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ADP Ribosylation Factor Is an Essential Protein in *Saccharomyces cerevisiae* and Is Encoded by Two Genes

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ADP ribosylation factor (ARF) is a ubiquitous 21-kDa GTP-binding protein in eucaryotes. ARF was first identified in animal cells as the protein factor required for the efficient ADP-ribosylation of the mammalian G protein G_s by cholera toxin in vitro. A gene (*ARF1*) encoding a protein homologous to mammalian ARF was recently cloned from *Saccharomyces cerevisiae* (Sewell and Kahn, Proc. Natl. Acad. Sci. USA, 85:4620–4624, 1988). We have found a second gene encoding ARF in *S. cerevisiae*, *ARF2*. The two *ARF* genes are within 28 centimorgans of each other on chromosome IV, and the proteins encoded by them are 96% identical. Disruption of *ARF1* causes slow growth, cold sensitivity, and sensitivity to normally sublethal concentrations of fluoride ion in the medium. Disruption of *ARF2* causes no detectable phenotype. Disruption of both genes is lethal; thus, *ARF* is essential for mitotic growth. The ARF1 and ARF2 proteins are functionally homologous, and the phenotypic differences between mutations in the two genes can be accounted for by the level of expression; *ARF1* produces approximately 90% of total ARF. Among revertants of the fluoride sensitivity of an *arf1* null mutation were *ARF1-ARF2* fusion genes created by a gene conversion event in which the deleted *ARF1* sequences were repaired by recombination with *ARF2*.

ADP ribosylation factor (ARF) was originally identified as the protein cofactor required for efficient in vitro ADP ribosylation of the stimulatory regulatory component of adenylate cyclase, $G_{\alpha s}$, by cholera toxin (17, 36). ARF is itself a 21-kDa GTP-binding protein and is active in the cholera toxin reaction only in the GTP-liganded state (18). For recent reviews on the role of ARF in the cholera toxin reaction, see reference 16 and R. Kahn, Methods Enzymol., in press. Based on the in vitro ADP-ribosylation assay (36), ARF has been purified from a number of tissues, including bovine brain, in which ARF represents as much as 1 to 2% of total cell protein.

ARF protein is encoded by at least two genes in both cows (26, 39) and humans (3). The bovine *ARF1* gene was used as a probe to isolate a homologous yeast gene, *ARF1* (39), and antibodies against a conserved region of the ARF protein have been used to detect ARF in many organisms (19), suggesting that ARF is a ubiquitous protein in eucaryotes. In all cases, the antibodies specifically recognize proteins with an apparent molecular weight of 20,000 to 24,000. In several samples, including purified bovine brain ARF, the anti-ARF-reactive species ran on sodium dodecyl sulfate (SDS) gels as a closely spaced doublet (17). Although this doublet might be due to differences in the migration of ARF proteins encoded by different genes, it could also be due to covalent modification of a population of the ARF proteins (e.g., myristylation [19]). No functional significance has been ascribed to the different forms of ARF.

The yeast gene *ARF1* encodes a protein highly homologous (74% amino acid sequence identity) to the bovine ARF protein (39). This level of sequence homology between ARF proteins from very divergent organisms suggests that ARF has a fundamental role in cellular physiology. We are interested in using yeast genetics to determine this role. Studies in yeasts on the function of other low-molecular-weight, GTP-binding proteins, e.g. *RAS1*, *RAS2* (45), *YPT1* (38), and *SEC4* (32), have allowed the initial description of mutant phenotypes as well as the identification of functionally related gene products. A role for the ARF protein in the protein secretory pathway has recently been described, deduced from the phenotype of yeast ARF mutants (43).

In this paper we report the presence of a second *ARF* gene in *Saccharomyces cerevisiae* that encodes a protein functionally homologous to that encoded by *ARF1* and demonstrate that at least one *ARF* gene is required for viability. These results are the first evidence that ARF has an essential function in normal cell growth.

MATERIALS AND METHODS

Strains and plasmids. The yeast strains and plasmids used are listed in Table 1. Strains bearing disruptions of the *ARF* genes were constructed by direct replacement of the wild-type chromosomal loci with disrupted loci constructed in vitro (31). *ARF1* was disrupted by replacing the *Bgl*II-*Xba*I fragment of *ARF1* (see Fig. 2) with either the *Bam*HI-*Spe*I fragment of the yeast plasmid YEp24, making *arf1*::*URA3* (pRB1292), or, after making the ends flush with T4 polymerase, with the *Eco*RV-*Hin*cII fragment of the yeast plasmid YEp6, making *arf1*::*HIS3* (pRB1293). *ARF2* was disrupted by replacing the *Xba*I-*Xba*I fragment of *ARF2* with the *Nhe*I-*Spe*I fragment of YEp24, making *arf2*::*URA3* (pRB1295). The *GAL1-ARF1* plasmids were constructed by cloning an *Nla*IV to *Hae*III fragment of *ARF1* into the pUC19 *Hin*cII site. This plasmid was then cut with *Bam*HI and *Hin*dIII and the *ARF1* fragment was ligated into the *Bam*HI and *Hin*dIII sites of pBM272 (15) to give pRB1301. The *Aat*II-*Hin*dIII fragment of pRB1301 was inserted into YCp402 (21) to give pRB1302.

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TABLE 1. Strain list

Strain or plasmid	Genotype
Strain	
DBY4974	*MATα his3-Δ200 leu2-3,112 lys2-801 ura3-52* Gal$^+$
DBY4975	*MATα ade2-101 his3-Δ200 leu2-3,112 lys2-801 ura3-52* Gal$^+$
DBY5305	*MATα his3-Δ200 leu2-3,112 lys2-801 ura3-52 arf1::URA3*
DBY5306	Ura$^-$ fluoride-resistant revertant of 5305
DBY5307	*MATα his3-Δ200 leu2-3,112 lys2-801 ura3-52 arf1::URA3*
DBY5308	Ura$^-$ fluoride-resistant revertant of 5307
DBY5403	*MATa his3-Δ200 leu2-3,112 lys2-801 ura3-52 arf1::his3 arf2::ura3* (pRB1302)
DBY5406	*MATa his3-Δ200 leu2-3,112 lys2-801 ura3-52* (pRB1301)
DBY5457	*MATa/MATα ade2-101/ADE2 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 ura3-52/ura3-52 arf1::URA3/ARF1*
DBY5458	*MATa/MATα ade2-101/ADE2 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/LYS2 trp1-1/trp1-1 ura3-52/ura3-52 arf1::HIS3/ARF1*
DBY5459	*MATa/MATα ade2-101/ADE2 his3-Δ200/his3-Δ200 leu2-3,112/leu2-3,112 lys2-801/LYS2 trp1-1/trp1-1 ura3-52/ura3-52 arf1::HIS3/ARF1 arf2::URA3/ARF2*
Plasmid	
pRB1292	*arf1::URA3*
pRB1293	*arf1::HIS3*
pRB1295	*arf2::URA3*
pRB1297	*ARF1* in YCp50
pRB1301	*gal1::ARF1 URA3 CEN*
pRB1302	*gal1::ARF1 LEU2 CEN*
pRB1306	*ARF2* in YCp50
pRB1344	*ARF1 Eco*RI-*Bgl*II fragment
pRB1345	Plasmid recovered from DBY5308
pRB1346	Plasmid recovered from DBY5306

Media, genetic techniques, and transformation. Media for yeast growth and sporulation were as described by Sherman et al. (40), except for 5-fluoro-orotic acid plates, described by Boeke et al. (5), and YEPGal, which is identical to YEPD except that 2% galactose is substituted for the glucose. Yeast growth, mating, sporulation, and tetrad analysis were performed as described by Sherman et al. (40). Growth on plates was scored by spotting suspensions of cells in sterile water onto plates with a 32- or 48-point inoculator. Yeast cells were transformed with DNA by the lithium acetate method of Ito et al. (14), modified as described before (42). Transformants were plated on synthetic complete (SC) medium lacking the appropriate nutrient to select cells carrying the transformation marker.

Preparation of antibodies and immunodetection of ARF. A peptide corresponding to amino acid residues 159 to 173 of *ARF1* and *ARF2* was purchased from Peptide Technologies, Washington, D.C. This peptide contains the sole cysteine in the *ARF1* sequence, allowing conjugation to carrier keyhole limpet hemocyanin as described by Green et al. (12). Rabbits were immunized with multiple intradermal injections of conjugated peptide in complete Freund adjuvant and were boosted after 2 weeks as described by Mumby et al. (24). The serum from rabbit R-16 was used in all experiments.

Total yeast protein extracts were prepared by harvesting approximately 2×10^7 cells by centrifugation, resuspending in 20 μl of Laemmli sample buffer, and immediately boiling for 3 min. Glass beads were then added and the samples were vortexed at high speed for 2 min. Fifty microliters of sample buffer was added, the supernatant fraction was removed and boiled again, and an aliquot was loaded on an SDS-polyacrylamide (15%) gel. Proteins were electrophoretically transferred to a nitrocellulose membrane, and ARF was detected by incubation of the membrane with anti-ARF primary serum followed by ^{125}I-labeled protein A (6). That antibody R-16 was functioning in the linear range in experiments in which relative amounts of the ARF1 and ARF2 proteins were estimated was tested with known amounts of bacterially expressed ARF1.

DNA hybridizations and sequencing. The *ARF2* gene was cloned by hybridization of a radiolabeled *ARF1* probe to a yeast genomic DNA library. Some 100,000 colonies of a YCp50-based yeast DNA library (29) were probed with the *Eco*RI-*Pst*I fragment of *ARF1* labeled to 10^6 cpm/ml by nick translation (27). Hybridization was at 37°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt solution–0.5% SDS–50% formamide–50 mM KPi–100 μg of carrier DNA per ml. The filters were washed at 37°C in 0.5× SSC–0.1% SDS. Twenty-six positive clones were analyzed by restriction digestion and DNA hybridization. Of these, 24 were *ARF1* and the remaining 2 were *ARF2*. Both strands of the *Nar*I fragment containing the entire coding region of *ARF2* were sequenced by the dideoxy-chain termination method (33).

Total yeast genomic DNA was prepared as described previously (9). Restricted DNA was transferred from agarose gels as described by Southern (41). Hybridizations with ^{32}P-labeled probes were performed under conditions described by Wahl et al. (46); washes were carried out in 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA [pH 7.7])–0.1% SDS at 65°C. ^{32}P-labeled probes were prepared by random hexamer labeling of restriction fragments in low-melting-point agarose (11).

Cloning of *arf1::URA3* intragenic revertant loci. The *ARF1* loci from two independent Ura$^-$ *arf1::URA3* revertants (DBY5306 and DBY5308) were cloned by gene eviction (28) as follows. Plasmid pRB1344 was made by inserting the *Eco*RI-*Bgl*II fragment of *ARF1* in the yeast-integrating plasmid YIp5. This plasmid was then cut with *Nhe*I and transformed into the two Ura$^-$ revertants, selecting for the *URA3* marker of the plasmid. DNA was then isolated from two transformants for each of the two revertants. This DNA was

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   1 GCCTGCAGGT ACGCCCCTTT ATTTGATCAG GAAGCCGTAT TGATTATCTA ATAGGGCCTA

  61 GTTATCCTAA TTGTGGGGAG TCGAGCAGTA CGGCTCTGAT GTTTTTCGAA CGAAGATAAG

 121 GAGTTGACAT ACAAAGTCAA CAGAAGTTCT TCTTGTTAGC GTCTCTGTGC TCAATATCTC

 181 TCTTTTTTTC TTTAAGTAGT AATTACTAAC ATCAGCCAAC CAATAGAGAT AAAAAAAAAA

 241 GGAATTAAGA TTTCATAGAG AAAAGATGGG TCTATACGCT TCTAAGTTAT TCAGCAATCT
   1                          M  G   L  Y  A   S  K  L  F   S  N  L
                                                 (F)
 301 TTTTGGCAAC AAAGAGATGC GTATACTTAT GGTTGGTCTA GATGGTGCCG GTAAGACCAC
  13  F  G  N   K  E  M  R   I  L  M   V  G  L   D  G  A  G   K  T  T

 361 CGTTTTGTAC AAGTTGAAGT TGGGCGAAGT TATCACTACC ATTCCAACCA TTGGTTTCAA
  33  V  L  Y   K  L  K  L   G  E  V   I  T  T   I  P  T  I   G  F  N

 421 CGTTGAGACT GTCCAATATA AGAACATTTC CTTCACTGTC TGGGACGTCG GTGGACAAGA
  53  V  E  T   V  Q  Y  K   N  I  S   F  T  V   W  D  V  G   G  Q  D

 481 CAGGATTAGA TCTTTATGGA GACACTACTA CAGAAACACC GAAGGTGTTA TTTTTGTCAT
  73  R  I  R   S  L  W  R   H  Y  Y   R  N  T   E  G  V  I   F  V  I
                                                                    (V)
 541 CGATTCCAAC GATAGATCGC GTATTGGTGA AGCCAGAGAA GTCATGCAGA GAATGCTGAA
  93  D  S  N   D  R  S  R   I  G  E   A  R  E   V  M  Q  R   M  L  N

 601 TGAAGATGAA TTGAGAAATG CTGTCTGGTT AGTCTTCGCT AACAAACAAG ATTTGCCAGA
 113  E  D  E   L  R  N  A   V  W  L   V  F  A   N  K  Q  D   L  P  E
                             (A)
 661 AGCCATGTCT GCTGCTGAAA TCACCGAAAA ATTAGGTTTA CATTCTATTA GAAACCGTCC
 133  A  M  S   A  A  E  I   T  E  K   L  G  L   H  S  I  R   N  R  P

 721 ATGGTTTATC CAGTCTACTT GTGCAACCTC GGGTGAAGGT CTGTACGAAG GTCTGGAGTG
 153  W  F  I   Q  S  T  C   A  T  S   G  E  G   L  Y  E  G   L  E  W
                  (A)
 781 GTTAAGCAAC AACTTGAAGA ATCAATCCTA ATCTAAATCT GTATAGAACG TTTAGTCATG
 173  L  S  N   N  L  K  N   Q  S
             (S)             (S)(T)
 841 CGCACCTTGT GTGTTTTGTT TCTAGATTGT TTTATTTTTA TGATTGTTGA AGATATAAAC

 901 CACTGTATAG TTGTATAAGA TAGGATAATG ATGGTGCACT GAAAATAAAC TTACTAGCTC

 961 TTTAATATTG CAACGGCTTG TAACGGGCGA CTCTAGAGGA TCCCCGGGTA CC
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FIG. 1. DNA sequence of the *ARF2* gene and the predicted protein sequence. The differences between the predicted *ARF1* and *ARF2* proteins are indicated in parentheses below the *ARF2* sequence. All other residues, as well as predicted total protein length, are identical to *ARF1*. The GenBank accession number for the *ARF2* sequence is M 35158.

cut with *Sph*I, diluted, ligated, and used to transform *Escherichia coli*, selecting for ampicillin resistance. In all cases the recovered plasmids (pRB1346 from DBY5306 and pRB1345 from DBY5308) had the expected structure. The *Eco*RI-*Bgl*II fragment of each plasmid was cloned into M13tg130 and sequenced by the dideoxy-chain termination method (33).

RESULTS

Two genes encode ARF in *S. cerevisiae*. The yeast gene *ARF1* was cloned by virtue of its homology to the bovine *ARF* gene and encodes a protein that is 74% identical to mammalian ARF (39). Two lines of evidence indicated the presence of a second *ARF* gene in yeast cells. First, DNA hybridization experiments showed a second locus that hybridized with an *ARF1* probe under stringent hybridization conditions (not shown). Second, in characterizing null mutations in *ARF1*, we found revertants of an *arf1*::*URA3* deletion-insertion mutation that appeared to have occurred at the *ARF1* locus (see below). The ARF$^+$ phenotype in these intragenic revertants was linked to the *ARF1* locus, and the revertants were phenotypically Ura$^-$. These results suggested that the reversion event might be gene conversion of the *arf1*::*URA3* mutation by homologous sequences in the yeast genome, possibly a second *ARF* gene, resulting in loss of the *URA3* insertion concomitant with gain of ARF function. To isolate this homologous gene, we used the *ARF1* gene as a probe of yeast genomic DNA. A labeled DNA fragment containing the entire *ARF1* coding sequence was used to screen a yeast genomic DNA library in plasmid YCp50 (29). Of 26 clones isolated in this way, 24 were found to contain inserts from the *ARF1* locus; 2 appeared to be different from *ARF1* but related to each other. The sequence of this *ARF1*-homologous locus was determined and found to contain an open reading frame that is 91% identical to *ARF1* at the nucleotide level and will be called *ARF2* (Fig. 1). There is no significant homology between the two genes outside of the coding sequences. It is worth noting that the homology between the *ARF1* and *ARF2* genes is such that

TABLE 2. Genetic mapping of *ARF1* and *ARF2*[a]

Interval	PD:	NPD:	TT
arf1-arf2	51	0	65
arf1-cdc2	12	1	17
arf2-cdc2	41	0	16
arf1-cdc9	44	0	21
arf2-cdc9	16	0	7
arf1-cdc36	36	0	12
arf2-cdc36	16	0	6
cdc2-cdc36	11	0	11
cdc9-cdc36	22	0	1

[a] PD, Parental ditype; NPD, nonparental ditype; TT, tetrad.

certain restriction sites within the coding sequences are conserved, possibly explaining the failure to identify *ARF2* in the experiments in which *ARF1* was originally cloned (39).

The predicted ARF2 protein is identical in length to the ARF1 protein and 96% identical in protein sequence, differing at only 7 of 181 residues (Fig. 1). Most of these seven differences are conservative amino acid changes, and three of the seven are at the C terminus of the protein, a region relatively divergent among other low-molecular-weight, GTP-binding proteins. The GTP-binding consensus regions and the putative myristylation site present in *ARF1* are conserved in *ARF2*.

Genetic mapping of *ARF1* and *ARF2*. A DNA blot of yeast chromosomes separated by CHEF electrophoresis was probed with a fragment of the *ARF1* gene to determine the chromosomal locations of the two *ARF* genes. The conditions used allowed hybridization of the probe to both *ARF1* and *ARF2*. The only hybridization signal observed in these experiments was to chromosome IV, indicating that both genes are on the same chromosome. Strains bearing genetically marked *ARF* loci were then crossed to each other and to strains bearing chromosome IV genetic markers. Segregants of the appropriate genotype from these crosses were then crossed to each other to order linked genes in three-point crosses. The tetrad data presented in Table 2 show that the two *ARF* genes are separated by approximately 28 centimorgans on the left arm of chromosome IV, on either side of the *ste7-cdc9-cdc36* gene cluster. *ARF2* is centromere proximal, between *cdc2* and *cdc36*, and *ARF1* is centromere distal. This arrangement is depicted in Fig. 2. The two *ARF* genes occupy map positions distinct from all other mapped yeast genes (23) and by that criterion represent newly identified genes.

At least one functional *ARF* gene is required for viability. As a first step in determining the function of the two *ARF* genes, we constructed deletion-insertion mutations in each of the genes. In each case a large portion of the coding sequence was replaced with a yeast marker gene (Fig. 3). Two *arf1* disruption constructions (*arf1::URA3* and *arf1::HIS3*), differing only in the inserted marker, were transformed separately into wild-type diploid strain DBY1830 such that they replaced the chromosomal *ARF1* locus (31). The resulting strains (DBY5457 and DBY5458) were sporulated and tetrads were dissected. All four spores were viable for both the *arf1::URA3* and *arf1::HIS3* constructions, and the disruption markers segregated $2^+:2^-$ in a total of 39 tetrads examined. DNA hybridization experiments showed that the Ura^+ and His^+ segregants lacked a wild-type copy of the *ARF1* locus (see below).

The *arf2::URA3* disruption construction was transformed into a diploid *ARF1/arf1::HIS3* strain, such that it replaced one of the wild-type *ARF2* loci, resulting in strain DBY5459.

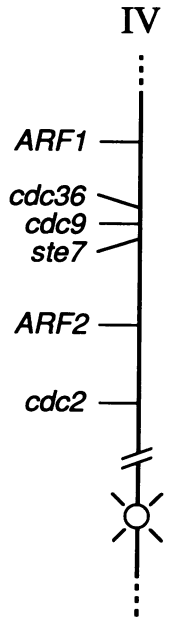

FIG. 2. Genetic map location of *ARF1* and *ARF2* on chromosome IV. Three-point crosses were used to establish the order of *ARF1*, *cdc9*, and *ARF2*; *ARF1*, *cdc9*, and *cdc36*; and *ARF2*, *cdc2*, and *cdc36*.

In this strain the two *arf* mutations are in *trans*: the *arf1::HIS3* mutation in one copy of chromosome IV in the diploid, *arf2::URA3* in the other. DBY5459 was sporulated and tetrads were dissected. Of 50 tetrads scored, 24 had four viable spores, with the *arf1::HIS3* and *arf2::URA3* mutations in the parental configuration, whereas 26 had three viable spores, with the markers in the tetratype configuration. In all cases the missing spore in the tetratype tetrads was the inferred double mutant, indicating that the *arf1 arf2* double null mutant is inviable. This ratio of parental to tetratype tetrads is consistent with the genetic distance between *ARF1* and *ARF2* determined in mapping crosses. A strain in which the two disruption mutations were in the *cis* configuration also produced parental and tetratype tetrads in the expected ratios (data not shown); in this case the parental tetrads had two viable spores. A parental ditype tetrad from DBY5459 was subjected to DNA blotting analysis, with probes against both *ARF1* and *ARF2* (Fig. 4). Each disruption mutation segregated $2^+:2^-$, and in each of the four spores one of the *ARF* genes was wild type whereas the other was disrupted.

The double-disruption experiment shows that a spore lacking a functional *ARF* gene is unable to form a colony, suggesting that ARF is required for growth. We sought to demonstrate this requirement for ARF independent of spore germination. A strain was constructed in which the only complete *ARF* gene was under control of the inducible *GAL1* promoter. The level of transcription from this promoter is high when galactose is the carbon source and very low when glucose is the carbon source; thus, growth should be carbon source dependent if ARF was required for mitotic growth. The yeast centromere plasmid pRB1302 contains the *ARF1* coding sequence and 3′ noncoding region fused to the *GAL1* promoter, as well as the *LEU2* gene. pRB1302 was transformed into a diploid strain that is phenotypically Gal^+ and has the *ARF* genotype *ARF1/arf1::HIS3 ARF2/arf2::URA3*. A transformant was sporulated and tetrads were dissected

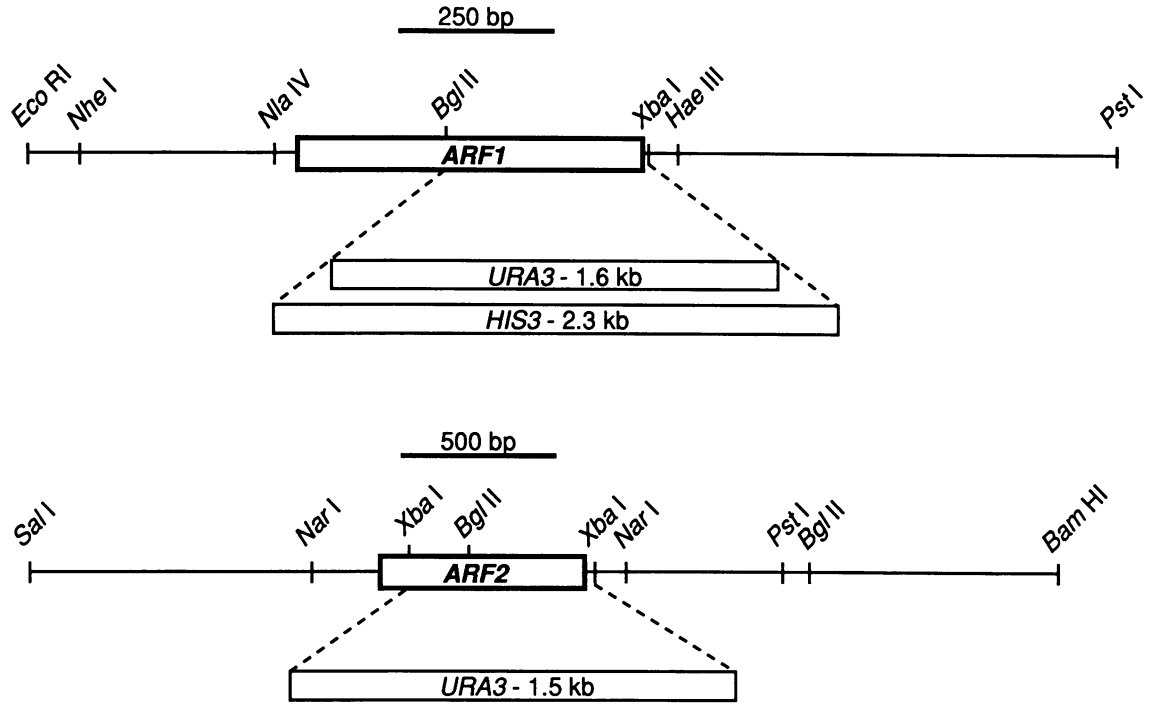

FIG. 3. Restriction maps of *ARF1* and *ARF2* and disruption constructions. The insert fragments (containing *URA3* and *HIS3*) are not to scale; their sizes are indicated separately.

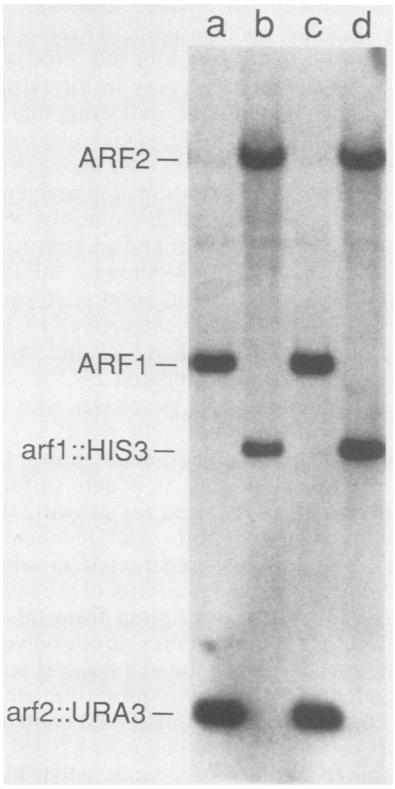

FIG. 4. DNA blot of *arf* disruption strains. Genomic DNA from strains of a parental ditype tetrad of DBY5459 was cut with *Pst*I and probed with a mixture of *ARF1* and *ARF2* probes.

on rich medium with galactose as the carbon source (YEPGal). These conditions should allow the growth of a haploid segregant lacking both chromosomal copies of *ARF*, but carrying the plasmid with the galactose-inducible *ARF1* gene. A segregant that was phenotypically His^+, Ura^+, and Leu^+ (DBY5403) was checked by DNA hybridization to confirm that the plasmid-borne *ARF1* gene was the only complete *ARF* gene (data not shown). The ability of this strain to grow on different carbon sources was tested. Growth on galactose medium appeared normal, though slower than that of wild type. Growth on glucose medium was completely inhibited (Fig. 5). We examined the growth of this strain in liquid medium and found that, when shifted from YEPGal to YEPD medium, growth continued for 7 h and then leveled off, presumably reflecting depletion of ARF protein. No morphological changes were observed in cells from these cultures. Most cells in the DBY5403 culture were inviable 8 h after the shift to glucose; wild-type cells continued to grow exponentially under the same conditions. We found that incorporating 0.2% glucose into the 2% galactose medium improved the growth rate of strain DBY5403. Presumably this improvement is due to a reduction in the level of expression from the *GAL1* promoter, as overexpression of the ARF1 protein from this promoter in wild-type cells is deleterious (see below).

Phenotypes of *arf* mutants. (i) Growth defect. Dissection of tetrads derived from diploids bearing *arf1* and *arf2* null mutations revealed an obvious growth defect in the *arf1* null segregants. Spore colonies of strains carrying the *arf1* disruption marker were much smaller than their *ARF1* siblings. In contrast, the *arf2* null spore colonies were indistinguishable in size from wild-type spore colonies. The growth rates of wild-type, *arf1* null, and *arf2* null strains in liquid medium confirmed these observations; wild-type and *arf2* null strains had a generation time of approximately 85 min at 30°C,

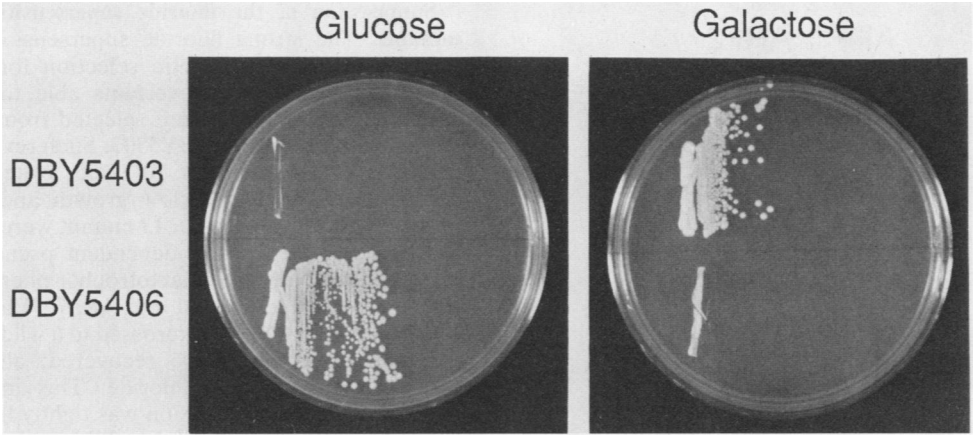

FIG. 5. Phenotype of *ARF1* depletion and overexpression. DBY5403 *arf1*::*HIS3 arf2*::*URA3* (pRB1302) and DBY5406 *ARF1 ARF2* (pRB1302) were inoculated on SC-ura medium containing the indicated carbon source and incubated for 3 days at 30°C.

whereas the *arf1* null strain had a generation time of 125 min. The *arf1* null phenotypic defect was strikingly accentuated by incubation at lower temperatures. At 14°C, wild-type and *arf2* null strains grow with a generation time of 8 h, whereas the *arf1* null strain doubled once in 17 h but did not double again during a further 72 h of incubation. Microscopic examination of the *arf1* null cells after 18 h at 14°C did not reveal any cell cycle arrest phenotype. Examination of the morphologies of the nucleus with the DNA-specific dye 4′,6-diamidino-2-phenylindole and the microtubule cytoskeleton by antitubulin immunofluorescence did not reveal any significant structural defects at either 30 or 14°C (not shown).

(ii) Sensitivity to fluoride ion. It has been observed that the trimeric GTP-binding proteins can be activated in vitro by a combination of aluminum and fluoride ions, although no such activation has been reported for monomeric GTP-binding proteins, including ARF. Interestingly, we found that *arf1* null mutants were sensitive to what are normally sublethal amounts of potassium fluoride in the medium. Wild-type haploid yeasts are typically resistant to approximately 60 mM KF in YEPD, whereas *arf1* null mutants were inhibited for growth by as little as 15 mM. The same result was obtained with sodium fluoride, while potassium chloride and sodium chloride had no effect. We found that 40 mM F^- was a reliable concentration for easily distinguishing *arf1* null mutants from wild type. No cell cycle-specific arrest could be detected either in a wild-type culture treated with 100 mM KF or an *arf1* null mutant culture treated with 40 mM KF. Addition of $AlCl_3$ in the range of 0 to 10 mM to plates containing various KF concentrations had no effect (other than an increasing amount of precipitate at higher concentrations). If aluminum ions are required for this effect, however, it is likely that a sufficient amount is already present in the media. *arf2* null mutants were indistinguishable from wild type in terms of sensitivity to fluoride ions.

YEPD plates containing 40 mM KF or NaF lost the ability to support growth of even wild-type strains about 5 to 7 days after preparation. The ability to support the growth of wild type, but not *arf1* null strains, could be restored by adding Mg^{2+} to the plate prior to use (typically 0.1 ml of 1 M $MgCl_2$ per 35-ml plate). The probable cause for this effect is the complexing and precipitation of Mg^{2+} ions by F^-. This raises the possibility that the *arf1* null defect is actually a sensitivity to reduced Mg^{2+} levels in the media. To test this possibility, the effect of EDTA on growth was examined. EDTA solutions of different concentrations were placed in wells in plates seeded with lawns of either wild-type or *arf1* null mutant cells. The zones of inhibition caused by the EDTA were dependent only on the concentration of EDTA used and not on the genotype of the cells in the lawn. Thus, lowering the extracellular magnesium concentration with EDTA does not mimic the effect of fluoride. Chelation of aluminum or formation of aluminum fluoride complexes over hours or days is also a possibility for the effect of age on the fluoride plates.

(iii) Overexpression lethality. Low-copy-number (*CEN*) and high-copy-number (2μm) plasmids that contained either the *ARF1* or the *ARF2* coding sequence were constructed and used to transform a wild-type strain. Although *ARF1* and *ARF2* centromere plasmids had no effect, the 2μm-based plasmids severely inhibited the growth rate of wild type. In particular, the *ARF1*-2μm plasmid (pRB1300) yielded transformants at a much lower frequency than the vector without an insert, and the transformants that did grow into colonies grew very slowly. The *ARF2*-2μm plasmid (pRB1307) had a similar, though not as severe, effect. A similar result was obtained with a centromere plasmid (pRB1301) carrying the *GAL1-ARF1* construction described above. A wild-type strain bearing this plasmid grew normally on medium containing glucose but was unable to grow when galactose was the sole carbon source, and the *GAL1* promoter is maximally induced (Fig. 5). The growth of a control strain bearing the *GAL* vector without an insert was not affected by the change in carbon source.

Antibodies against the *ARF* gene products. Antibodies against yeast *ARF* were generated by immunizing rabbits with a synthetic peptide coupled to keyhole limpet hemocyanin. The sequence used for immunization is identical in *ARF1* and *ARF2*; thus, the antibodies generated should recognize both proteins equally well. The polyclonal antiserum derived from rabbit R-16 used in these experiments recognized a ~21-kDa protein in whole yeast extract, as well as one other protein of higher molecular weight. Peptide competition experiments showed that only the 21-kDa signal is diminished by peptide. *ARF* protein is expressed at a level of 0.03 to 0.1% of total cell protein as determined by quantitation of immunoblots with purified recombinant *ARF1* as the standard. The R-16 polyclonal antiserum was used to examine the relative contributions of *ARF1* and *ARF2* to the total amount of *ARF* in the cell. Although *ARF1* and *ARF2* are of identical length and nearly identical sequence, they have slightly different mobilities under appro-

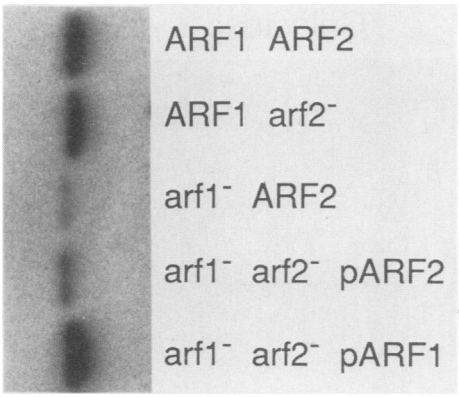

FIG. 6. ARF protein levels in wild-type and mutant strains. Whole protein extracts from strains of the indicated genotypes were subjected to electrophoresis, blotted, and probed with anti-ARF antibody as described in Materials and Methods. The antibody used is directed against a peptide epitope of ARF that is identical in the *ARF1* and *ARF2* proteins.

priate electrophoretic conditions. The three viable spores from a tetratype tetrad of DBY5459 were examined by immunoblotting (Fig. 6). The *arf1*::*HIS3* spore clearly lacked the major band of *ARF* reactivity, but retained a minor band that migrates slightly faster than *ARF1*. This minor band reproducibly appeared to be missing in the *arf2*::*URA3* strain, although its proximity to the major band made this difficult to resolve. The minor band was overproduced severalfold in a strain carrying *ARF2* on a centromere plasmid (presumably present in more than single copy, although not tested), demonstrating that the minor band is the product of the *ARF2* gene. Assuming that the R-16 antibody recognizes both forms of ARF equally, the minor band was estimated to constitute approximately 10% of the total ARF protein. There did not appear to be an increase in the expression of the *ARF2* protein in *arf1* disruption mutants. These findings have been confirmed with *ARF1*- and *ARF2*-specific antibodies (R. Kahn, unpublished observation).

The *ARF1* and *ARF2* proteins are functionally homologous. The near identity of the *ARF1* and *ARF2* proteins suggested that they might perform identical functions and that the genetic difference between the two genes could be accounted for by the lower level of expression from the *ARF2* gene. In this case, simply increasing the copy number of the *ARF2* gene should suppress the phenotypic defects of an *arf1* null mutant. This hypothesis was tested by transforming *arf1* null mutant strains with plasmids bearing the *ARF2* gene. First, a yeast-integrating plasmid bearing *ARF2* was integrated at the chromosomal *ARF2* locus of an *arf1* null strain by homologous recombination, resulting in a duplication of *ARF2*. Transformants in which only a single copy of the plasmid had integrated were identified by DNA blotting. Second, a yeast centromere plasmid bearing *ARF2* (pRB1306) was introduced into an *arf1* null strain; centromere plasmids are typically maintained at 1 to 2 copies per cell (8). In both cases, the increased *ARF2* gene dosage was sufficient to completely suppress the three *arf1* null phenotypes of slow growth, cold sensitivity, and fluoride supersensitivity. Thus, when the *ARF2* copy number is as little as doubled, there is sufficient *ARF2* protein to perform all ARF functions in the cell, indicating that the *ARF1* and *ARF2* proteins are functional homologs.

Suppression of the fluoride supersensitivity of *arf1* null mutants. The strong fluoride supersensitivity of *arf1* null mutants provided a genetic selection for increased ARF function. Spontaneous revertants able to grow on plates containing 40 mM KF were selected from the *arf1*::*URA3* mutants DBY5305 and DBY5307. Such revertants arose at a frequency of about 1 in 10^7 cells plated. In all of the revertants isolated, the slow growth and cold sensitivity phenotypes of the *arf1*::*URA3* mutant were also suppressed. Surprisingly, 29 of 102 independent pseudorevertants isolated had become uracil auxotrophs, phenotypically losing the *URA3* marker inserted into *ARF1*. When three of these Ura$^-$ revertants were backcrossed to a wild-type strain, only parental ditype asci were recovered; all spores showed wild-type resistance to fluoride. This indicated that the mutation causing suppression was tightly linked to *ARF1* or was in the *ARF1* gene itself. In addition, the suppression was dominant; when Ura$^-$ pseudorevertants were crossed to an *arf1*::*URA3* strain, the resulting diploids were fluoride resistant. The simplest explanation of these results is that the disrupted *arf1* gene was repaired by recombination with *ARF2* sequences. This seemed unlikely, however, as the *arf1*::*URA3* construction deleted sequences past the 3′ end of the *ARF1* coding sequence and thus lacked any homology with the *ARF2* locus at the 3′ end.

To determine the event that occurred in the Ura$^-$ revertants, genomic DNA from wild type, *arf1*::*URA3*, and three Ura$^-$ revertants was subjected to DNA blot analysis. The probe in these experiments was the *Eco*RI-*Pst*I fragment of *ARF1* containing the entire coding sequence that, under the hybridization conditions used, hybridizes to both *ARF1* and *ARF2*. An internal *Bgl*II site present at a homologous location in both *ARF1* and *ARF2* (Fig. 3) was destroyed in the *arf1*::*URA3* allele by ligation with a *Bam*HI end of a fragment carrying *URA3*. The *Bam*HI/*Bgl*II junction formed cannot be cut by either enzyme. The pattern of hybridization confirmed that the *ARF1* *Bgl*II site was missing in the *arf1*::*URA3* strain but surprisingly reappeared in the revertants (not shown). Hybridization of the *ARF1* probe to a number of genomic digests revealed that the restored *Bgl*II site in the revertants was derived from homologous DNA at the *ARF2* locus. The resulting genes must then encode fusion proteins with an amino-terminal portion derived from *ARF1* and the remainder derived from *ARF2*. In all digests, the *ARF2* gene remained unchanged in the revertants. These results suggested that the repair of *arf1*::*URA3* and the loss of the *URA3* marker were accomplished by a gene conversion event: a nonreciprocal transfer of information from *ARF2* to *arf1*::*URA3*.

There must be two crossover points that define the extent of the replacement of *arf1*::*URA3* DNA with *ARF2* DNA, one on each side of the nonhomologous *URA3* insert. DNA blot analysis revealed that the converted *arf1* loci in strains DBY5306 and DBY5308 do not have the *Xba*I site located near the 5′ end of the *ARF2* reading frame (Fig. 3), while both have the *Bgl*II site from *ARF2* (see above). The site of the 5′ crossover then must be in the 151-bp region between these two restriction sites. The site of the 3′ crossover could not be determined but must lie further than an *Xba*I site located 52 bp from the 3′ end of the *ARF2* reading frame as this site was acquired in the revertant loci.

To determine directly the structure of the revertants, the *arf1* loci from two revertant strains were cloned by integrating a plasmid carrying *ARF1* homology immediately adjacent to the converted *arf1*, isolating genomic DNA from transformants, and cutting and ligating the DNA so that the con-

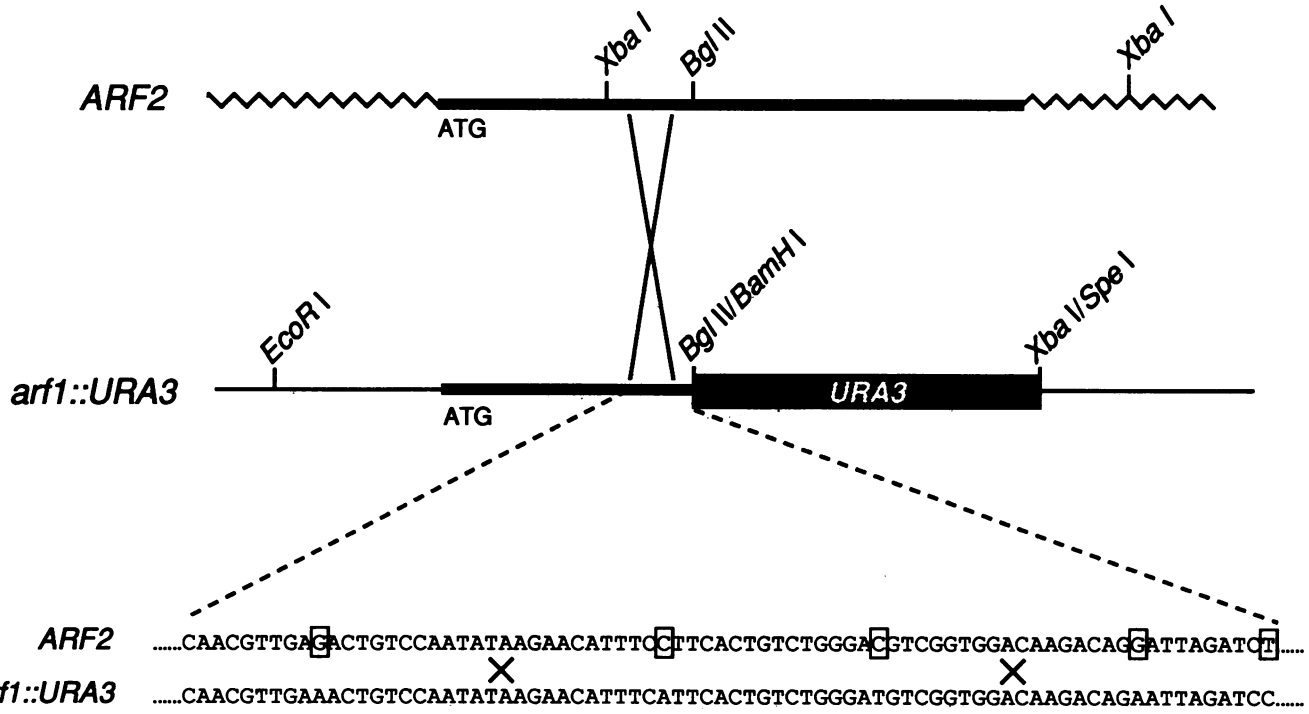

FIG. 7. Structure of two *ARF1/ARF2* fusions. The genomic DNA at the *ARF1* locus of two intragenic *arf1::URA3* revertants (DBY5306 and DBY5308) that had undergone a recombination event between *arf1::URA3* and *ARF2* was cloned and sequenced. The locations of the 5′ crossover events in the two revertants are shown as X's between the *ARF2* and *arf1::URA3* sequences (the DBY5306 site is the more 5′ of the two). The boxed bases in the *ARF2* sequence shown are those that differ from *ARF1*.

verted loci would be recovered in *E. coli* on the plasmid (see Materials and Methods). The DNA sequence of these loci revealed that, as predicted, they are *ARF1/ARF2* fusion genes, with the crossover points in the expected region (Fig. 7). The actual site of the crossover could only be approximated due to the high degree of sequence identity between *ARF1* and *ARF2* in this region. For strain DBY5306, the crossover point could be narrowed to a region 39 to 61 bp 5′ of the *Bgl*II site, and for strain DBY5308, it could be narrowed to a region 6 to 22 bp 5′ of the *Bgl*II site (Fig. 7).

DISCUSSION

In this paper we demonstrate that ARF is encoded by two genes in *S. cerevisiae*, that at least one of these genes must be functional for viability, and that the ARF proteins encoded by these two genes are functionally homologous. These results are the first evidence of an essential role for ARF in cellular physiology. We have also begun to analyze the phenotypes of *arf* mutations, gaining information about the function of ARF not available from the previous studies on the in vitro properties of this protein.

The *ARF1* and *ARF2* genes encode proteins that are 96% identical, yet mutations in the two genes resulted in different phenotypes. It is likely that this genetic difference is due to a difference in the level of expression of the two *ARF* proteins. We found that approximately 90% of total ARF protein is produced by the *ARF1* gene, and the remainder is produced by *ARF2*; a single extra copy of *ARF2* could suppress the phenotypic defects caused by deletion of *ARF1*. This raises a number of points concerning the two *ARF* genes. First, there is likely to be excess ARF in yeast cells grown under standard laboratory conditions, as *arf1 arf2* double null mutant strains that have plasmid-borne copies of *ARF2* and are phenotypically wild type still have less ARF protein than an *ARF1 ARF2* cell (Fig. 6). An alternative explanation of these results is that the *ARF2* protein is more active in some way than *ARF1* protein and is able to perform all necessary functions at lower protein levels. Second, the function of *ARF2* is obscure, as *arf2* null mutants are phenotypically indistinguishable from wild type, and the *ARF1* and *ARF2* proteins appear to be functionally homologous. Among the known examples in yeast cells of duplicated genes are those in which the two genes are regulated differently, such as cytochrome *c* (20), and those in which the proteins are directed to different cellular compartments, such as citrate synthase (30). Although we presently lack information on the regulation of the *ARF* genes and the localization of the ARF proteins in *S. cerevisiae*, the *arf1* suppression results indicate that any difference in regulation or localization can be overcome by simple duplication of the *ARF2* gene. The duplicated yeast α-tubulin genes, *TUB1* and *TUB3*, closely resemble the *ARF* genes in a number of respects. The two α-tubulin proteins are functionally homologous, but disruption of *TUB1*, which makes the majority of α-tubulin, is lethal, whereas disruption of *TUB3* has only minor phenotypic consequences (35). Strikingly, *TUB1* and *TUB3*, like *ARF1* and *ARF2*, are genetically linked to each other, separated by approximately 38 centimorgans on chromosome XIII (35).

The two *ARF* genes are located on chromosome IV, about 28 centimorgans apart, and can recombine with each other. This is clearly demonstrated by the recovery of *ARF1/ARF2* fusion proteins created by in vivo recombination in strains selected for suppression of the *arf1::URA3* null phenotype. The two independent recombinants that were sequenced shared common features. The recombination events were

nonreciprocal; the *ARF2* locus remained unchanged, while the fusion protein was created at the *arf1* locus. The 5′ ends of the recombination events (relative to the coding strand of *ARF1*) were in a region of DNA homology between the *ARF1* and *ARF2* coding sequences, the exact location differing in the two recombinants. Third, the 3′ ends of the recombination events were not determined but must have been beyond the end of the *ARF1* coding sequence. This 3′ crossover is interesting because there is no detectable homology in the sequenced 3′ untranslated regions of the two *ARF* genes. It is possible that the 3′ crossover occurred in repeated DNA, such as Ty, ∂, or σ elements (4).

What is the nature of the essential function of ARF in *S. cerevisiae*? In separate experiments (44) we have found that ARF is involved in protein secretion in yeast cells and that ARF protein is localized to the Golgi of mammalian cells. The phenotypes that we report here for *arf1 arf2* double null mutants are consistent with this; there is no specific cell cycle defect associated with loss of ARF function and cells depleted of ARF by repression of a *GAL1-ARF1* construction arrest growth at all points in the cell cycle. The phenotypes of the *arf1* null mutant, slow growth, cold sensitivity, and fluoride sensitivity, are genetically useful but not revealing of function, even in light of the role of ARF in secretion (see below).

As ARF was originally identified as a cofactor in the covalent modification of the G protein $G_{\alpha s}$ in animal cells, it is perhaps most interesting to note what phenotypes were not observed in the yeast *arf* mutants. There are two known yeast G_{α} proteins; *GPA1* is involved in mating pheromone signal transduction (10, 22), whereas the function of *GPA2* is unknown, and mutations in *GPA2* result in no discernible phenotype (25). If *ARF* were required for *GPA1* function, then sterility or constitutive activation of the mating pathway might be expected, but neither of these phenotypes was observed in either *arf1* null mutants or under conditions of ARF depletion (see Results; also, unpublished observation). A caveat of this interpretation is that the known secretion defect of *arf* mutants might prevent the observation of the morphological changes associated with activation of the mating pathway. If *ARF* were required solely for *GPA2* function, then *ARF* might be expected to be dispensable, but we have shown that *ARF* is essential. We have not ruled out the possibility that *ARF* is involved with *GPA2* function, only that this cannot be the sole function of *ARF*, nor have we addressed whether the in vitro $G_{\alpha s}$ ADP-ribosylation assay is actually relevant to in vivo function. Further, cholera toxin has been reported to have no effect on yeast adenylate cyclase and no protein acceptor of ADP-ribose has been identified in yeast extracts (7). Our results do clearly demonstrate that the level of ARF protein is critical for proper function. Either underexpression or overexpression of ARF protein results in impaired growth or complete cessation of growth, depending on the degree of alteration.

The fluoride sensitivity of *arf* mutants is intriguing. Although a model for the mechanism of activation of the α subunits of the trimeric G proteins by fluoride complexes has been proposed (2), there is no evidence that fluoride or aluminum fluoride complexes bind to or activate any of the low-molecular-weight GTP-binding proteins. For the trimeric G proteins, it is believed that AlF_4^- is the active species for the activating effect (44), resulting from the resemblance of this compound to the gamma phosphate of GTP. Thus, liganded GDP plus AlF_4^- is the equivalent of liganded GTP (1). Whereas F^- concentrations in the millimolar range are required for the in vitro effects, Al^{3+} is only required in the micromolar range. Often, exogenous Al^{3+} need not be added as it is a common contaminant of most aqueous solutions (44). In contrast to trimeric G proteins (13), tests conducted with purified bovine ARF show no effect of fluoride on ARF activity or intrinsic fluorescence, using conditions in which activating guanine nucleotides dramatically increased each (17). Thus, a direct effect of a fluoride complex with ARF appears unlikely. A plausible model for the fluoride sensitivity of *arf* mutants is that fluoride activates a previously uncharacterized trimeric G protein in *S. cerevisiae* that is distinct from those containing *GPA1* and *GPA2* as α subunits. If ARF normally acted to inhibit this G protein, then in the absence of ARF, fluoride might overactivate it. Alternatively, millimolar concentrations of fluoride are also known to affect protein phosphatases, phosphorylases, ATPases, and free-metal concentration (34). The last is interesting in light of the ability of magnesium to restore growth of wild-type cells on agar plates containing fluoride and the reported effects in yeast cells of *ypt1* mutations on Ca^{2+} uptake and rescue from growth arrest by increased extracellular calcium (37). *YPT1* is also a 21-kDa GTP-binding protein with subcellular distribution and apparent role in protein secretion (38) similar to those recently described for ARF (43). Ultimately, knowledge of the basis of the increased fluoride sensitivity of *arf1* null mutants may provide insight into the function of ARF in the secretory pathway.

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T.S., R.A.K., and M.A.H. contributed equally to this work.

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