> [pMR1752: pCGS42 <i>kar1-Δ13</i>]	
MS2127	<i>MATα ura3-52 leu2-3 leu2-112 trp1-Δ1 kar1-Δ13</i> [pCGS42]	
MS2133	<i>MATα ura3-52 leu2-3 leu2-112 trp1-Δ1 kar1-Δ13</i> [pMR1713: pCGS42 <i>kar1-S72</i>]	
MS2135	<i>MATα ura3-52 leu2-3 leu2-112 trp1-Δ1 kar1-Δ13</i> [pMR1552: YCp50 <i>kar1-Δ13</i>]	
MS2989	<i>MATα ura3-52 leu2-3 leu2-112 trp1-Δ1 kar1-Δ13</i> [pMR1426: pSB32 <i>kar1-P240</i>]	
MS2990	<i>MATα ura3-52 leu2-3 leu2-112 trp1-Δ1 kar1-Δ13</i> [pMR2399: pCGS42 <i>kar1-P240</i>]	
MS2991	<i>MATα ura3-52 leu2-3 leu2-112 trp1-Δ1 kar1-Δ13</i> [pMR2323: pSB32 <i>kar1-P231</i>]	
MS2992	<i>MATα ura3-52 leu2-3 leu2-112 trp1-Δ1 kar1-Δ13</i> [pMR2400: pCGS42 <i>kar1-P231</i>]	
MY603	<i>MATa ura3-52 ino1-1 SUP^{UGA} kar1-103</i>	Rose and Fink (1987)
MY606	<i>MATa ura3-52 ino1-1 SUP^{UGA} kar1-104</i>	Rose and Fink (1987)
MY749	<i>MATa ura3-52 ino1-1 SUP^{UGA} kar1-105</i>	Rose and Fink (1987)
BWG1-7A	<i>MATa ura3-52 leu2-3 leu2-112 his4-519 ade1-100 GAL⁺</i>	L. Guarente
MY913	<i>MATa ura3-52 leu2-3 leu2-112 his4-519 ade1-100 GAL⁺</i> [pMR260: YCp50 p <i>GAL1-KAR1</i>]§	
TD28	<i>MATa ura3-52 ino1-1 SUP^{UGA}</i>	Rose and Fink (1987)
8960-11b	<i>MATα ura3-52 leu2-3 kar1-1</i>	Rose and Fink (1987)
XW2-20A	<i>MATa his4 leu1 can1 nys^R thr kar1-2</i>	Polaina and Conde (1982)
JPI1	<i>MATα ade2-1 his4-15 can1 nys^R kar1-5</i>	Polaina and Conde (1982)
JPJ1	<i>MATα ade2-1 his4-15 can1 nys^R kar1-3</i>	Polaina and Conde (1982)
JPK2	<i>MATα ade2-1 his4-15 can1 nys^R kar1-4</i>	Polaina and Conde (1982)

* Unless otherwise noted, all strains are from this study.

‡ Meluh and Rose (1990). pMR1300 contains the amino-terminal 309 codons of *KAR3* fused to the 5' end of the *lacZ* gene.

§ Rose and Fink (1987).

formed into MS136 and assayed for complementation in the spore viability test and transformed into MS117 to assay for complementation of the karyogamy defect.

Mapping of Mutations and DNA Sequence Analysis

Temperature sensitive and karyogamy deficient alleles of *KARI* were mapped by the method of gap repair (Orr-Weaver et al., 1983). Plasmids containing the deletion alleles of *KARI* described above were linearized with *Sal*I or *Xho*I at the site of the deletion. Linearized plasmids were transformed into the mutant strain, and transformants were screened either for temperature resistance by replica plating to 37°C, or for karyogamy proficiency by the limited mating plate assay (Rose and Fink, 1987). Transformation by gapped plasmids is dependent upon repair templated by the chromosomal *karl* allele. If the mutation was in the deleted region, all transformants repaired the gap using the mutated DNA and displayed the mutant phenotype. However, if the mutation was not within the deleted region, repair from the chromosome usually reconstituted a wild type copy of the gene. By this analysis, temperature sensitive alleles *karl-103*, *karl-104*, and *karl-105* (Rose and Fink, 1987) were mapped between amino acids 247 and 288. Karyogamy defective alleles *karl-1*, *karl-2*, *karl-3*, *karl-4*, *karl-5* (Polaina and Conde, 1982), and *karl-106* (Kadish, D., and M. Rose, unpublished observations) were mapped between amino acids 118 and 191.

Regions genetically defined to contain mutations were sequenced by the dideoxy method of Sanger et al. (1977) using Sequenase (USB, Cleveland, OH). Temperature sensitive *karl* alleles were sequenced from pMR266, pMR267, and pMR268 (Rose and Fink, 1987) as double-stranded supercoiled plasmid templates. For the karyogamy defective *karl* alleles, single-stranded DNA from the region containing the mutation was isolated by an asymmetric PCR amplification using a 100:1 ratio of primers (Kreitman and Landweber, 1989). The single-stranded DNA produced in this reaction was then sequenced using the limiting primer in the PCR reaction to prime synthesis in the sequencing reaction.

Immunological Techniques

Rabbit anti-KAR1 serum was prepared against a fusion protein containing the amino-terminal 190 amino acids of *Karlp* fused to *E. coli* *trpE* protein as described (Rose et al., 1989).

For Western blots, total yeast proteins were extracted by the method of Ohashi et al. (1982) and separated by SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose (Burnette, 1981; Towbin et al., 1979). Nonspecific antibodies were reduced by preadsorbing diluted anti-KAR1 serum (1:150) to a filter containing electrophoretically transferred proteins from a strain carrying *karl-Δ13* (deleted for the epitopes used for immunization). Antibody binding was visualized using ¹²⁵I-labeled protein A (Amersham Chemical Corp., Arlington Heights, IL) and autoradiography. Immunofluorescent staining of yeast cells was performed by a modification of the methods of Adams and Pringle (1984) and Kilmartin and Adams (1984), as described by Rose and Fink (1987). Rabbit antiserum (RAP 124) directed against yeast β -tubulin was a generous gift from F. Solomon (M.I.T., Cambridge, MA). The rat anti-yeast α -tubulin mAb YOL1/34 (Kilmartin et al., 1982) was obtained from Accurate Chemical & Scientific Corp. (Westbury, NY). Mouse anti- β -galactosidase mAb was from Promega Biotec (Madison, WI). Rabbit anti- β -galactosidase polyclonal antibody was from Cappel Research Reagents (Malvern, PA). FITC and rhodamine-conjugated secondary antibodies were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). The fluorescent DNA-specific dye 4',6'-diamidino-2-phenylindole (DAPI) was used to visualize yeast nuclei.

Wild type cells and cells containing karyogamy deficient *karl* alleles were grown to mid-logarithmic phase at 30°C in YPD or SC lacking uracil for plasmid-containing strains. In either case media were adjusted to pH 4.0 with HCl. For α -factor arrest, cultures were diluted 1:1 with fresh media, supplemented with α -factor (Sigma Chemical Co., St. Louis, MO) to 5 μ M and incubated at 30°C for 3 h.

Construction of Strains Containing Karyogamy Deficient Alleles of *KARI*

Isogenic strains that carry chromosomal copies of the karyogamy deficient *karl* deletions as well as *karl-1* were constructed. For alleles $\Delta 13$ and $\Delta 15$, YCp50-based plasmids containing the mutations were digested with *Tth*III and religated, thereby deleting *CEN4* and *ARS1* and converting them into YIp plasmids. These plasmids were linearized with *Bgl*II to direct integration into the *KARI* locus (Orr-Weaver et al., 1983) and transformed into yeast. Purified transformants were subsequently treated with 5-FOA to se-

lect cells in which the plasmid had excised via homologous recombination between the two repeated copies of the *KARI* gene. The 5-FOA^R colonies were then screened by mating assays and Southern blots to identify strains carrying the karyogamy defective deletion alleles. The *karl-1* allele was isolated by integrating pMR36 in strain 8960-11B (Rose and Fink, 1987). A transformant that had gene converted both copies of *KARI* to the *karl-1* allele was isolated. Total DNA was prepared, digested with *Bgl*II, ligated, and subsequently used to transform *E. coli* to Amp^R. The presence of the *karl-1* allele on plasmid pMR722 and the isolation of an isogenic *karl-1* strain was confirmed as described above for the integration of deletion alleles of *KARI*.

Results

The Hydrophobic Carboxyl Terminus of *Karlp* Is Important for Mitotic Function

To identify functional domains in the 433 residue *Karlp* protein, we constructed a series of linker insertions within the coding region. One set introduced two or more codons and did not disrupt the reading frame (Barany 1985a,b, 1988) (Table II and Fig. 1 A). A second set of linker insertions were among those previously constructed by Rose and Fink (1987) (Table II and Fig. 1 A). The effects of the various mutations were tested by the "plasmid shuffle" or the "spore viability" test as described in Materials and Methods.

Of 14 linkers tested, only 3 linker insertions resulted in a complete loss of function at all temperatures tested. All three mapped to the carboxyl terminus, which is comprised of a 20-residue hydrophobic region (412–431) showing the characteristics of a type II membrane spanning domain (Hartmann et al., 1989). The most distal in-frame linker insertion, *karl-P240* at position 421, introduced a positively charged residue, arginine, and a putative alpha-helix breaker, glycine (Fig. 2). These changes would be expected to severely affect the function of a membrane spanning domain. A second linker insertion, *karl-S72*, introduced a frameshift mutation at residue 426 (Rose and Fink, 1987). Linker *karl-S72* truncated the hydrophobic region and substituted a novel carboxyl terminus (GRPYMKKCVQLWQ) containing several charged and polar residues. The third linker, *karl-P231*, was constructed to introduce a termination codon at residue 413, truncating the protein at the beginning of the hydrophobic region. These data defined the hydrophobic carboxyl terminus as being essential for viability.

One other mutation caused a detectable mutant phenotype. The linker insertion *karl-P230* deleted Tyr⁴¹² and replaced it with Ser-Ala-Asp (Fig. 2, Table II). Linker *P230* is at the beginning of the hydrophobic stretch and caused a 15% increase in the doubling time compared to wild type.

We were surprised that the remaining linker insertions outside of the small hydrophobic tail region had no detectable effect on *KARI* function. Some insertions (e.g., *P210* and *P220*) introduced prolines and would be expected to alter the structure of the protein. Moreover, insertions containing multiple linkers were obtained at two sites (Table II). Like the two amino acid insertions, and the two small in-frame deletions previously isolated (Rose and Fink, 1987), these multiple linker insertions had no effect on *KARI* function.

Large Duplications within *Karlp* Cause No Mutant Phenotype

As the small in-frame insertions did not produce obvious

Table II. Amino Acids Added by Linker Insertions

Allele	Plasmid	Residue	Sequence at wild type locus	Altered sequence generated by insertion or deletion	Mitotic phenotype*
Q250	pMR1071	14	SK	SSTSTK	+
P210	pMR1420	71	NI	NPRI	+
Q270	pMR1295	112	FR	FRRR	+
Q271	pMR1074	112	FR	FRRRRR	+
C281	pMR1451	190	KSSS	KRSTAS	+
S96	pMR99	288-294	DNSFKISTP	DGRPP	+
Q260	pMR1294	349	IE	IVDE	+
Q261	pMR1075	349	IE	IVDVDE	+
S88	pMR97	385-392	EQMVNKGWRK	ERSTK	+
P220	pMR1422	404	NI	NPRI	+
P230	pMR1424	411	EYF	ESADF	+/-
P231	pMR2323	411	EYF	ESterm	-
P240	pMR1426	421	IL	IRGL	-
S72	pMR95	427-433	NIYVYRFterm	NGRPYMKKCQVLWQterm	-

* Mutations carried on low copy plasmids were assayed for phenotypes in the plasmid shuffle and spore viability assays described in Materials and Methods. (+) Wild type growth; (+/-) decreased growth rate compared to wild type; (-), complete loss of function; (term) carboxy terminus.

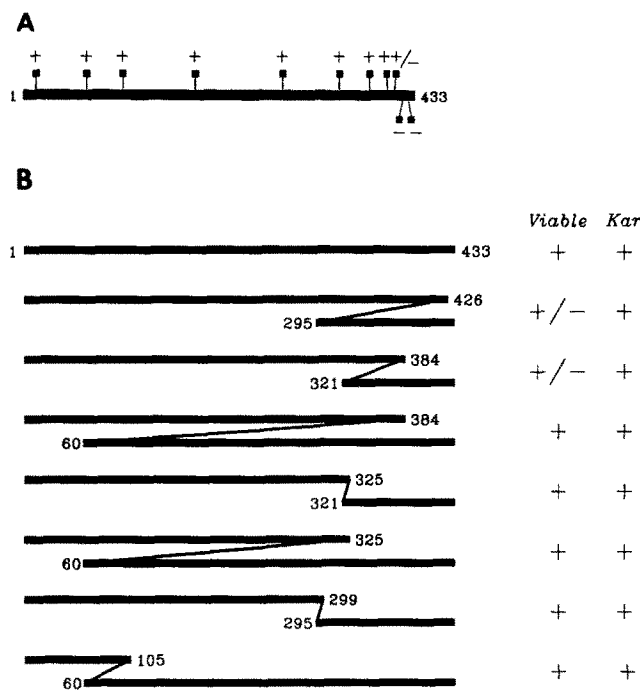


Figure 1. Insertion mutations in the *KAR1* gene. (A) Small insertions and deletions. Plasmids containing *KAR1* were mutagenized in vitro by the insertion of synthetic oligonucleotide linkers at the sites shown. See Table II for the specific sequences inserted and deleted. (B) Duplications within the *KAR1* gene. Plasmids containing duplications of internal sequences within the *KAR1* gene were constructed by ligating together various fragments of the *KAR1* gene. The amino acids contained on each fragment are shown; the duplicated region consists of those sequences contained on both fragments. The mutagenized, low copy number plasmids were transformed into yeast strains and assayed for phenotypic effects after plasmid shuffling or in the spore viability test. All plasmids were tested for nuclear fusion by the plate mating assay. All the viable insertion mutations in A were Kar⁺. (+) Wild type growth; (+/-) decreased growth rate compared to wild type; (-), complete loss of function.

mutant phenotypes, we constructed large duplications of the coding region. These might be expected to cause more drastic changes in the protein structure. Such duplications have previously been reported to cause nonconditional loss of function and temperature-sensitive mutant phenotypes in β -lactamase (Barany, 1985a). Duplications were constructed by ligating together different amino-terminal and carboxyl-terminal fragments from various linker insertions (Fig. 1 B). The duplications ranged in size from 30 to 300 amino acids. The structures of the duplications in the yeast transformants were confirmed by Southern blot hybridization (data not shown). In some cases Western blots were performed to verify the size of the mutant protein (data not shown).

Two of the duplications caused a slight decrease in growth rate compared to wild type. The doubling times for strains containing duplications of amino acids 295-426 and 321-384 (Fig. 1 B) were 8 and 25% longer, respectively. In both of these duplications, both sides of the fusion joint were near the carboxyl terminus. No effects on nuclear fusion were observed. The remaining duplications had no detectable effect on either *KARI* function.

A Second Region of *Kar1p* Important for Mitosis

From the above, it appears that *KAR1* tolerates many different changes that should produce significant perturbations in protein structure. Therefore, to use a more rigorous means to identify regions that are important for function, we constructed a series of large in-frame deletions of *KAR1*. The deletions were constructed from appropriate pairs of the linker insertions. None encroached upon the hydrophobic carboxyl terminus. Plasmids were then assayed for complementation of a large chromosomal deletion using the spore viability test.

Remarkably, most of the *KAR1* coding sequence could be deleted with no effect on viability (Fig. 3). In the amino-terminal half, as much as 41% of the protein (residues 15-192) was dispensable. Likewise, in the carboxy-terminal half, 39% of the coding sequence (residues 235-404), was not essential. However, deletion of the region between

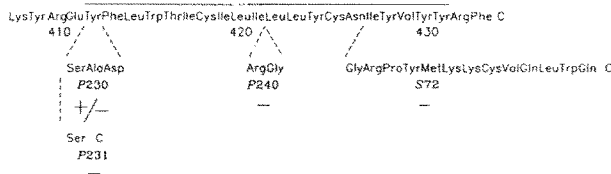


Figure 2. Mutations of the carboxyl terminus of Karlp. The hydrophobic carboxyl terminus of Karlp is shown. The putative membrane spanning region is overscored. Residues inserted by the *karl-P230* and *karl-P240* linker insertion mutations are indicated. The termination codon inserted in *karl-P231* occurs at position 413. The frameshift mutation *karl-S72* occurs at residue 426 and inserts the residues shown. (C) Carboxyl terminus; (+) wild type growth; (+/-) decreased growth rate compared to wild type; (-), complete loss of function. The growth phenotypes were assayed when the mutations were carried on low copy number plasmids.

residues 191 and 246 ($\Delta 17$) created a temperature-sensitive growth defect. All strains containing larger deletions (e.g., $\Delta 18$, $\Delta 19$, and $\Delta 20$) that span this interval were inviable at all temperatures tested (13, 23, 30, and 37°C). Therefore, these mutations defined a second important region in the *KARI* gene, which we will refer to as region I.

Mapping and Sequence Analysis of *karl*⁻ Alleles

Given that only an extremely small portion of *KARI* was found to be required for its mitotic function, it was of interest to determine the position of three previously isolated temperature-sensitive mutations (Rose and Fink, 1987). All three independently isolated mutations were genetically mapped between residues 247 and 288, a region which deletion analysis (see above) had shown to be nonessential. Se-

quence analysis demonstrated that all three alleles contained the same mutation, changing Trp²⁴⁸ to a UGA nonsense codon. As the carboxyl terminus plays a significant role in Karlp's mitotic function, it seemed unlikely that the truncated protein fragment would be functional. Genetic analysis of the strain in which the conditional alleles were isolated, TD28, demonstrated the presence of a weak temperature-sensitive UGA-suppressor. To date, no single point mutations have been isolated that create temperature-sensitive forms of Karlp. However, multiple mutations within region I do lead to a temperature-sensitive phenotype (Vallen, E., and M. Rose, unpublished observations).

The Viable Deletion Mutants Define a Region Required for Nuclear Fusion

Strains carrying viable in-frame deletions were tested for nuclear fusion to ascertain whether a specific region of Karlp is required for karyogamy. Most of the viable deletions were Kar⁺. However, all strains bearing deletions including residues 118–191 were found to be Kar⁻ (Fig. 3). To accurately measure the karyogamy defect, Kar⁻ alleles $\Delta 13$ and $\Delta 15$ were integrated at the *KARI* locus. Both deletions caused a large decrease in the formation of diploids, and a concomitant increase in the formation of cytoductants (Table III). Strains containing the deletion alleles were as deficient for nuclear fusion as isogenic strains carrying the *karl-1* allele. The residual nuclear fusion seen in these strains may represent the null phenotype for *KARI*'s karyogamy function.

The locations of six independently isolated Kar⁻ point mutations (including *karl-1*) were genetically mapped and found to lie within the region defined by deletion analysis as

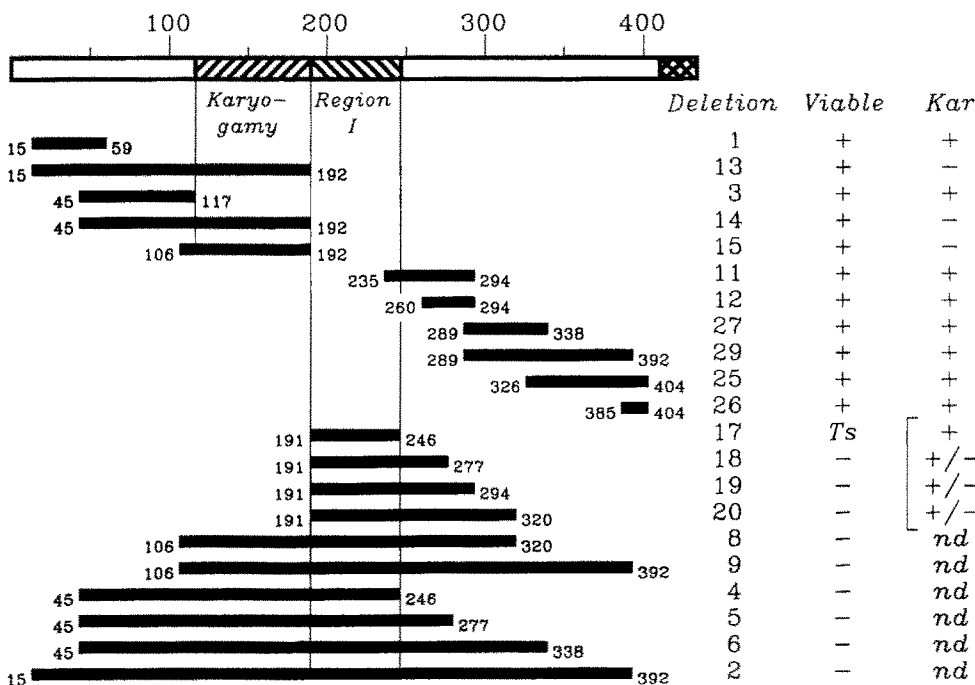


Figure 3. Deletions within the *KARI* gene. Plasmids containing deletions of internal sequences within the *KARI* gene were constructed by the ligation of pairs of fragments of the *KARI* gene. The allele numbers corresponding to each deletion are shown. The region deleted in each allele is shown by a solid line; numbers flanking the solid bars correspond to the missing residues in each deletion. The mitotic function of each deletion plasmid was assayed using the spore viability test. (+) Wild type growth; (Ts) temperature sensitive growth; (-) inviability. Alleles were also tested for nuclear fusion defects: (+) wildtype karyogamy proficiency; (+/-) intermediate karyogamy proficiency; (-) karyogamy defective. The bracketed results were obtained by complementation of the karyogamy defect conferred by *karl- $\Delta 13$* ; in these cases, the phenotype was assayed when the mutations were carried on multicopy, 2 μ m-based plasmids. The Kar1 protein (*top*) is numbered according to amino acid sequence. Region I (////) and the region required for karyogamy (///) are demarcated by hatching. The hydrophobic tail (XXX) required for both functions is indicated by crosshatching.

tained by complementation of the karyogamy defect conferred by *karl- $\Delta 13$* ; in these cases, the phenotype was assayed when the mutations were carried on multicopy, 2 μ m-based plasmids. The Kar1 protein (*top*) is numbered according to amino acid sequence. Region I (////) and the region required for karyogamy (///) are demarcated by hatching. The hydrophobic tail (XXX) required for both functions is indicated by crosshatching.

being required for karyogamy. DNA sequence analysis revealed that all of the mutations were the result of the same single base change which converts Pro¹⁵⁰ to Ser. Thus, the viable deletions and the missense mutations define a region of *Karl*p necessary for nuclear fusion.

Intragenic Complementation between Deletion Alleles

The inviability of some of the deletion mutants prevented a direct determination of their ability to perform nuclear fusion. To test whether these proteins retained karyogamy function, strains containing chromosomal *Kar*⁻ deletion alleles ($\Delta 13$ and $\Delta 15$) were transformed with plasmids containing some of the inviable and temperature-sensitive deletion alleles. Transformants were assayed for karyogamy proficiency by replica print plate matings and quantitative filter assays (see Materials and Methods). If the karyogamy region is a domain distinct from that required for viability, these deletions should restore nuclear fusion capability.

Representative data for the transformants of $\Delta 13$ are shown in Table III. Results for $\Delta 15$ were essentially identical. Both high and low copy number plasmids containing $\Delta 17$ promoted wild type levels of nuclear fusion. On low copy number plasmids, three Region I mutations, $\Delta 18$, $\Delta 19$, and $\Delta 20$, only partially restored nuclear fusion function to the $\Delta 13$ strain. However, nuclear fusion was markedly improved when these deletions were carried on high copy number plasmids. In contrast, a high copy number plasmid carrying a karyogamy region deletion ($\Delta 13$) did not restore nuclear fusion. Clearly, the region I deletions retain karyogamy function. Therefore, the karyogamy function and the mitotic function of *KAR1* are at least partially mediated by separate protein domains.

The carboxy-terminal mutations were also defective for complementation of $\Delta 13$. The defects associated with two mutations, (*kar1-P231* and *kar1-S72*), were at least partially suppressed by expression of the mutant proteins from high copy plasmids, whereas the defect associated with another mutation, (*kar1-P240*), was not suppressed. One explanation for these results is that the severity of the defect correlates with the steady-state levels of the mutant proteins (see below). However, high copy expression of *Kar1-P231* protein results in only partial complementation of $\Delta 13$, although steady-state protein levels are comparable to single copy wild type *Karl*p (data not shown). These results imply that the hydrophobic tail may have an additional function beyond the simple requirement for protein stability.

Decreased Steady-State Levels of Mutant Proteins

The interpretation of deletion mutations that are functionally defective is complicated by the fact that negative results can arise either from loss of function or from decreased protein stability or expression. In this regard, the karyogamy proficiency conferred by $\Delta 17$ in single copy provided functional proof for the presence of mutant protein. However, several other mutations showed little or no complementation of the karyogamy defect unless they were present on high copy number plasmids. One likely explanation of these results is that the mutant proteins were unstable.

Western blot analysis was performed on strains containing the region I deletion alleles ($\Delta 17$, $\Delta 18$, $\Delta 19$, $\Delta 20$) and the frameshift mutation in the carboxy terminus (*S72*) to deter-

Table III. Nuclear Fusion Defects of *KAR1* Mutants

Strain	Relevant genotype	Quantitative matings		Plate assay
		Diploids	C/D	
		%		
MS52	<i>KAR1</i>	69	0.001	+
MS739	<i>kar1-1</i>	1.0	8.3	-
MS1113	<i>kar1-$\Delta 15$</i>	2.3	3.7	-
MS1117	<i>kar1-$\Delta 13$</i>	0.6	3.2	-
MS1461	<i>kar1-$\Delta 13$ [YCp<i>kar1-$\Delta 17$</i>]</i>	30	0.028	+
MS1463	<i>kar1-$\Delta 13$ [YCp<i>kar1-$\Delta 18$</i>]</i>	3.7	1.9	-/+
MS1465	<i>kar1-$\Delta 13$ YCp<i>kar1-$\Delta 19$</i>]</i>	4.4	1.1	-/+
MS1467	<i>kar1-$\Delta 13$ YCp<i>kar1-$\Delta 20$</i>]</i>	9.5	0.49	-/+
MS1473	<i>kar1-$\Delta 13$ [YCp<i>KAR1</i>]</i>	33	0.026	+
MS1475	<i>kar1-$\Delta 13$ [YCp50]</i>	2.0	4.3	-
MS2111	<i>kar1-$\Delta 13$ [2μ<i>kar1-$\Delta 17$</i>]</i>			+
MS2113	<i>kar1-$\Delta 13$ [2μ<i>kar1-$\Delta 18$</i>]</i>			+/-
MS2115	<i>kar1-$\Delta 13$ [2μ<i>kar1-$\Delta 19$</i>]</i>			+/-
MS2117	<i>kar1-$\Delta 13$ [2μ<i>kar1-$\Delta 20$</i>]</i>			+/-
MS2127	<i>kar1-$\Delta 13$ [2μ]</i>			-
MS2059	<i>kar1-$\Delta 13$ [YCp<i>kar1-S72</i>]</i>			-/+
MS2133	<i>kar1-$\Delta 13$ [2μ<i>kar1-S72</i>]</i>			+
MS2991	<i>kar1-$\Delta 13$ [YCp<i>kar1-P231</i>]</i>			-
MS2992	<i>kar1-$\Delta 13$ [2μ<i>kar1-P231</i>]</i>			-/+
MS2989	<i>kar1-$\Delta 13$ [YCp<i>kar1-P240</i>]</i>			-
MS2990	<i>kar1-$\Delta 13$ [2μ<i>kar1-P240</i>]</i>			-
MS2135	<i>kar1-$\Delta 13$ [YCp<i>kar1-$\Delta 13$</i>]</i>			-
MS2124	<i>kar1-$\Delta 13$ [2μ<i>kar1-$\Delta 13$</i>]</i>			-

For quantitative matings, strains were mated to a *MATa KAR1* [ρ^{ho}] *cyh*^R strain (MS147) for 5 h at 30°C. After mating, cells were plated to determine the number of viable cells, diploids, and cytoductants. Cytoductants contain a haploid nucleus from one parent and a cytoplasmic genetic element from the other parent. An increase in the cytoductant to diploid ratio (C/D) indicates a decrease in the frequency of nuclear fusion.

For the plate assay, a modification of the standard mating type test protocol was used (Rose et al., 1990). Strains were replica plated together, allowed to mate for 3 h at 30°C, and then replica printed to select for diploid cells. Diploid formation was compared to *KAR1* and *kar1-1* controls on the same plate. For the same mutations on *CEN*-based vs. 2 μ m-based plasmids, strains were tested together on the same mating plate.

The approximate frequency of diploid and cytoductant formation can be defined by comparison of plate mating data to quantitative mating data for known controls. + indicates the wildtype *KAR1*⁺ phenotype, >25% diploid formation and a C/D ratio of <0.05; +/- corresponds to 11-25% diploid formation and C/D ratio of 0.05-0.4; -/+ corresponds to 3-10% diploid formation and C/D ratio of 0.4-3; - is the phenotype caused by the *kar1-1* mutation, <3% diploid formation and C/D ratio of >3.

mine the steady state levels of the mutant proteins. To specifically monitor the plasmid encoded proteins, the chromosome carried the $\Delta 13$ allele whose protein product does not contain the epitopes that react with the antibody. With the possible exception of $\Delta 17$, the levels of the mutant proteins were significantly less than that of wild type (Fig. 4). When the $\Delta 18$, $\Delta 19$, $\Delta 20$, and *S72* mutations were carried on high copy number plasmids, protein levels were similar to that of wild type protein expressed from a low copy number plasmid. These data support the suggestion from the complementation data that the mutant proteins were unstable.

In contrast to the karyogamy results, $\Delta 18$, $\Delta 19$, and $\Delta 20$ on high copy number plasmids remained defective when assayed by the spore viability test. Moreover, overexpression did not suppress the temperature sensitivity of $\Delta 17$. Thus, although some of the mutant proteins appeared to be less sta-

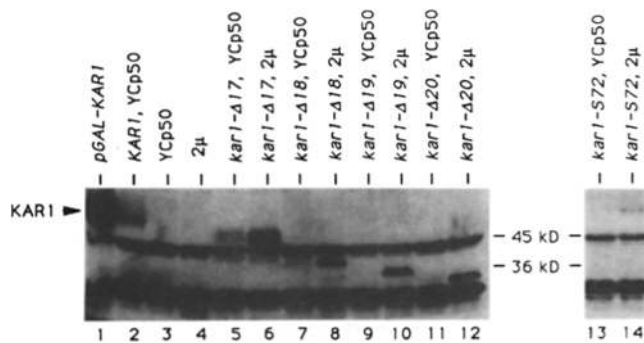


Figure 4. Steady-state levels of Karlp in deletion mutants. MS1117 (*karl-Δ13*) was transformed with various plasmids and the resulting strains were subjected to Western blot analysis. The *karl-Δ13* allele is deleted for the epitopes recognized by the Karlp antibodies. (Lane 1) MY913 (*pGAL-KARI*) grown on galactose; (lane 2) MS1473 (*KARI* on YCp50); (lane 3) MS1475 (YCp50); (lane 4) MS2127 (2 μ m); (lane 5) MS1461 (*karl-Δ17* on YCp50); (lane 6) MS2111 (*karl-Δ17* on 2 μ m); (lane 7) MS1463 (*karl-Δ18* on YCp50); (lane 8) MS2113 (*karl-Δ18* on 2 μ m); (lane 9) MS1465 (*karl-Δ19* on YCp50); (lane 10) MS2115 (*karl-Δ19* on 2 μ m); (lane 11) MS1467 (*karl-Δ20* on YCp50); (lane 12) MS2117 (*karl-Δ20* on 2 μ m); (lane 13) MS2059 (*karl-S72* on YCp50); (lane 14) MS2133 (*karl-S72* on 2 μ m). Lanes 13 and 14 are from a separate gel. The band corresponding to the wild type Karlp is indicated. The sizes of the deletion proteins vary between strains. Other bands are due to cross-reacting proteins and are not derived from Karlp.

ble than wild type, increasing their steady-state levels was not sufficient to restore viability. One explanation for the inability of the overexpressed mutant proteins to restore viability is that the intact or partially degraded mutant proteins might titrate other essential components. We believe this explanation to be unlikely because it predicts that the mutant proteins would confer a dominant negative phenotype which we have not detected. Therefore, these deletions must inactivate a domain that is required for the essential mitotic function.

In contrast to the region I deletions, *karl-S72* on a high copy number plasmid allowed the recovery of viable strains containing a complete deletion of the chromosomal copy of *KARI*. Thus, overexpression of Kar1-S72p restored mitotic function as well as karyogamy function. Since the *S72* allele removes only part of the hydrophobic tail, overexpression may enhance its residual function.

Unlike *karl-S72*, two other carboxy-terminal mutations, *karl-P231* and *karl-P240*, did not support growth when present on high copy number plasmids. Because Kar1-P231p and Kar1-S72p are expressed to comparable levels from high copy number plasmids (data not shown), Kar1-P231p must be defective for some essential mitotic function other than simple stabilization of the protein. It seems likely that the tail serves to localize or anchor the protein in the nuclear envelope; decreased stability might arise secondarily from mislocalization of the mutant protein.

Phenotypes of Karyogamy Deficient Alleles

karl-1 mutant shmooos and zygotes have aberrantly long cytoplasmic microtubules (Rose and Fink, 1987). We therefore examined the microtubules in cells containing the karyog-

amy defective deletions to determine whether their phenotype was similar. Wild type cells, or cells containing the *Kar⁻* alleles were treated with the mating pheromone α -factor for 3 h to induce shmoo formation and then examined by immunofluorescent staining. Like the *karl-1* mutation, the karyogamy defective *karl* deletions caused abnormally elongated cytoplasmic microtubules in shmooos (Fig. 5, compare A and B).

Based on two observations, it seemed possible that *KARI* might exert its function in karyogamy via *KAR3*. First, long cytoplasmic microtubules are often found in mating cells containing either *KAR3-lacZ* hybrids or mutations in *KAR3* (Meluh and Rose, 1990). Second, although *Kar3-lacZ* protein also localizes to the distal end of cytoplasmic microtubules, both *Kar3-lacZ* and *Kar1-lacZ* hybrid proteins localize to the SPB in pheromone-treated cells. We therefore analyzed the localization pattern of *Kar3-lacZ* hybrid protein in *Kar⁻ Δ13* mutant cells. In wild type shmooos, ~40% of the cells showed *Kar3-lacZp* staining at both the SPB and the distal tip of the microtubules (Fig. 5, C and D). In the remaining 60% of the cells, the SPB staining was too faint to be discernible, and only the brighter staining at the tip of the microtubules was evident. In strains containing $\Delta13$, *Kar3-lacZp* staining at the SPB was not observed (<0.5%, $n = 200$, six cells could not be scored due to an unfavorable orientation of the cell with respect to the observer), although it was readily observed at the distal end of the cytoplasmic microtubules (Fig. 5, E and F). The combination of the hybrid protein and $\Delta13$ caused formation of greatly elongated cytoplasmic microtubules, which were often longer than the cell and curved along the interior margin. As was seen for the *Kar⁻* mutation alone, these microtubules were often, but not always, dissociated from the SPB. In contrast, *Kar3-lacZ* protein localization was unaffected by mutations in *KAR2* (data not shown) or *KAR3* (Meluh and Rose, 1990), thereby demonstrating that the effect is specific to mutations in *KARI*. Conversely, the SPB localization of *Kar1-lacZ* hybrid protein is not affected by deletion of *KAR3* (data not shown).

Thus, the localization of *Kar3-lacZp* to the SPB is dependent upon the integrity of the *KARI* karyogamy domain. These results suggest that the SPB localization of *Kar3-lacZp* is functionally significant for nuclear fusion.

Discussion

The *KARI* gene is required for at least two distinct functions in yeast. First, during vegetative growth, *KARI* is essential for cell viability; loss of function prevents SPB duplication. Consistent with this, immunofluorescent staining demonstrated that *Kar1-lacZ* hybrid proteins associate with the SPB that is directed into the bud during cell division (Vallen et al., 1992). Second, *KARI* is also required for efficient nuclear fusion. During mating, the karyogamy defective *karl* mutants are characterized by the presence of abnormally long cytoplasmic microtubules emanating from the SPB (Rose and Fink, 1987). Together, these data suggested that *Kar1* protein is a functional component of the yeast SPB for at least a portion of the cell cycle. In this paper, we have used a series of internal in-frame insertions, deletions and duplications to define the regions of *Kar1* protein required for its activities.

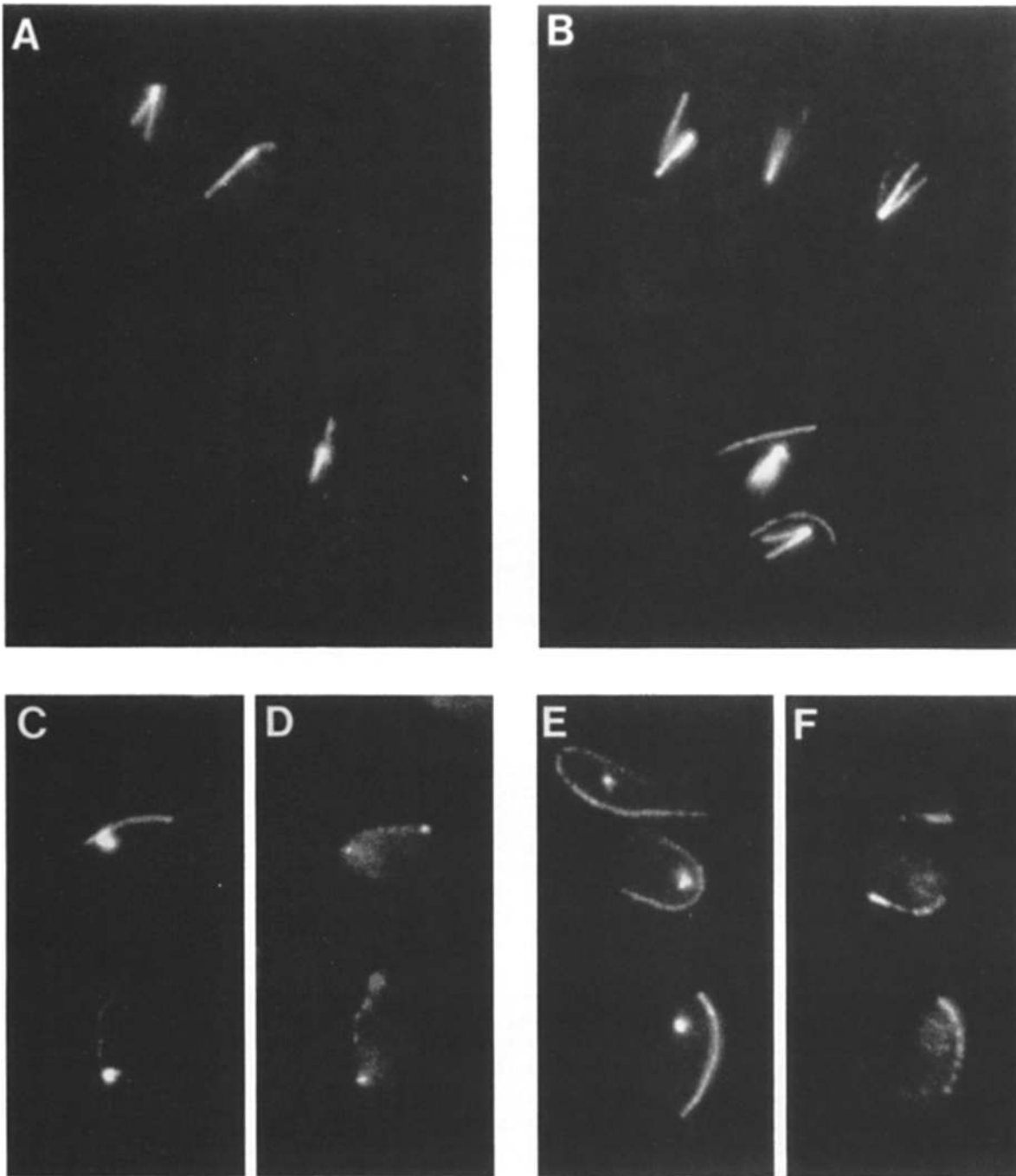


Figure 5. *KAR1* mutations affect cytoplasmic microtubules and Kar3-lacZ hybrid protein localization. *A*, *B*, *C*, and *E* show staining with rabbit anti- β -tubulin; *D* and *F* show staining with mAb against β -galactosidase to determine the localization of the Kar3-lacZ hybrid protein. Wildtype strain MS10 (*A*) and *kar1- Δ 13* strain MS1274 (*B*) were treated with α -factor for 3 h and prepared for immunofluorescent staining of the microtubules. In the wildtype strain, the brightest staining is observed for the nuclear microtubules. The cytoplasmic microtubules are short faint extensions, in some cases intersecting the nuclear microtubules at an obtuse angle. In the mutant strain, note the presence of long, detached cytoplasmic microtubules. In *C-F*, strains containing pMR1300 (*KAR3-lacZ*) were treated with α -factor. A wildtype strain is shown in *C* and *D* and a *kar1- Δ 13* strain is shown in *E* and *F*. In the wildtype strain, the Kar3-lacZ hybrid protein is associated with both the SPB and the distal tips of the cytoplasmic microtubules. In the *kar1* mutant, the Kar3-lacZ hybrid protein is associated only with the cytoplasmic microtubules.

A Model for Kar1 Protein

Surprisingly, the results of our analysis showed that only two small discrete regions were uniquely important for the mi-

totic function of *KARI*. One region, located at the extreme carboxyl terminus, is important for viability and karyogamy. This region contains an uninterrupted stretch of 20 hydro-

phobic and uncharged residues, in contrast to the rest of the protein which is highly charged and hydrophilic. Insertion of Arg-Gly into this region resulted in loss of Karlp function, suggesting that the hydrophobicity of this region is essential.

Minimally, the hydrophobic tail serves to stabilize the Karl protein; mutations in this region resulted in lower steady-state levels of the protein. Although we have not ruled out the possibility that these mutations affect gene expression, their location at the 3' end of the open reading frame seems more consistent with an effect on protein stability.

Several observations suggest a more specific function for the hydrophobic tail. First, its length is sufficient to span a lipid bilayer. Second, it is flanked by charged residues as is frequently observed for membrane spanning domains. Indeed, the distribution of charged residues predicts that Karl protein would be a type II membrane protein (Hartmann et al., 1989) with the protein exposed to the cytoplasm. Third, a similar hydrophobic carboxy terminus has been described for several putative ER membrane proteins (Orlean et al., 1988; Shim et al., 1991; Ferro-Novick, S., personal communication). Fourth, a β -galactosidase hybrid protein containing Karlp residues 393–433 at its carboxy terminus localizes to the nuclear periphery (Vallen et al., 1992). Moreover, upon fractionation, the hybrid protein behaves as an integral membrane protein (Scherson, T., and M. Rose, unpublished observations). Finally, a serendipitous gene fusion between *KARI* and the pBR322 tetracycline resistance gene is functional for both mitosis and nuclear fusion (Vallen, E., and M. Rose, unpublished observation). In this hybrid, Karlp's hydrophobic tail is deleted and replaced by sequences from the integral membrane protein encoded by *ter^R*. These observations suggest that the hydrophobic tail of Karlp is a membrane spanning domain, perhaps serving to localize or anchor the protein to the nuclear envelope during both vegetative growth and karyogamy. The mutant proteins might be unstable as a consequence of their mislocalization.

A second region, located near the center of the *KARI* coding sequence (region I; between residues 191 and 246), is essential for cell growth. Larger deletions that include this region are inviable, whereas a precise deletion of this interval ($\Delta 17$) causes slow growth at low temperatures and inviability at high temperatures. Accurate definition of this region is difficult because deletions that are contiguous to or partially overlap this region have no mitotic phenotype ($\Delta 11$, $\Delta 13$, $\Delta 14$, $\Delta 15$). One explanation for the complexity is that loss of function might require the deletion of more than one domain. The larger inviable deletions produce lower steady-state levels of protein, but overexpression on high copy plasmids does not suppress the inviability associated with them. Therefore, loss of function is not simply the result of decreased levels of protein.

Experiments using β -galactosidase hybrid proteins showed that residues 190–259 are both necessary and sufficient for SPB localization (Vallen et al., 1992). It is striking that the region defined genetically to be essential for the mitotic function of Karlp is also necessary for SPB localization. One possibility is that this region of the protein interacts with other SPB components thereby allowing localization of the hybrid proteins. Alternatively, this region might include a specific SPB localization signal that targets Karlp protein to the SPB.

Phenotypic analysis of the deletion mutants also defined a

region of Karlp required for nuclear fusion (118–191). All of the karyogamy defective point mutations alter the same amino acid in this interval, Pro¹⁵⁰ to Ser. The karyogamy region is adjacent to, but separate from, region I, as demonstrated by two observations. First, karyogamy defective mutations have no perceptible effect on the mitotic function of *KARI*. Second, deletions of region I can provide nuclear fusion function to a karyogamy region deletion in trans. The simplest interpretation of these results is that the karyogamy region and region I form discrete structural domains with distinct and separate functions during mitosis and nuclear fusion.

The large variety of insertions, duplications, and deletions that retain function indicate that Karl protein can endure a remarkable degree of alteration. The extreme structural plasticity is reminiscent of eukaryotic transcriptional activators in which a small DNA binding domain and short regions responsible for activation are joined by large nonessential linker regions (e.g., *GAL4*; Ma and Ptashne, 1987). By analogy, Karlp may contain distinct protein binding domains, one for association with the SPB and one for interaction with the nuclear fusion machinery, both flexibly connected to a membrane anchor. In principle, the remainder of the protein might be concerned with other nonessential cellular processes, or essential processes masked by functionally redundant domains in Karlp or other proteins.

Role for Karlp in Nuclear Fusion

Nuclear fusion involves at least two steps. The first concerns the microtubule-dependent movement of nuclei towards each other in the zygote. The second step involves the fusion of the spindle plaques and the nuclear envelopes. The karyogamy defective *karl* mutations have clear effects on the structure of the cytoplasmic microtubules making it most likely that *KARI* is involved with the first step in the pathway. One possibility is that the karyogamy domain is required for the binding of a specific protein that is required for microtubule-dependent nuclear fusion.

Recently, the *KAR3* gene has been discovered to be related to the microtubule motor protein, kinesin (Meluh and Rose, 1990). Hybrid proteins containing the amino-terminal portion of Kar3 protein fused to β -galactosidase localize to the distal ends of the cytoplasmic microtubules and the SPB in α -factor-treated cells. From the expected polarity of the cytoplasmic microtubules (plus end out) and the normal polarity of kinesin movement (towards the plus end), a model was described in which the force required for pulling the nuclei together is generated at the SPB. The cytoplasmic microtubules would be pulled into the SPB, coupled to depolymerization at the minus end. The model made two clear predictions. First, the length of the cytoplasmic microtubules should be partly determined by proteins physically present at the SPB. Second, the localization of Kar3-lacZ chimeric proteins to the SPB should depend on SPB proteins that are also required for nuclear fusion. Consistent with the model, the cytoplasmic microtubules in *karl* mutant shmoo are much longer than those in wild type cells and frequently dissociate from the SPB. Stronger support for the model comes from the observation that in *karl* mutants the Kar3-lacZ chimeric protein fails to localize to the SPB but still localizes to the distal ends of the cytoplasmic microtubules. Thus

Kar3 protein localization to the SPB is dependent upon Karl protein.

The directionality of Kar3p movement has not yet been determined and Kar3p may yet prove to be like *ncd*, a minus end-oriented kinesin-like motor protein. Nevertheless, the localization of the hybrid protein to the SPB seems more consistent with Kar3p being a plus end-oriented motor.

The model specifically suggests that Karl1 protein exerts its effects in nuclear fusion solely via an interaction with Kar3 protein. The model provides an explanation for one of the striking genetic peculiarities of the *kar1* mutants. Although the karyogamy defective mutations are recessive, it is sufficient for one parent to be mutant for nuclear fusion to fail (a "unilateral" defect). Nevertheless, the wild type gene product is both in excess in wild type cells and accessible to the mutant nucleus (Dutcher and Hartwell, 1982). In contrast, for recessive *kar3* mutations both parents must be defective (a "bilateral" defect). Immunofluorescent staining of hybrid proteins indicates that Karl protein is assembled into the SPB before cell fusion (Vallen et al., 1992). The preassembly of the SPB may preclude assembly of the wild type Karl protein derived from the other parent. In the absence of the functional karyogamy domain the diffusible Kar3 protein would lack a binding site at the SPB. In a heterozygous *KAR1/kar1* mutant the presence of the wild type Karl protein at the SPB would allow binding of Kar3 protein in spite of the presence of the mutant Karl protein.

The identification of specific domains in the *KAR1* gene product has demonstrated the existence of distinct functions for the protein. Characterization of the proteins that interact with the separate domains of Karlp should define other components of the SPB and serve to elucidate their roles in mitosis and nuclear fusion.

We wish to thank the members of this laboratory, particularly Pam Meluh and Joe Vogel, for many stimulating discussions and for critically reading this manuscript.

This research was supported by grants from the National Institutes of Health (NIH) (GM37739), the National Science Foundation (Presidential Young Investigator Award DCB8657497), and the James S. McDonnell Foundation. E. A. Vallen was supported by an NIH training grant for cancer research. M. A. Hiller was supported by a grant from the Charles A. Dana Foundation.

Received for publication 26 December 1991 and in revised form 18 March 1992.

References

- Adams, A. E. M., and J. R. Pringle. 1984. Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic mutant *Saccharomyces cerevisiae*. *J. Cell Biol.* 98:934-945.
- Barany, F. 1985a. Single-stranded hexameric linkers: a system for in-phase insertion mutagenesis and protein engineering. *Gene.* 37:111-123.
- Barany, F. 1985b. Two codon insertion mutagenesis of plasmid genes by using single stranded hexameric oligonucleotides. *Proc. Natl. Acad. Sci. USA.* 82:4202-4206.
- Barany, F. 1988. Procedures for linker insertion mutagenesis and use of new kanamycin resistance cassettes. *DNA Prot. Eng. Tech.* 1:29-44.
- Boeke, J. D., F. Lacroute, and G. R. Fink. 1984. A positive selection for mutants lacking orotidine 5'-phosphate decarboxylase activity in yeast: 5-fluoroorotic acid resistance. *Mol. Gen. Genet.* 181:288-291.
- Boeke, J. D., J. Trueheart, G. Natsoulis, and G. R. Fink. 1987. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods Enzymol.* 154:164-175.
- Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellu-

lose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195-203.

- Byers, B. 1981. Cytology of the yeast life cycle. In *The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance*. Strathern, J. N., E. W. Jones, and J. R. Broach, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 59-96.
- Byers, B., and L. Goetsch. 1974. Duplication of spindle plaques and integration of the yeast cell cycle. *Cold Spring Harbor Symp. Quant. Biol.* 38:123-131.
- Byers, B., and L. Goetsch. 1975. Behavior of spindles and spindle plaques in the cell cycle and conjugation in *Saccharomyces cerevisiae*. *J. Bacteriol.* 124:511-523.
- Conde, J., and G. R. Fink. 1976. A mutant of *Saccharomyces cerevisiae* defective for nuclear fusion. *Proc. Natl. Acad. Sci. USA.* 73:3651-3655.
- Delgado, M. A., and J. Conde. 1984. Benomyl prevents nuclear fusion in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* 193:188-189.
- Dutcher, S. K., and L. H. Hartwell. 1982. The role of *Saccharomyces cerevisiae* cell division cycle genes in nuclear fusion. *Genetics.* 100:175-184.
- Dutcher, S. K., and L. H. Hartwell. 1983. Genes that act prior to conjugation to prepare the *Saccharomyces cerevisiae* nucleus for karyogamy. *Cell.* 33:203-210.
- Fink, G. R., and J. Conde. 1976. Studies on *KAR1*, a gene required for nuclear fusion in yeast. In *International Cell Biology 1976-1977*. Brinkley, B. R., and K. R. Porter, editors. The Rockefeller University Press, New York. 414-419.
- Hartmann, E., T. A. Rappoport, and H. F. Lodish. 1989. Predicting the orientation of eukaryotic membrane-spanning proteins. *Proc. Natl. Acad. Sci. USA.* 86:5786-5790.
- Hoffman, C. S., and F. Winston. 1987. A ten minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *E. coli*. *Gene.* 57:267-272.
- Holmes, D. S., and M. Quigley. 1982. A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem.* 114:193-197.
- Huffaker, T. C., J. H. Thomas, and D. Botstein. 1988. Diverse effects of β -tubulin mutations on microtubule formation and function. *J. Cell Biol.* 106:1997-2010.
- Ito, H., Y. Fukada, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells with alkali cations. *J. Bacteriol.* 153:163-168.
- Kilmartin, J. V., and A. E. M. Adams. 1984. Structural rearrangements of tubulin and actin during the cell cycle of the yeast *Saccharomyces*. *J. Cell Biol.* 98:922-933.
- Kilmartin, J. V., B. Wright, and C. Milstein. 1982. Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line. *J. Cell Biol.* 93:576-582.
- Kreitman, M., and L. H. Landweber. 1989. A strategy for producing single-stranded DNA in the polymerase chain reaction: a direct method for genomic sequencing. *Gene Anal. Tech.* 6:84-88.
- Ma, J., and M. Ptashne. 1987. Deletion analysis of *GAL4* defines two transcriptional activating segments. *Cell.* 48:847-853.
- Meluh, P. B., and M. D. Rose. 1990. *KAR3*, a kinesin-related gene required for yeast nuclear fusion. *Cell.* 60:1029-1041.
- Normington, K., K. Kohno, Y. Kozutsumi, M.-J. Gething, and J. Sambrook. 1989. *S. cerevisiae* encodes an essential protein homologous in sequence and function to mammalian BiP. *Cell.* 57:1211-1221.
- Ohashi, A., J. Gibson, I. Gregor, and G. Schatz. 1982. Import of proteins into mitochondria. *J. Biol. Chem.* 257:13042-13047.
- Orlean, P., C. Albright, and P. W. Robbins. 1988. Cloning and sequencing of the yeast gene for dolichol phosphate mannose synthase, an essential protein. *J. Biol. Chem.* 263:17499-17507.
- Orr-Weaver, T. L., J. W. Szostak, and R. J. Rothstein. 1983. Genetic applications of yeast transformation with linear and gapped plasmids. *Methods Enzymol.* 101:228-245.
- Polaina, J., and J. Conde. 1982. Genes involved in the control of nuclear fusion during the sexual cycle of *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* 186:253-258.
- Rose, M. D., and G. R. Fink. 1987. *KAR1*, a gene required for function of both intranuclear and extranuclear microtubules in yeast. *Cell.* 48:1047-1060.
- Rose, M. D., L. Misra, and J. P. Vogel. 1989. *KAR2*, a karyogamy gene, is the yeast homolog of the mammalian BiP/GRP78 gene. *Cell.* 57:1211-1221.
- Rose, M. D., F. Winston, and P. Hieter. 1990. *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 198 pp.
- Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463-5467.
- Shim, J., A. P. Newman, and S. Ferro-Novick. 1991. The *BOS1* gene encodes an essential 27-kD putative membrane protein that is required for vesicular transport from the ER to the golgi complex in yeast. *J. Cell Biol.* 113:55-64.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
- Vallen, E. A., T. Y. Scherson, T. Roberts, K. van Zee, and M. D. Rose. 1992. Asymmetric mitotic segregation of the yeast spindle pole body. *Cell.* In press.
- Vogel, J. P., L. Misra, and M. D. Rose. 1990. Loss of BiP/GRP78 function blocks translocation of secretory proteins in yeast. *J. Cell Biol.* 110:1885-1895.