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DAF1, a Mutant Gene Affecting Size Control, Pheromone Arrest, and Cell Cycle Kinetics of *Saccharomyces cerevisiae*

FREDERICK R. CROSS

Division of Basic Sciences, Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, Washington 98104

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The mating pheromone α-factor arrests *Saccharomyces cerevisiae MAT*a cells in the G1 phase of the cell cycle. Size control is also exerted in G1, since cells do not exit G1 until they have attained a critical size. A dominant mutation (*DAF1-1*) which causes both α-factor resistance and small cell size (volume about 0.6-fold that of the wild type) has been isolated and characterized genetically and by molecular cloning. Several α-factor-induced mRNAs were induced equivalently in *daf1*$^+$ and *DAF1-1* cells. The *DAF1-1* mutation consisted of a termination codon two-thirds of the way through the *daf1*$^+$ coding sequence. A chromosomal deletion of *DAF1* produced by gene transplacement increased cell volume about 1.5-fold; thus, *DAF1-1* may be a hyperactive or deregulated allele of a nonessential gene involved in G1 size control. Multiple copies of *DAF1-1* also greatly reduced the duration of the G1 phase of the cell cycle.

The main regulatory point of the *Saccharomyces cerevisiae* cell cycle identified in genetic and physiological studies is START in G1 phase (53). Cells arrest at START if they are starved for nutrients (possibly via a lowering of cyclic AMP levels [37]) or if they are exposed to mating pheromone. While starved cells arrest in a quiescent growth-arrested state, mating pheromone-arrested cells continue to grow, producing large cells which fail to enter the division cycle (6, 42, 43, 53). Mutations in various genes which cause arrest at START appear to mimic one or the other of these two forms of arrest (54).

Cells also fail to pass START (or have a low probability of passing START [69]) if they have not attained a sufficient mass or critical size; thus, START is considered the point in the cell cycle at which growth and division are coordinated (31, 53). This idea is consistent with the finding that limiting growth by various means preferentially expands the pre-START portion of the cell cycle (21, 25, 53). Mutations in two genes in *S. cerevisiae* have been shown to reduce cell size. The semidominant *whi1* mutation reduces the size of cells in log and stationary phase, whereas the recessive *whi2* mutation only makes cells small as they enter stationary phase (8, 65). In the fission yeast *Schizosaccharomyces pombe*, size control (operating at the G2-M boundary rather than at START [G1/S]) involves essential cell cycle control genes as well as nonessential modulatory functions (1, 13, 48, 56, 57, 67).

Mating pheromones cause G1 arrest as part of the pathway of mating between MATa and MATα cells to produce MATa/MATα zygotes. The pheromones (**a**- and α-factor made by **a** and α cells, respectively) initiate mating by binding to receptors on cells of the opposite mating type (66), encoded by *STE2* in MATa cells and *STE3* in MATα cells (7, 17, 27, 28, 45). Other genes may have a role in maintaining the level of cell type-specific mRNA (14, 15, 19, 29) and in signal transduction (29, 30). A protein with homology to mammalian G proteins may play a role in the pheromone response pathway (11, 26, 40). The expression of various genes is induced by pheromone binding (18, 19, 30, 32, 36, 38, 41, 45, 61, 63, 68), and the G1-arrested cells become large and pear-shaped (6, 41–43).

I have isolated and characterized a mutant defective in pheromone arrest and in size control. The phenotype of this mutant suggests a close connection between these two aspects of G1 regulation.

MATERIALS AND METHODS

Mutant isolation and strain constructions. All strains were derived from strains congenic with 381G (20); parent strains were provided by the laboratory of L. Hartwell. All strains carried the *bar1* mutation to prevent α-factor degradation (9), except when *sst2* (9) was present. The *scg1*::*lacZ6-LEU2* allele (11) and the *hml*α-2::*LEU2* allele (62) were introduced into the 381G background by transformation. (*HML*α strains can become α-factor resistant by switching mating type.) To enrich for dominant mutations, I selected α-factor-resistant mutants derived from **a/a** diploids (constructed as described before [20]). **a/a** *hml*α diploids exhibited a spontaneous frequency of α-factor-resistant mutants of about 3×10^{-7}, compared to about 10^{-4} for **a** haploids. Unmutagenized **a/a** diploids were spread on Yc plates (3×10^6 cells per plate) containing 10^{-7} M α-factor (Sigma) and incubated for 4 to 5 days at 20°C. α-Factor-resistant mutants were sporulated (following introduction of the *MAT*α gene on a plasmid [23, 47]). For most of the mutants, each meiosis generated two α-factor-resistant segregants and two α-factor-sensitive segregants, indicating a single dominant mutation. Only mutants showing such 2:2 segregation were characterized further. The mutations were sorted into linkage groups by constructing doubly heterozygous diploids, sporulating, and testing for recovery of wild-type progeny. At least two linkage groups of mutations have been found. The mutant genes have been designated *DAF* (for dominant α-factor resistance).

a- and α-factor resistance was assayed by replica-plating patches of cells, followed by replica-plating the primary replica to secondary plates containing 0.5 ml of supernatant from MATa cultures or 10^{-7} M α-factor. Mating was assayed by replica-plating patches to YEPD plates prespread with 0.2 ml of stationary YEPD cultures of mating type testers, incubating, and replica-plating to medium selective for diploids.

Some transformants with the integrating *DAF1-1*-containing plasmid pFC101-1 (see below) contained multiple copies of the integrated plasmid (50) (from serial dilution in Southern blots, I estimate a *DAF1-1* gene dosage of two [2×]

in one transformant and about eight [8×] in another; data not shown). The higher copy number resulted in a more extreme phenotype (see text). The 8× *DAF1-1* strains also contained the *dafl*$^+$ gene; the 2× *DAF1-1* strains did not.

Measurements of size and cell cycle parameters. Log-phase YEPD cultures were fixed in 10% Formalin, sonicated to break up aggregates, spun down and suspended in phosphate-buffered saline, and observed and photographed by phase microscopy for quantitation of cell number, budding index, and cell size. These preparations were also analyzed in a Coulter channelizer to determine the distribution of cell volumes.

For flow cytometry, cells were fixed in 70% ethanol, washed in phosphate-buffered saline, sonicated, incubated in RNase A (1 mg/ml) for 10 min at 37°C, washed, and stained with propidium iodide (50 μg/ml). They were washed once in propidium iodide (5 μg/ml) and analyzed by flow cytometry. (This procedure was suggested by T. Weinert.) These preparations were also analyzed by fluoresence microscopy to confirm nuclear staining and to quantitate the proportion of binucleate cells. An approximate quantitation of the proportion of G1 cells was obtained by cutting out the peaks from histograms such as those shown in Fig. 8 and weighing the paper. (The G1 peak was assumed to be symmetrical and was reduced slightly on the right to account for spillover from S-phase cells.) It should be noted that the positions of the two peaks (presumably G1 and G2 cells) in Fig. 8 differed between strains; the *dafl-1* strain appeared to contain more DNA than the *dafl*$^+$ strain, which appeared to contain more DNA than the *DAF1-1* strains. A similar artifact, such that larger cells appear to contain more DNA, was noted in studies of *cdc* mutants of *S. pombe* (2).

Nucleic acids. Standard procedures were used to analyze nucleic acids (35). The genomic library containing *DAF1-1* was constructed by using a phosphatase-treated *Bam*HI-cut YCp50 vector and partial *Sau*3A fragments of *DAF1-1* yeast DNA from 9 to 15 kilobases (kb) long.

For DNA sequencing, the *Sal*I-*Bgl*II fragment and the *Sal*I-*Hpa*I fragment of *DAF1-1* were subcloned into plasmid vectors containing M13 origins. Nested deletions were constructed as described previously (22). Single-stranded DNA was recovered and sequenced by using Sequenase and dITP instead of dGTP (United States Biochemical Co.). Sequencing was done on both strands except for nucleotides 1 to 282 and 2637 to 2737, which were both read only from left to right (Fig. 1) due to deficiencies in one set of nested deletions. The sequence in these regions was clear on the strand sequenced. Any ambiguities elsewhere in the sequence were resolved by using clear sequence from the other strand.

The plasmids derived from the gap repair experiment (see below) were sequenced by digestion at a unique *Sal*I site, exonuclease III digestion to produce single strands, and priming with oligonucleotides hybridizing near the regions of interest. Oligonucleotides were synthesized on an Applied Biosystems automated synthesizer. 20-mers priming 26 nucleotides (nt) 3′ to the *Xho*I site and 93 nt 3′ to the *Eco*RI site were used in this experiment. About 150 nt of sequence was obtained from each of the six plasmids sequenced with each primer.

Yeast RNA was isolated by disrupting cells with glass beads (59) in guanidine buffer (24). Formaldehyde gels were run, transferred, and hybridized as described before (35).

Plasmids, transplacements, and gap repair. pFC101 was a YCp50 (51) clone containing *DAF1-1* (it included about 2.7 kb 5′ to the *Sal*I site and less than 500 base pairs [bp] 3′ to the *Bgl*II site in Fig. 1). *DAF1-1* was mapped by cloning subfragments of pFC101 back into YCp50.

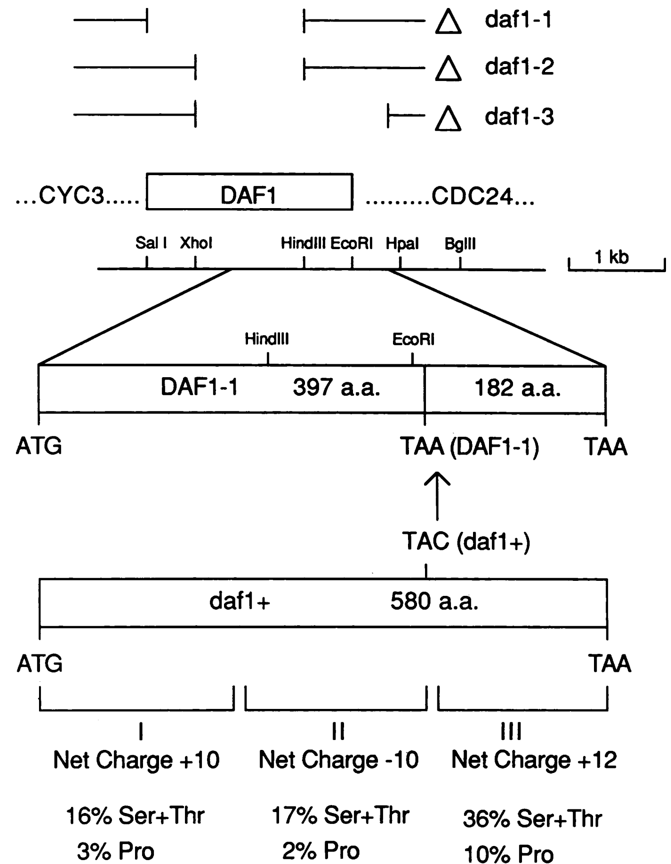

FIG. 1. *DAF1-1* structure. The restriction map of the *DAF1-1* region is shown, as deduced from two overlapping clones containing *DAF1-1*. *DAF1* is on chromosome I adjacent to *CDC24*. The minimum fragment subcloned into YCp50 conferring α-factor resistance comparable to that conferred by chromosomal *DAF1-1* is the indicated *Sal*I-*Eco*RI fragment. Note that this region does not contain the complete *DAF1-1* coding sequence (see Fig. 2). The *Sal*I-*Hin*dIII fragment was inactive, and the *Xho*I-*Eco*RI fragment exhibited greatly reduced activity. At top are shown the locations of three deletions transplaced into the genome; see Materials and Methods. Data from DNA sequence analysis of *DAF1-1* (see Fig. 2) and partial sequence analysis of *dafl*$^+$ are summarized. A division of the *dafl*$^+$ coding sequence into three domains is indicated. Boundaries: I, 1 to 189; II, 190 to 410; III, 411 to 580. Net charge is the number of Lys and Arg residues minus the number of Glu and Asp residues. a.a., Amino acids.

The centromere sequence of pFC101 was removed by *Sma*I and partial *Hpa*I digestion and ligation to produce pFC101-1. This plasmid could be linearized at the unique *Hpa*I site 3′ to *DAF1-1* in yeast DNA to give an integrating plasmid. For transplacements (55), a YIp5 plasmid lacking an *Eco*RI site and containing the *URA3* gene was substituted for *DAF1-1* DNA between the *Sal*I or the *Xho*I site and the *Hin*dIII site (Fig. 1). These plasmids were digested with *Eco*RI and used to construct the *dafl-1* and *dafl-2* deletion alleles, respectively, by transforming an **a**/α *DAF1-1*/*dafl*$^+$ *ura3* strain, followed by sporulation and tetrad analysis (see Results). A deletion from the *Xho*I site to an *Nco*I site in the *dafl*$^+$ coding sequence 35 amino acids from the termination codon (Fig. 1 and 2) was constructed and transplaced into *dafl-2* cells by cotransformation with a *TRP1* plasmid, followed by selection for loss of *URA3* (in the *dafl-2* allele)

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/GTCGACGTGCTGCGGTGCATGGCTGTTTTACCCGTTTAGGAAAAAACTCGGCGGGTTTT   60
 SalI
CTTGACGGGCAAATGTCGCCATTCGTCTCGTTTGAACGCTTGGCCTGTAAAACAGCTAAC   120

TCATTCACTATCTCTATCCTGCGACCGTGATGATACCGAAAGTATGCTGATACAGATCGG   180

TCCCTCATATCGCGATAGGGCTTTCTGAGCTCTCCTCCCCCTTCTTCCCTCCCCAGCCAA   240

AGAGGCCGGTTTTCCTTCTGGGGAAGTGTCGCAACCAAACGCTGTGCTCAAACCCTAAAT   300

TGTGAACTTTCAAGAAAAAAAAAAAAGAAAAAGTGAAAAATTATCAGGCAAGAAAAAGAAA   360

TTACCAAGCCTGCTCTCACTGTAATGATCAAGTTACATAAATTTACTATCGGATTAGTGT   420

GTCCCTCTGCCACATTTCCATATTTGGCCTTGGGTTTTTGCCCTCATCTTTTTTTTTTCT   480

TCCTCCTACCTATTTATAATTTGTATATCTGTACTTTCCTCGAGCTTTTAATCTTCTTCT   540
                                       XhoI
TAAACATTTTCTTGTGTAGTATACTTTCACACAATTTCTTTCTTGATTTTTTCTTCTACT   600

ACTGAGTCTGCCAGTCAAATGGATTTCTGAGGAAAGAGGACTATACCCATTAGGAAACGA   660

ATTGCCCGAGTAGTCTCCTCTGCCGACTTAAACCAACCTTTTTCTATTTCTCTTTTCTTT   720

TCTCCCTCTTTTTTCTCTGTACTAGCATCCAAAAGCAAGCATCCATCCGAGTCCCAGTCG   780

CAATCTCACATCTCCAATTTAACGTATCCATTGCATTTCCTCATTCGGTTTAACTCCTCT   840

GCATTTCTTTTCTGACCCATAGCATTTCTTACATTCCATTGCATCTCCCTTTTACTCTCG   900

                                   MetAlaIleLeuLysAspThrIleIle
TTCAAGACACTGATTTGATACGCTTTCTGTACGATGGCCATATTGAAGGATACCATAATT   960

ArgTyrAlaAsnAlaArgTyrAlaThrAlaSerGlyThrSerThrAlaThrAlaAlaSer
AGATACGCTAATGCAAGGTATGCTACCGCTAGTGGCACTTCCACCGCCACTGCCGCCTCT   1020

ValSerAlaAlaSerCysProAsnLeuProLeuLeuLeuGlnLysArgArgAlaIleAla
GTCAGCGCTGCCTCATGTCCTAATTTGCCCTTGCTCTTGCAAAAGAGGCGGGCCATTGCT   1080

SerAlaLysSerLysAsnProAsnLeuValLysArgGluLeuGlnAlaHisHisSerAla
AGTGCAAAGTCTAAAAACCCTAATCTCGTTAAAAGAGAATTGCAAGCACATCACTCAGCG   1140

IleSerGluTyrAsnAsnAspGlnLeuAspHisTyrPheArgLeuSerHisThrGluArg
ATCAGCGAATACAATAATGATCAATTGGACCACTATTTCCGTCTTTCCCACACAGAAAGG   1200

ProLeuTyrAsnLeuThrAsnPheAsnSerGlnProGlnValAsnProLysMetArgPhe
CCGCTGTACAACCTGACTAACTTCAACTCTCAGCCACAAGTTAATCCGAAGATGCGTTTC   1260

LeuIlePheAspPheIleMetTyrCysHisThrArgLeuAsnLeuSerThrSerThrLeu
TTGATCTTTGACTTCATCATGTACTGTCACACAAGACTCAATCTATCGACCTCGACTTTG   1320

PheLeuThrPheThrIleLeuAspLysTyrSerSerArgPheIleIleLysSerTyrAsn
TTCCTTACTTTCACTATCTTGGACAAGTATTCCTCGCGGTTCATTATCAAGAGTTACAAC   1380

TyrGlnLeuLeuSerLeuThrAlaLeuTrpIleSerSerLysPheTrpAspSerLysAsn
TACCAGCTCTTGTCCTTGACCGCGCTTTGGATTTCGTCCAAATTTTGGGACTCCAAGAAT   1440

ArgMetAlaThrLeuLysValLeuGlnAsnLeuCysCysAsnGlnTyrSerIleLysGln
AGAATGGCCACTTTGAAAGTCTTGCAAAACTTGTGTTGCAATCAATATTCTATAAAGCAA   1500

PheThrThrMetGluMetHisLeuPheLysSerLeuAspTrpSerIleCysGlnSerAla
TTCACGACTATGGAAATGCATCTTTTCAAATCACTCGATTGGTCCATCTGTCAGTCGGCA   1560

ThrPheAspSerTyrIleAspIlePheLeuPheGlnSerThrSerProLeuSerProGly
ACATTCGACTCCTACATCGACATCTTCTTGTTCCAATCTACGTCCCCGTTATCGCCTGGC   1620

ValValLeuSerAlaProLeuGluAlaPheIleGlnGlnLysLeuAlaLeuLeuAsnAsn
GTTGTCCTTTCTGCCCCTTTGGAAGCTTTCATTCAACAGAAACTGGCCTTATTAAATAAC   1680
                     HindIII
AlaAlaGlyThrAlaIleAsnLysSerSerSerSerGlnGlyProSerLeuAsnIleAsn
GCTGCTGGTACTGCTATTAATAAATCGTCCTCTTCTCAAGGCCCCTCTTTGAACATCAAC   1740

GluIleLysLeuGlyAlaIleMetLeuCysGluLeuAlaSerPheAsnLeuGluLeuSer
GAGATCAAATTGGGTGCCATTATGTTGTGCGAGTTAGCTTCCTTCAATCTCGAATTATCA   1800

PheLysTyrAspArgSerLeuIleAlaLeuGlyAlaIleAsnLeuIleLysLeuSerLeu
TTTAAATATGATCGTTCACTAATTGCGCTGGGTGCAATTAACCTCATCAAATTATCTTTG   1860

AsnTyrTyrAsnSerAsnLeuTrpGluAsnIleAsnLeuAlaLeuGluGluAsnCysGln
AACTACTATAATTCAAACCTTTGGGAAAATATCAATCTGGCTTTGGAGGAAAACTGCCAA   1920

AspLeuAspIleLysLeuSerGluIleSerAsnThrLeuLeuAspIleAlaMetAspGln
GACCTAGATATTAAATTGTCAGAAATCTCTAATACTTTATTGGATATAGCAATGGACCAA   1980

AsnSerPheProSerSerPheLysSerLysTyrLeuAsnSerAsnLysThrSerLeuAla
AATTCTTTCCCCTCCAGTTTCAAATCAAAATATTTGAATAGCAATAAGACATCTTTAGCA   2040

LysSerLeuLeuAspAlaLeuGlnAsnTyrCysIleGlnLeuLysLeuGluGluPheTyr
AAATCTCTCTTAGACGCATTACAAAACTATTGTATTCAATTGAAACTGGAAGAATTCTAC   2100
                          Tyr                    EcoRI
ArgSerGlnGluLeuGluThrMet***AsnThrIlePheAlaGlnSerPheAspSerAsp
CGTTCACAAGAATTGGAAACCATGTAAAATACTATCTTTGCTCAGTCCTTTGACAGCGAT   2160
                          C
SerLeuThrCysValTyrSerAsnAlaThrThrProLysSerAlaThrValSerSerAla
TCATTGACTTGTGTTTACTCAAATGCTACTACTCCAAAGAGCGCTACGGTTTCATCTGCG   2220

AlaThrAspTyrPheSerAspHisThrHisLeuArgArgLeuThrLysAspSerIleSer
GCCACAGACTATTTCTCGGATCACACTCATTTAAGAAGGTTGACCAAAGATAGCATTTCT   2280

ProProPheAlaPheThrProThrSerSerSerSerSerProSerProPheAsnSerPro
CCACCATTTGCCTTCACTCCAACCTCATCTTCATCCTCTCCATCTCCATTCAATTCCCCT   2340

TyrLysThrSerSerSerMetThrThrProAspSerAlaSerHisHisSerHisSerGly
TACAAGACTTCAAGTTCAATGACGACCCCAGACTCTGCATCACACCATTCACATTCAGGT   2400

SerPheSerSerThrGlnAsnSerPheLysArgSerLeuSerIleProGlnAsnSerSer
TCGTTCTCTTCTACCCAAAATTCTTTTAAAAGGTCACTGAGCATCCCACAAAATTCAAGC   2460

IlePheTrpProSerProLeuThrProThrThrProSerLeuMetSerAsnArgLysLeu
ATCTTTTGGCCAAGCCCACTAACTCCCACCACCCCATCTCTAATGTCAAATAGAAAATTA   2520

LeuGlnAsnLeuSerValArgSerLysArgLeuPheProValArgProMetAlaThrAla
TTACAAAATTTATCTGTGCGTTCAAAAAGATTATTTCCTGTTAGACCCATGGCCACTGCT   2580

HisProCysSerAlaProThrGlnLeuLysLysArgSerThrSerSerValAspCysAsp
CACCCATGCTCTGCCCCCACCCAACTGAAAAAGAGATCAACTTCCTCTGTGGATTGTGAT   2640

PheAsnAspSerSerAsnLeuLysLysThrArg***
TTTAATGATAGTAGCAACCTCAAGAAAACTCGCTGAAACGACAAAAAAAAAAATGCATTTA   2700

ACAACAAATAAATTAAAATTTGCAAAGCAAATACGTT/                         2737
                                 (HpaI?)
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FIG. 2. Sequence of *DAF1-1*. The sequence of *DAF1-1* from the *Sal*I site to the *Hpa*I site was determined (all sequence was determined from both strands except for some terminal sequences; see Materials and Methods). The location of the *Hpa*I site is not definitive, since this site was cloned as a blunt end into the sequencing vector, leaving only half the site. Two consecutive open reading frames are translated; these are not separated by a stop codon in *daf1*$^{+}$ (see text), and a TAC tyrosine codon is substituted for the ochre stop as indicated. A potential polyadenylation signal (4) (AATAAA) is underlined.

by fluoro-orotic acid selection (5). This procedure efficiently replaced the *daf1-2* deletion with the larger deletion, named *daf1-3*.

The gap repair experiment (33, 50) to rescue the *daf1*$^{+}$ gene was performed by digestion of a *DAF1-1–CEN–TRP1–ARS1* plasmid (the vector was pSE358, obtained from C. Mann) with *Xho*I and *Eco*RI, deleting from about 450 bp 5′ of the *DAF1-1* ATG to 30 bp 5′ to the *DAF1-1* termination codon (Fig. 1 and 2). The gapped plasmid was purified by gel electrophoresis and used to transform a *trp1 daf1*$^{+}$ strain. About 10% of the transformants were α-factor resistant, consistent with the mutation being near one of the gap termini (33). Recovered plasmids (three causing α-factor resistance and three not) were sequenced in these terminal regions by using oligonucleotide primers (see above). The TAA to TAC change described in the text cosegregated with the inability to cause α-factor resistance (see text).

RESULTS

Rationale. I have selected dominant α-factor resistance mutations, since dominance might be a characteristic of mutations in genes which either are the target of the α-factor-induced negative signal or can act to bypass α-factor arrest by altering cell cycle regulation. In contrast, α-factor resistance mutations in genes required for signal generation (e.g.,

STE2, the α-factor receptor), are recessive (20). Mutations in the *DAF1* linkage group make cells both α-factor resistant and smaller than wild type; this pleiotropy suggested that *DAF1* might be involved in START control. The gene nomenclature used is as follows. The dominant α-factor resistance mutation is referred to as *DAF1-1* (or *DAF1* in figures, for convenience). The wild-type gene is referred to as *daf1*$^+$ (since it is recessive to *DAF1-1* with respect to α-factor resistance). The deletion alleles *daf1-1*, *daf1-2*, and *daf1-3* (Fig. 1), which are recessive to the wild type with respect to size (see below) are collectively referred to as *daf1*.

Cloning and map position of *DAF1*. A genomic library constructed from *DAF1-1* yeast DNA in the low-copy-number yeast vector YCp50 (51) was transformed into *daf1*$^+$ cells. Two plasmids with overlapping inserts that made cells α-factor resistant and small were isolated from the library by α-factor selection of transformants.

Linkage of the cloned gene with *DAF1-1* was shown by integration into the genome, followed by tetrad analysis of integrants (50, 55, 59). *DAF1-1* is within 4 centimorgans (cM) of the cloned DNA with 95% certainty (no recombinants detected in 98 tetrads). A transplaced deletion of the cloned DNA (see below) reverted *DAF1-1* in *cis* but not in *trans*. Therefore, the cloned DNA included *DAF1-1*.

Hybridization of *DAF1* DNA to *Sfi*I and *Not*I digests of yeast DNA, combined with a *Sfi*I and *Not*I restriction map of the yeast genome, placed *DAF1* within 60 kb of *CDC19* on chromosome I (A. Link, personal communication). Comparison of the *DAF1* restriction map with that of chromosome I clones (10) combined with deletion mapping allowed the mapping of *DAF1* to a 2.2-kb *Sal*I-*Eco*RI fragment, adjacent to *CDC24* (10, 44) (Fig. 1). A 2-kb transcript from this region, *FUN10* (for function unknown), was detected previously (10).

***DAF1-1* sequence and identification of the activating mutation.** The *DAF1-1* region contained a 397-amino-acid open reading frame (Fig. 1 and 2). Upstream of the coding sequence were a number of poly(dA) and poly(dT) stretches, which may promote constitutive transcription (64). The *DAF1-1* termination codon was immediately followed by another 182 amino acids of open reading frame in the same frame, before another termination codon.

The *daf1*$^+$ gene was recovered by gap repair (see Materials and Methods). The *daf1*$^+$ gene did not make cells α-factor resistant when it was present on low-copy-number plasmids or integrated into the chromosome, unlike the *DAF1-1* gene (although one or a few extra copies of *daf1*$^+$ did reduce cell size, to a lesser extent than *DAF1-1*; data not shown). Limited sequencing showed the substitution of a TAC tyrosine codon in *daf1*$^+$ for the *DAF1-1* TAA termination codon. C-terminal switches constructed between *DAF1-1* and *daf1*$^+$ at the *Eco*RI site (Fig. 1) showed that the *DAF1-1* C-terminus activated *daf1*$^+$ and the *daf1*$^+$ C-terminus inactivated *DAF1-1* with respect to size and α-factor resistance. Therefore, the termination codon of *DAF1-1* is the only mutation required to convert the *daf1*$^+$ coding sequence to a form causing α-factor resistance.

No strong sequence similarity between the *daf1*$^+$ gene and any protein-coding sequence in the GenBank data base was detected. The sequence can be roughly divided into a basic N-terminal third, an acidic middle third, and a basic C-terminal third containing a very high proportion of serine, threonine, and proline residues; the latter region was removed by the *DAF1-1* termination codon (Fig. 1).

***DAF1-1* prevents α-factor-induced division arrest.** At concentrations of α-factor sufficient to completely inhibit the growth of *daf1*$^+$ strains, the growth of *DAF1-1* strains continued, although at a lower rate (Table 1), resulting in haloes of lighter lawn growth near an α-factor source (Fig. 3A). This partial sensitivity to α-factor converted to nearly complete resistance when the *DAF1-1* gene dosage was increased about eightfold (Table 1, Fig. 3A). (Note that this high-copy *DAF1-1* allele also contained the *daf1*$^+$ gene [see Materials and Methods]. While the presence of the *daf1*$^+$ gene probably does not contribute significantly to the phenotype, this has not been rigorously established. Throughout this paper the allele is simply referred to as 8× *DAF1-1* or high-copy *DAF1-1*.) Following α-factor addition (10^{-7} M) to liquid cultures (Fig. 3B), *daf1*$^+$ cells arrested as unbudded G1 cells (6, 53). *DAF1-1* cells only arrested transiently; their recovery was not due to α-factor degradation (data not shown). High-copy *DAF1-1* cells failed to show this transient arrest (Fig. 3B, upper). *daf1*$^+$ cells showed a similar transient arrest and adaptation in response to low α-factor concentrations ($\leq 10^{-9}$ M) (42, 43).

TABLE 1. Cell cycle parameters[a]

Allele	Mother cell size (arbitrary units)	% UB	Doubling time (h)	
			YEPD	YEPD + α-factor
daf1$^+$	1.00 ± 0.20 (30)	43 ± 4 (7)	1.8	N.A.[b]
DAF1-1	0.55 ± 0.17 (33)	42 ± 3 (9)	1.8	3
8× *DAF1-1*	0.59 ± 0.21 (34)	38 ± 4 (8)	1.8	2.2
daf1-1	1.52 ± 0.50 (30)	48 ± 5 (6)	1.8	N.A.

[a] Cell cycle parameters for strains in YEPD medium, 30°C. Relative mother cell volume was calculated from photomicrographs by the formula volume = $K \times L \times W^2$ (31), where K is a constant, L is length, and W is width. Doubling time in YEPD or YEPD with 10^{-7} M α-factor was determined by following the OD_{600}. Cell size and doubling times were determined for a single representative strain of each genotype. These strains were matched at all loci except *DAF1* and *URA3*. % UB, Percentage of unbudded cells in log-phase cultures. The indicated number of independent strains were assayed in parallel. Mean ± standard deviation (number of replicates: individual cells for size measurements, number of strains for % UB) are presented. The doubling time results shown were from a single representative experiment. Two-tailed t tests were performed pairwise on the data in the first two columns. All comparisons showed significant differences ($P < 0.001$) except for % UB for *daf1*$^+$ and *DAF1-1*.

[b] N.A., Not applicable; cultures did not enter log phase in this medium.

DAF1-1* reduces mating efficiency in both mating types.** As might be predicted from their reduced pheromone sensitivity, *DAF1-1* cells had a reduced mating efficiency, although they produced near-normal levels of the appropriate mating pheromone (Fig. 4). A slight reduction in **a**-factor production by *MATa** *DAF1-1* strains was not sensitive to *DAF1-1* dosage, unlike the mating efficiency.

mRNA induction by α-factor occurs efficiently in *DAF1-1* cells. A number of mRNAs are induced by pheromones. Induction may not require G1 division arrest (18, 38). If these mRNAs are induced equally well in *DAF1-1* and *daf1*$^+$ cells, this result would support the idea that α-factor binding to *DAF1-1* cells produces the normal signal and that a transcriptional branch of the response pathway is intact, but that division arrest in response to this signal is defective (see Fig. 9). A similar induction of *STE2* RNA by a saturating dose of α-factor was observed in *daf1*$^+$, *DAF1-1*, and high-copy *DAF1-1* strains (Fig. 5A) (*STE2* [α-factor receptor] mRNA is induced about fourfold by α-factor [19, 30, 45]). Similar dose-response kinetics were observed for *daf1*$^+$ and high-copy *DAF1-1* strains for α-factor induction of the *FUS1* RNA (39, 68) (Fig. 5B). In several experiments, the difference in *FUS1* induction at various doses from 10^{-11} to

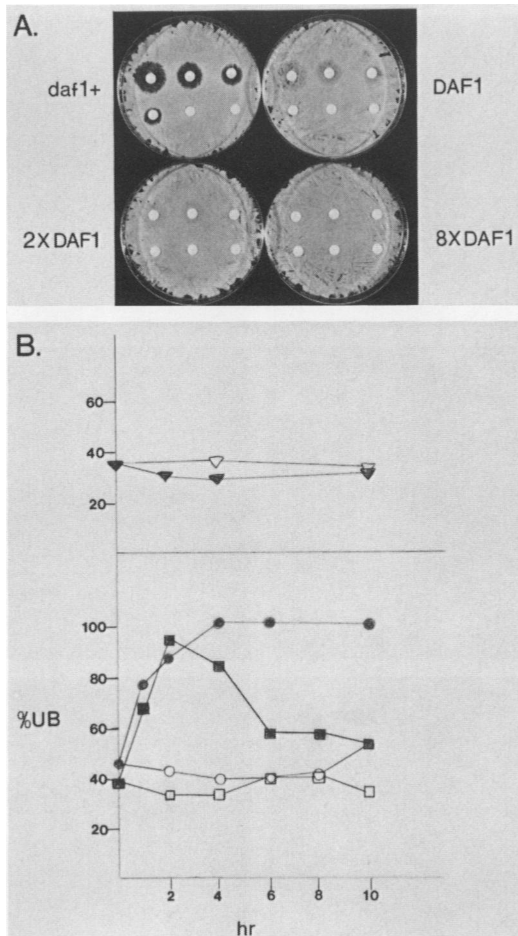

FIG. 3. Assays of α-factor resistance of *DAF1-1* and *dafl*$^+$ strains. (A) Disks (Difco) containing threefold serial dilutions of α-factor (10 μl, starting with 10^{-4} M) were placed on YEPD plates prespread with 0.2 ml of stationary-phase YEPD cultures of the indicated *DAF1* genotype. 2× *DAF1* is *DAF1::DAF1-URA3*; 8× *DAF1* is *dafl*$^+$::8× *DAF1-URA3* (see Materials and Methods). (B) Liquid YEPD cultures in early log phase (30°C) were split, and α-factor was added to half to 10^{-7} M. At intervals, portions were removed, fixed, and sonicated, and the percentage of unbudded cells (% UB) was scored. Open symbols, No α-factor; solid symbols, with α-factor. Circles, *dafl*$^+$ (strain 34-10D); squares, *DAF1-1* (strain 34-9C); triangles, 8× *DAF1-1* (strain 121-3-4D). *DAF1-1* strains (especially high-copy *DAF1-1* strains) grew in a clumpy manner in the presence of α-factor; although sonication effectively dissociated the cells, some clumpiness may have remained, possibly causing slight underestimation of the fraction of unbudded cells in these populations.

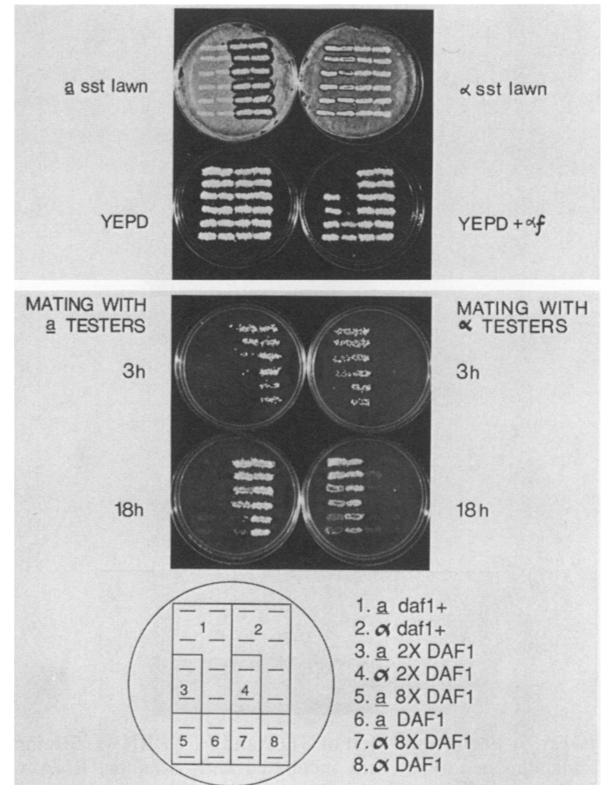

FIG. 4. Pheromone production, α-factor sensitivity, and mating of *DAF1-1* strains. Tetrads from strains X120 (*dafl*$^+$/*dafl*$^+$ *ura3*/$^+$) (top two rows in each dish), X121-2 (*dafl*$^+$/*DAF1-1::DAF1-1-URA3 ura3/ura3*) (second two rows), and X121-3 (*DAF1-1/dafl*$^+$::8× *DAF1-1-URA3 ura3/ura3*) (third two rows) were patched and replica-plated to test for pheromone production with supersensitive tester (sst) strains for α-factor resistance and for mating. Tetratype tetrads for *MAT* and *URA3* were chosen and arranged (left to right) *URA*$^+$ MAT**a**, *ura* MAT**a**, *URA*$^+$ MATα, and *ura* MATα. A key to the genotypes is shown at bottom.

10^{-7} M between *dafl*$^+$ and high-copy *DAF1-1* strains was never more than approximately twofold (although where differences existed the level for the *DAF1-1* strain was slightly lower). This result implies that the number and activity of α-factor receptors are similar between *dafl*$^+$ and *DAF1-1* strains with respect to *FUS1* induction.

***DAF1-1* suppresses *sst2* and *scg1*.** Mutations in the *SST2* gene result in supersensitivity to pheromones in both **a** and α cells (9). *SST2* may be an antagonist to the pheromone signal. If *DAF1-1* works by superactivating *SST2*, then *DAF1-1* should be suppressed by an *sst2* mutation. If *DAF1-1* acts independently of *SST2*, then *DAF1-1* should suppress the pheromone sensitivity of an *sst2* mutation. MAT**a** and MATα *sst2* strains were transformed with YCp50 (low-copy-number) plasmids containing *DAF1-1*. The supersensitive phenotype was suppressed by the *DAF1-1* plasmid in all transformants, although some partial sensitivity remained.

Disruption of the *SCG1* gene results in a phenotype similar to constitutive pheromone signalling, leading to nearly complete division arrest without added pheromone (11, 26, 40). A diploid strain with the genotype **a**/α *scg1::LACZ6-LEU2/SCG1*, when sporulated to produce haploids, yielded wild-type, *leu2* colonies and very slow growing, putative *LEU2*$^+$ colonies containing large, shmoolike cells (the *scg1* phenotype [11]). When the strain was transformed before sporulation with a YCp50 (*URA3*) plasmid containing the *DAF1-1* gene, fast-growing *LEU2*$^+$ *URA3*$^+$ colonies (both **a** and α) were efficiently recovered; the YCp50 vector alone was inactive. *LEU2*$^+$ colonies rescued with the plasmid were unable to lose the plasmid after extended mitotic growth, unlike their *leu2* (*SCG1*$^+$) siblings. Since *SCG1* disruption bypasses the requirement for pheromone receptor interaction for the normal pheromone response (11, 26, 40), this result and the dose-response experiment for *FUS1* induction (Fig. 5) provide independent evidence that the *DAF1-1*-conferred α-factor resistance is not due to a defect in pheromone-receptor interaction, but that *DAF1-1* acts at a later step (after the presumed inactivation of *SCG1* [11, 26, 40]).

***DAF1-1* cells are small.** *DAF1-1* cells were smaller than

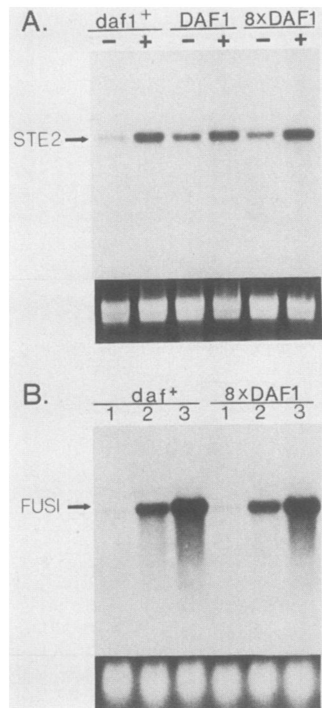

FIG. 5. α-Factor induction of *STE2* and *FUS1* RNAs. Strains of the indicated genotype were incubated with α-factor. RNA was isolated, run on formaldehyde-agarose gels, and transferred to nylon membranes, which were hybridized with RNA probes to *STE2* (7) or *FUS1* (38). (A) Incubation with 0 or 10^{-7} M α-factor for 3 h. (B) Incubation with 0, 10^{-10} M, or 10^{-9} M α-factor for 20 min (lanes 1 to 3, respectively). Ethidium bromide staining of the total RNA samples used (run on a parallel miniagarose gel) is shown below each lane.

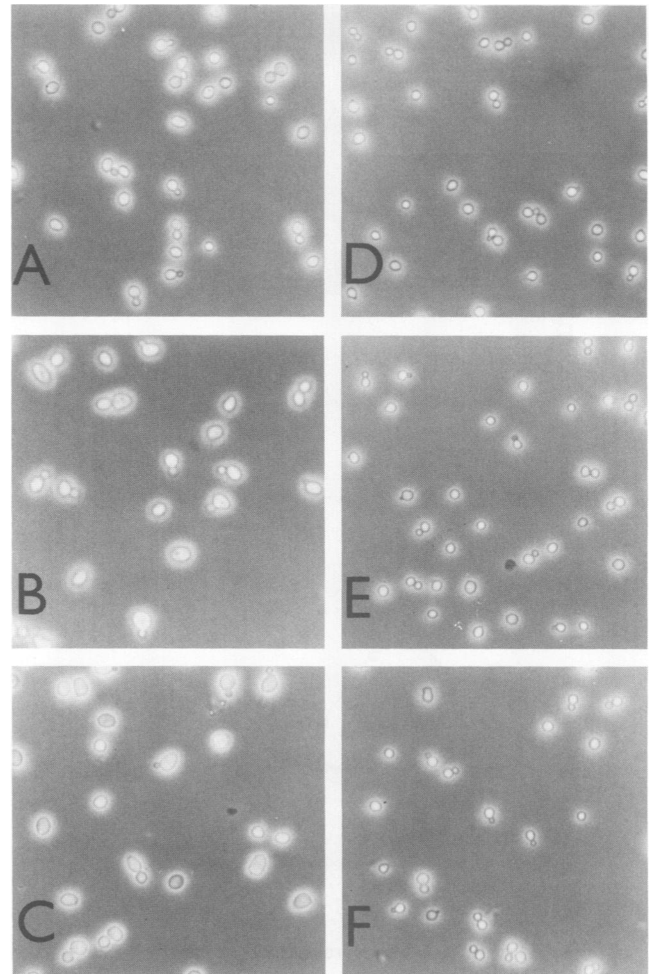

FIG. 6. Photomicrographs of *dafl*$^+$, *DAF1-1*, and *dafl* strains. Cells of differing *DAF1* genotype were harvested in the log phase in YEPD, fixed, sonicated, and photographed by phase-contrast microscopy. A, *dafl*$^+$; B, *dafl-1*; C, *dafl-2*; D, *DAF1-1*; E, 2× *DAF1-1*; F, *dafl*$^+$::8× *DAF1-1*.

wild-type cells (Fig. 6, Table 1). Coulter channelizer volume distribution of log-phase cells (Fig. 7) showed size shifts due to *DAF1-1* alleles that were approximately consistent with the volume determinations in Table 1. Despite the more extreme α-factor resistance phenotype of *dafl*$^+$::8×*DAF1-1* strains, cells with this allele were slightly larger than *DAF1-1* cells (Table 1; Coulter channelizer data not shown).

***dafl*$^+$ is not required for viability, but *dafl* cells are large.** Three deletions in the *DAF1* region were constructed and introduced into the genome by gene transplacement (Fig. 1; see Materials and Methods). An **a**/α *DAF1-1*/*dafl*$^+$ strain was transformed with transplacement constructions to produce strains heterozygous for *dafl-1* and *dafl-2*. Ten tetrads each from five transformants (two with the *dafl-1* allele and three with the *dafl-2* allele) were analyzed. The overall spore viability (50 tetrads) was 89% (the range for individual transformants was 83% to 98%). *URA3*$^+$ segregated 2:2 in complete tetrads, and the expected deletions cosegregated with *URA3*$^+$ by Southern blot analysis (not shown). *dafl* strains had doubling times similar to that of the wild type (Table 1) (although some *dafl* segregants had a mild slow-growth or delayed-germination phenotype).

The *dafl-3* allele (Fig. 1) was introduced by allele replacement of *dafl-2* by selection for fluoro-orotic acid resistance (5) (see Materials and Methods). This deletion removed all but the C-terminal 35 amino acids of the *dafl*$^+$ coding sequence. Cells with this deletion were viable, with growth rates similar to that of the *dafl-2* parent. This result rules out the possibility that the viability of *dafl-1* and *dafl-2* cells is due to expression of the residual C-terminal information in these alleles.

The high spore viability and ease of recovery of *dafl* strains from these transformants is strong evidence that *dafl*$^+$ is not an essential gene. The hypothesis that the viability of *dafl* strains is due to the generation and selection of extragenic suppressor mutations (as occurs in diploids heterozygous for α-tubulin mutations [58]) appears unlikely in this case, but the possibility has not been rigorously excluded. (Genetic tests to exclude such a hypothesis have been described and performed in the case of deficiency for the clathrin gene [52]).

The volume of *dafl* cells averaged about 1.5 times that of wild-type cells (Fig. 6 and 7, Table 1). A *dafl*$^+$ *cdc24* temperature-sensitive (ts) chromosome complemented a *dafl-1 CDC24* chromosome with respect to size at 37°C (data not shown), suggesting that the effect of the deletion on cell size is not due to a *cis* effect on the expression of *CDC24* (e.g., by deleting a promoter element or UAS [16]). In addition, *dafl-1* cells were as respiration competent as *dafl*$^+$ cells, suggesting that the deletion did not interfere with *CYC3* expression (10). Thus, the large-size phenotype is unlikely to be due to effects of the deletion on neighboring

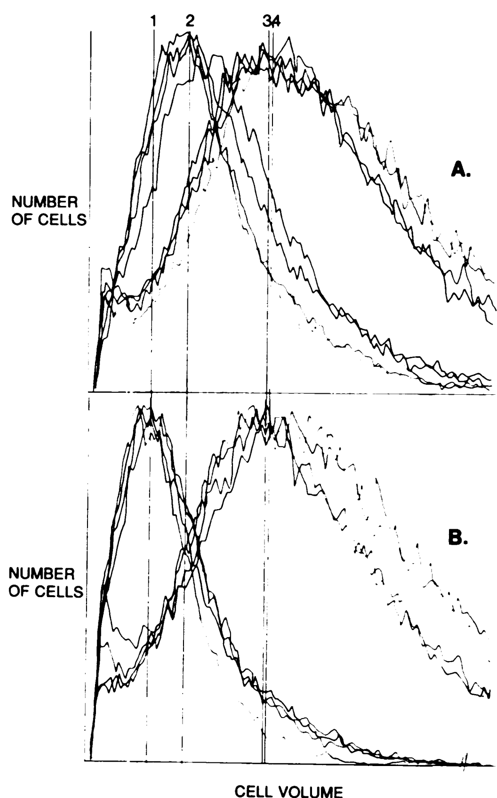

FIG. 7. Volume distributions of *dafl*$^+$, *DAF1-1*, and *dafl* strains. X121 (*MATa/MATα DAF1-1/dafl*$^+$) was transformed with transplacement constructions which produce *dafl-1* (A) and *dafl-2* (B) (see Fig. 1). (A) *DAF1-1* gene deleted; (B) *dafl*$^+$ gene deleted. The transformants were sporulated and tetrads were dissected. Log-phase YEPD cultures from all segregants from two tetrads from each transformant were fixed and sonicated, and the distribution of cell volumes was measured in a Coulter counter channelizer. Tetratype tetrads for *MAT* and the alleles at *DAF1* were analyzed. (A) *dafl*$^+$ (peak 2) and *dafl-1* (peak 3) segregants. (B) *DAF1-1* (peak 1) and *dafl-2* (peak 4) segregants.

genes, assuming that there are no other genes between *CDC24* and *CYC3* except *DAF1*; no other mRNAs were detected from this region (10).

The *dafl-1* large-cell phenotype reverted when a fragment of DNA containing *dafl*$^+$ was integrated into the *TRP1* locus. (The fragment used extended from an *Eco*RI site about 2.5 kb upstream of *dafl*$^+$ to a *Bgl*II site inside the *CDC24* gene [10] [Fig. 1]). A similar fragment, but with a deletion of the *dafl*$^+$ gene, had no effect; data not shown.)

The fraction of unbudded and G1 cells in log-phase cultures of *dafl* strains was slightly increased (Table 1, Fig. 8). Most or all of the increased volume of *dafl* cells can be accounted for by increased size of the mother cell at budding (Table 1, Fig. 6). These data are consistent with the idea that *dafl* cells are bigger specifically because they do not pass START until they reach a cell size larger than the normal critical size (53). These results imply that *dafl*$^+$ has a role in setting the critical size.

***DAF1* size phenotypes are cell type independent.** **a** and α strains have similar sizes dependent on their genotype at *DAF1* (Fig. 7). The effects of *DAF1* alleles on the size of **a**/α diploid cells are similar to their effects on the size of **a** and α cells, as determined by Coulter channelizer analysis (not shown). **a**/α cells are not responsive to pheromones (23, 47), so the effects of *DAF1* alleles are not mediated directly through the pheromone response pathway.

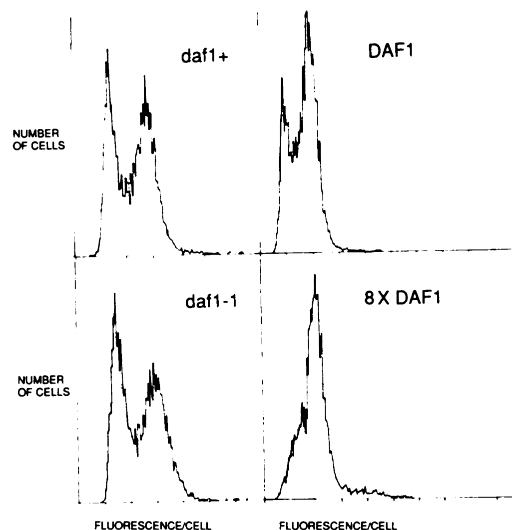

FIG. 8. Distribution of DNA in log-phase cells. Strains of the indicated genotype were harvested in log phase in YEPD, 30°C, and processed for flow cytometry as described in Materials and Methods. Peak positions did not align precisely between different strains; see Materials and Methods. Assuming that in all cases the first peak is G1 cells and the second is G2 cells, an approximate quantitation of the data in Fig. 9 and two other similar experiments yielded the following fractions of G1 cells: *dafl-1*, 0.45; *dafl*$^+$, 0.39; *DAF1-1*, 0.29; 8× *DAF1-1*, approx. 0.1?. (In some experiments, a small G1 peak was more clearly resolved in 8× *DAF1-1* samples; its small magnitude and poor resolution made quantitation difficult.)

***DAF1-1* advances the initiation of DNA synthesis.** The alterations in cell size caused by various alleles at *DAF1* suggested the possibility that *DAF1* was altering cell cycle kinetics. A difference in the timing of DNA synthesis was observed by quantitation of DNA content in individual cells by flow cytometry. *DAF1-1* reduced the proportion of G1 cells, especially at high copy number; *dafl-1* slightly increased the proportion of G1 cells.

Neither the doubling time nor the proportion of unbudded cells in log phase was much altered by the allele at *DAF1* (Table 1). The proportion of binucleate cells in *dafl*$^+$ and high-copy *DAF1-1* cultures was similar (data not shown). Thus, relative to the cell cycle events of bud emergence, nuclear division, and cell separation, initiation of DNA synthesis was specifically advanced by the *DAF1-1* mutation. By elimination, high-copy *DAF1-1* cells must be spending a disproportionate amount of their nuclear cycle after the initiation of DNA synthesis (and probably after completion of DNA synthesis; Fig. 8) but before nuclear division.

DISCUSSION

The *DAF1-1* mutation is a C-terminal truncation in the *dafl*$^+$ gene, resulting in small cell size, α-factor resistance, and a shortened G1 period. I discuss below how these phenotypes might be related to each other and to the activity of the *dafl*$^+$ gene.

Relationship between *dafl*$^+$ and *DAF1-1*. The deletion of *dafl*$^+$ produces a phenotype opposite to the *DAF1-1* phenotype: larger cells than *dafl*$^+$ instead of smaller and two- to threefold more α-factor sensitive (not shown) instead of resistant. Therefore, it is likely that the *DAF1-1* allele is a

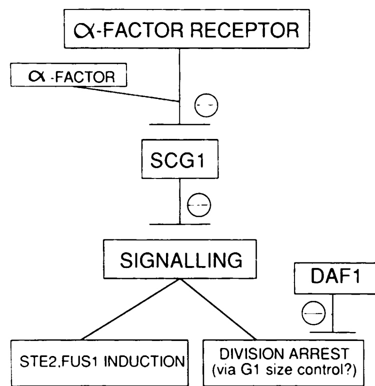

FIG. 9. Model for the induction of mRNAs and division arrest by α-factor and for the position of action of *DAF1*. Following binding of α-factor to its receptor (encoded by *STE2* [27, 28]), the *SCG1* product is inactivated; this step can be mimicked by mutational inactivation of this gene (11, 26, 40). *SCG1* is a negative regulator of signalling; following its inhibition, signalling leads independently to induction of various mRNAs (e.g., *STE2* and *FUS1*) and to division arrest. *DAF1* interferes specifically with division arrest, possibly because both the α-factor signal and *DAF1* affect the G1 size control pathway.

hyperactive or deregulated version of *dafl*$^+$, not an allele expressing any intrinsically novel function. Consistent with this idea, increasing the *dafl*$^+$ gene dosage from one to two reduced cell size slightly (data not shown). Therefore, the normal function of the *dafl*$^+$ product is probably related to G1 size control.

The C-terminal third of *dafl*$^+$, removed by the *DAF1-1* truncation, might be a regulatory domain modulating the activity of *dafl*$^+$. This idea also implies that the intrinsic activity of *dafl*$^+$ and *DAF1-1* is the same. One might expect *trans*-acting modulators of *dafl*$^+$ activity to interact with it via the C-terminus.

Relationship between *DAF1-1* activity, size control, and α-factor induced division arrest. *DAF1-1* cells are resistant to division arrest but not mRNA induction caused by α-factor. Inactivation of the *SCG1* product may be an early step in pheromone signal transduction (11, 26, 40). Since *DAF1-1* suppresses the mitotic defect of an *scg1* mutation, it acts downstream of this step. Assuming that a single intracellular signal is generated in response to α-factor binding and *SCG1* inactivation, normally leading to both mRNA induction and division arrest, *DAF1-1* cells are specifically defective in division arrest at START in response to this signal. A schematic outline of this idea is shown in Fig. 9. Consistent with this scheme, *DAF1-1 scg1* strains are constitutive for a level of *FUS1* RNA almost as high as that seen in *dafl*$^+$ or *DAF1-1 SCG1*$^+$ strains induced with α-factor (data not shown).

All five independent alleles of *DAF1* have an unselected small-cell phenotype (although the alleles differ in the strength of their size and α-factor resistance phenotypes). This finding suggests that G1 pheromone arrest and G1 size control may be mechanistically coupled, since one mutant gene product can perturb both. Suppose that α-factor arrests cells by increasing the setpoint of G1 size control. α-Factor division arrest may really be division delay, with the extent of the delay proportional to the number of receptors occupied (42, 43). Since cell growth continues in α-factor (6, 31), the longer the division delay the larger the cell; perhaps the eventual adaptation is due to the large size attained. The *DAF1-1* phenotype could be explained in several ways: if α-factor works by inactivating the *dafl*$^+$ product (e.g., via its C-terminus), then the *DAF1-1* product could escape this regulation. Alternatively, *DAF1-1* could simply be a hyperactive version of *dafl*$^+$, with both working in opposition to the effect of α-factor on the size control setpoint. Similar possibilities exist to explain the effects of *DAF1* alleles on size control in the absence of pheromones.

***DAF1-1* and cell cycle kinetics.** *DAF1-1* changes the timing of initiation of DNA synthesis but has little effect on the timing of bud emergence and nuclear division (Table 1, Fig. 8). The temporal uncoupling of bud emergence and initiation of DNA synthesis is consistent with the view of the cell cycle derived from the study of *cdc* mutants, in which these events are on independent functional pathways (53).

The *CDC28* gene is required for START and initiation of DNA synthesis in normal cells (39, 52, 54). There are several indications that *DAF1-1* or *dafl*$^+$ and *CDC28* act in the same pathway: a *cdc28* ts allele partially suppresses *DAF1-1* at permissive temperature, and *dafl cdc28* ts double mutants show a lower semipermissive temperature than *dafl*$^+$ *cdc28* ts strains (data not shown). Further work is required to evaluate the specificity of these interactions.

High-copy *DAF1-1* cells probably spend a disproportionate amount of their cycle between completion of DNA synthesis and nuclear division. Assuming that growth and division are coordinated in *DAF1-1* strains, this coordination could occur by G2 size control over nuclear division. This G2 size control could be present but cryptic in wild-type *S. cerevisiae*; its relationship to G1 size control would be similar to the relationship of cryptic G1 size control to the predominant G2 size control in *S. pombe* (46, 49). The *S. pombe weel* mutation results in small size through the loss of a mitotic inhibitor (56). *weel* alters the timing of a number of cell cycle events (12) and also causes a shift in the mode of size control from G2/M to G1/S (49, 56).

***DAF1* and *whil* are probably allelic.** Mutations in two genes have previously been shown to alter cell size in *S. cerevisiae* (8, 65). The *whi2* mutation appears to cause small size only as cells enter stationary phase, not in log phase growth, unlike *DAF1*. Like *DAF1*, the *whil* mutation causes small size in log-phase growth. Recent genetic and molecular data show that *whil* is probably allelic to *DAF1*; the *whil* allele causing small size is dominant and also causes α-factor resistance (B. Futcher, personal communication). It seems reasonable to suppose that the ability to sense cell size should be critical for coordinating growth and division (31). The dispensability of *dafl*$^+$/*whil*$^+$ might therefore indicate that there are other genes involved in the process of sensing cell size and transducing this information to the START apparatus. These genes might function in a way similar to *dafl*$^+$, or *dafl*$^+$ might be a unique and dispensable function modulating the functions of a more basic size control system.

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