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Asparagine-Linked Glycosylation in *Saccharomyces cerevisiae*: Genetic Analysis of an Early Step

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Asparagine-linked glycosylation is a form of covalent modification that distinguishes proteins that are either membrane bound or are in cellular compartments topologically outside of the cell from those proteins that remain soluble in the cytoplasm. This type of glycosylation occurs stepwise, with core oligosaccharide added in the endoplasmic reticulum and subsequent modifications occurring in the golgi. We used tunicamycin, an inhibitor of one of the earliest steps in the synthesis of *N*-linked oligosaccharide, to select for mutants that are resistant to this antibiotic. Genetic, biochemical, and physiological experiments led to the following conclusions. The synthesis of *N*-linked oligosaccharide is an essential function in cells. In contrast to mammalian cells, yeast cells do not transport tunicamycin by a glucosamine transport function. We identified a gene, *ALG7*, that is probably the structural gene for UDP-*N*-acetylglucosamine-1-P transferase, the enzyme inhibited by tunicamycin. Dominant mutations in this gene result in increased activity of the transferase and loss of the ability of the cell to sporulate. In addition, we identified another gene, *TUN1*, in which recessive mutations result in resistance to tunicamycin. The *ALG7* and *TUN1* genes both map on chromosome VII.

All eucaryotic cells synthesize a class of glycoproteins in which the oligosaccharide side chain is attached to asparagine residues in the polypeptide chain (reviewed in reference 8). These *N*-linked glycoproteins are either exported from the cell or are inserted into membranes or membrane-bound compartments that are topologically equivalent to the outside of a cell. Conversely, all proteins that remain soluble in the cytoplasm lack *N*-linked oligosaccharide. Therefore, *N*-glycosylation appears to be an important label that distinguishes two major classes of proteins. However, the role(s) that *N*-linked glycosylation plays in the cell remains unclear. The synthesis of *N*-linked oligosaccharides occurs through a complex series of steps, the earliest of which occur in the endoplasmic reticulum, with subsequent steps occurring in the Golgi. The high-mannose core oligosaccharide, consisting of nine mannose and two *N*-acetylglucosamine molecules, is synthesized on the lipid carrier dolichol (reviewed in reference 18). As the nascent polypeptide crosses the endoplasmic reticulum into the lumen, the core oligosaccharide is transferred en masse to an asparagine residue (21, 22).

One of the earliest steps in the synthesis of glycoproteins is the transfer of *N*-acetylglucosamine phosphate from the nucleotide sugar UDP-*N*-acetylglucosamine to dolichol phosphate, generating *N*-acetylglucosamine PP_i dolichol (17). The enzyme that catalyzes this step, *N*-acetylglucosamine-1-P transferase, is a membrane-bound protein located in the endoplasmic reticulum (19). We shall refer to this enzyme as the transferase.

Tunicamycin is an inhibitor of the transferase and has been widely used to inhibit glycoprotein biosynthesis (11). The effectiveness of tunicamycin probably stems from the fact that different portions of the molecule resemble either *N*-acetylglucosamine or dolichol, the two substrates of the reaction catalyzed by the transferase (5, 12, 29). Being a natural product antibiotic from *Streptomyces lysosuperficus*, tunicamycin is not a single compound but, rather, is a mixture of homologs that differ in the length and saturation of the isoprene chain. This mixture has been resolved into its major components, and each component has been analyzed for biological activity. Some homologs affect glycoprotein biosynthesis exclusively, whereas others affect protein synthesis in addition to glycoprotein biosynthesis (4, 13). Furthermore, there may be as yet unidentified effects of some homologs on some cellular processes. Therefore, it has been difficult to interpret the in vivo effects caused by the treatment of cells with tunicamycin. This difficulty is particularly evident when considering complex effects such as cell cycle-specific arrest and block in DNA synthesis caused by tunicamycin treatment of yeast cells (1).

Genetic investigations into the effects of tunicamycin have been limited, although tunicamycin-resistant variants have been isolated in Chinese hamster ovary (CHO) cell lines (28). Some of these variants appear to have altered levels of transferase activity, whereas others apparently affect the transport of tunicamycin and glucosamine (2, 24). The difficulty in interpreting these experiments involving somatic cell genetics is that variants arise after many generations in increasing levels of tunicamycin, so that one cannot be certain about how many different mutations a single variant may contain.

We have initiated a study of the transferase in the eucaryote *Saccharomyces cerevisiae*, taking advantage of the well-developed biochemical and genetic methodology available in this organism. In a previous report, we described the isolation of recombinant clones of *S. cerevisiae* DNA that overproduce transferase activity approximately eightfold (20). In this report, we genetically characterize mutations conferring resistance to tunicamycin and examine aspects of the physiology and biochemistry of these mutations.

MATERIALS AND METHODS

Strains and genetic methods. Strains and genotypes are presented in Table 1. Standard genetic manipulations were performed as previously described (15). Yeast cells were transformed by the procedure of Hinnen et al. (7) except that spheroplasts were prepared with lyticase (23) (a gift from the Schekman laboratory).

Media and growth conditions. Yeast rich medium (YPD) consisted of 1% yeast extract, 2% Bacto-Peptone (Difco Laboratories, Detroit, Mich.), 2% glucose, and, for solid

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TABLE 1. Strains

Strain	Alias	Genotype	Source
JRY9		α *leu2 ura3-52 trp1 his4*	
JRY22		**a** *ade2 ura3 trp1 his3-581*	
JRY89	X1266-1d	α *met10 thr4 trp1 ade1 ade2 ura1 gal1 gal7*	Yeast genetics stock center
JRY279		α *met⁻ leu2 his3 trp1 ura3 kar1*	
JRY138		**a** *trp1-289 ura3-52 his3Δ1 ade2 rad52*	
JRY183	CMY47	α *leu2-3,112 his3-11,15 trp1 ura3 tcm1*	C. Mann
JRY321	XW270-2d	α *gcn1 lys2-2*	L. Ballou
JRY311		**a** *his4-260,39 leu2-1 ura2-9,15,30 SUCX*	
JRY318		α *tun1-1 his4-260,39 leu2-1 ura3 thr4 met SUCX*	
JRY319		α *tun1-1 thr4 ura3 thr4 ade2 met⁻ suc⁰*	
JRY357		α *his4-260,39 leu2-1 ura2-9,15,30 can1 HOL1*	
JRY398	XS214-1B	**a** *rad52-1 leu2 trp5 arg4 his6 ilv3 ura1 lys9 ade2 met2*	D. Schild
JRY399	XS194-23C	**a** *rad52-1 ade2 trp1 ura3 his2 leu1 arg4 aro7*	D. Schild
JRY400	XS195-23B	**a** *rad52-1 his7 leu2 trp1 ade5 arg4 ilv3 lys7 met5*	D. Schild
JRY401	XS122-49C	α *rad52-1 leu2*	D. Schild
JRY402	(XS95-6C)	α *rad52-1 ura3-52 trp1-289 his3Δ1 leu2-3,112*	D. Schild
JRY475	LBL1/n	α *ade2-1 ade5 met13-c cyh2 trp5-d ade6 cly8-1 ura3-1 lys2-2 tyr1-2 his7-1*	M. Esposito

medium, 2% agar (Difco). Yeast minimal medium consisted of 0.17% yeast nitrogen base without amino acids or ammonium sulfate (Difco) and 5% ammonium sulfate. Two types of sporulation medium were used. Type A consisted of 1% potassium acetate, 0.25% yeast extract, and 2% agar supplemented with amino acids and bases as needed. Type B consisted of 0.3% potassium acetate, 0.022% raffinose, 2% agar, and appropriate supplements of amino acids and bases. BCP SUC medium was made by adding 8 ml of a 4% solution of bromcresol purple in ethanol to YP SUC medium (1% yeast extract, 2% Bacto-Peptone, 2% sucrose). Amino acids and bases were added at 30 mg/liter. Tunicamycin was added to either yeast minimal medium or YPD at 5 μg/ml (ca. 6 μM). Glucosamine was added to media at 10 mg/ml (56 mM). Yeast cells were grown at 30°C. *Escherichia coli* cells were grown in L broth (1% tryptone, 0.5% yeast extract, 1% NaCl) at 37°C with vigorous agitation. All percentages are expressed as weight/volume.

Chemicals. Glucosamine, amino acids, bases, and bromcresol purple were obtained from Sigma Chemical Co., St. Louis, Mo. Tunicamycin was obtained both as a gift from Matthew Suffness, Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, and from Calbiochem-Behring, La Jolla, Calif. Mithramycin was from Polysciences, Inc., Warrington, Pa.

Enzyme assays. UDP-*N*-acetylglucosamine-1-P transferase was assayed as previously described (20).

In vitro recombination. Plasmid preparation, restriction enzyme digestion, gel electrophoresis, gel transfer hybridizations, and bacterial transformations were performed as previously described (3). Enzymes were purchased from commercial sources.

Fluorescence microscopy. Mithramycin (25) was dissolved in aqueous 22.5 mM $MgCl_2$ at 0.6 mg/ml. A loopful of sporulating culture was added to 0.1 ml of 70% ethanol for 10 min. Mithramycin solution (0.2 ml) was then added, and the cultures were left undisturbed for 20 min. The cells were then observed at ×630 magnification with a Zeiss fluorescence photomicroscope III. Fluorescence illumination was at 455 to 490 nm for excitation and 520 to 560 nm for emission. The microscope was equipped with a Zeiss epifluorescence condenser 111 R.S.

Efficiency of sporulation. Efficiency of sporulation was monitored by suspending cells in sterile water, followed by visual inspection with a hemacytometer at ×300 magnification. The criterion for sporulation was two or more spores per ascus. Most asci had three or four spores.

rad52 chromosome loss mapping. *tun1* and *ALG7* mutants were isolated in a *rad52-1* strain (JRY401). These mutations were mapped to a chromosome by taking advantage of the high frequency of chromosome loss in *rad52/rad52* diploids (14) in a method developed by D. Schild and R. K. Mortimer (personal communication). The tunicamycin-resistant mutants isolated in strain JRY401 were mated to three mapping strains provided by D. Schild (JRY398, JRY399, and JRY400). These diploids were plated onto YPD plates and X-irradiated on a Machlett OEG-60 X-ray source operated at 50 kV and 25 mA. The exposure rate was 240 roentgens/s at an *f* factor of 0.90 rad/roentgen. The *tun1*/+ diploids received 10 kilorads of irradiation, and the *ALG7-x*/+ diploids received 5 kilorads of irradiation. The surviving colonies were patched onto master plates to allow more even growth of the aneuploids.

RESULTS

Isolation of mutants. Spontaneous mutants resistant to tunicamycin were isolated from four strains, two *RAD52* and two *rad52*, by plating ca. 10^8 stationary-phase cells on supplemented yeast minimal plates containing tunicamycin. Resistant colonies appeared after 7 days at 30°C. The mutant frequency for *RAD52* strains was 7×10^{-8}, and the frequency for *rad52* strains was 1.6×10^{-5}. Elevated mutation rates in *rad52* mutants have been previously noted by others (D. Schild, personal communication). The mutants were isolated at room temperature and tested for viability at 37°C. Twelve mutants were unable to grow at elevated temperature, but these mutants proved to be unstable and difficult to work with. In the two cases that were examined, the temperature sensitivity for growth was genetically unlinked to the mutation that conferred resistance. Only nonconditional mutants were used in the experiments described below.

Dominance tests. Each mutant and its parent strain were mated to a tunicamycin-sensitive strain of the opposite mating type, and diploids were selected and purified by prototrophic selection. The diploids were tested for resistance to tunicamycin by replica plating the diploids from a YPD master plate to a supplemented minimal plate containing tunicamycin at 5 μg/ml. For the mutants isolated in strain

JRY311 or JRY401, the diploids were clearly either as resistant as the haploid mutant or as sensitive as the parent strain. In the case of the mutants isolated in JRY22, the majority of the mutants were clearly dominant or recessive, but there were six that formed diploids of intermediate drug resistance. The frequency of dominant mutants was comparable to the frequency of recessive mutants in each of three strains tested.

Recessive mutants. To determine how many genes were affected in the tunicamycin-resistant mutants, one recessive mutant isolated in JRY311 was crossed to a wild-type parent (JRY89), and the segregation of tunicamycin resistance was measured among the progeny spores. The results indicate that tunicamycin resistance segregates as a lesion in a single nuclear gene in each of 19 tetrads. This result has been confirmed in a number of crosses (see Table 6). We refer to the locus affected by this mutation as *TUN1* and this allele as *tun1-1*. Table 2 summarizes the results of the mutant isolations and initial characterization.

We determined the number of different genes in which recessive mutations could lead to tunicamycin resistance by performing complementation tests. An α *tun1-1* segregant (JRY318) was mated to all of the recessive tunicamycin-resistant mutants isolated from strain JRY22. Each of 21 diploids from nine independent mutant isolations was resistant to tunicamycin, whereas the *tun1-1*/+ heterozygote was sensitive. Therefore, each of the 21 recessive mutations is in the same complementation group as *tun1-1*.

Since mutations of the *TUN1* locus that confer resistance are recessive, we infer that the loss of some function in these mutants is responsible for the resistant phenotype. A simple hypothesis consistent with this view is that these cells have lost some transport function that allows tunicamycin into the cell. In fact, tunicamycin-resistant mutants in CHO cells are reported to be deficient in the transport of glucosamine (24), a molecule structurally similar to one portion of the tunicamycin molecule. We tested the hypothesis that the *tun1* mutants were deficient in glucosamine transport in two ways. First, we determined whether glucosamine in molar excess would compete for a transport function and confer partial resistance to tunicamycin. Figure 1 represents a growth curve of strain JRY22 in the presence and absence of tunicamycin. These results demonstrate that in concentrations of tunicamycin capable of arresting the growth of cells, a 400-fold molar excess of glucosamine offered no relief of growth inhibition. Glucosamine, by itself, had no effect on growth. The continued increase in optical density in tunicamycin-treated cells did not reflect an increase in cell number. Rather the tunicamycin-treated cells arrested in G1 and continued to increase in size without dividing, as has been previously reported by others (1, 31). The arrest of cell division by tunicamycin was reversible for at least 24 h (data not shown).

TABLE 2. Isolation of Tunicamycin-resistant mutants

Strain	No. of:			
	Independent isolations	Mutants	Dominant[a]	Recessive
JRY22	9	37	10	21
JRY401	3	10	4	6
JRY311	1	6	4	2

[a] For the dominance test, mutations isolated in strain JRY311 were mated to strain JRY183; mutations isolated in strain JRY22 were mated to strain JRY357; and mutations isolated in strain JRY401 were mated to strain JRY22. Six mutants isolated in JRY22 were ambiguous with respect to dominance.

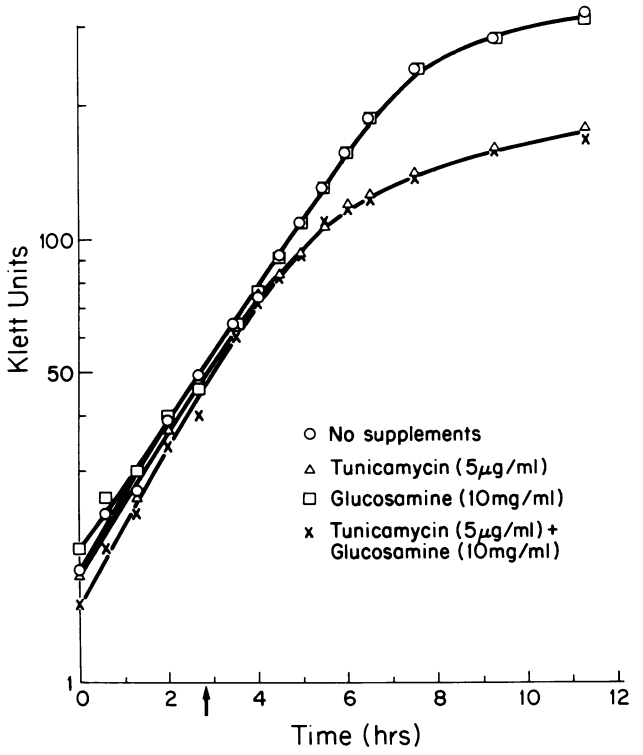

FIG. 1. JRY22 was grown to mid-logarithmic stage and diluted into yeast minimal medium plus essential bases and amino acids. At the time indicated by the arrow, various additions were made, and subsequent growth was monitored with a Klett-Somerson colorimeter. The continued increase in optical density of the cells treated with tunicamycin is due to an increase in cell size, not an increase in cell number. Examination of the tunicamycin-treated cultures indicated that the cells were approximately twice the diameter of control cells and were arrested in G1.

In a second test of the glucosamine transport hypothesis for the *tun1* mutants, we mated a *tun1-1* mutant (JRY318) to a *gcn1* mutant (JRY321), forming diploid X2. *gcn1* is a mutation that renders yeast cells unable to synthesize their own glucosamine and hence are dependent on exogenous glucosamine for viability (30). We reasoned that this diploid, when sporulated, should segregate recombinants that were *tun1 gcn1* double mutants. If *tun1* mutations result in the loss of a glucosamine transport system, then the double mutant should be unable to grow even in the presence of exogenous glucosamine. Asci from this diploid were dissected on YPD supplemented with glucosamine. The spore viability was good, with 39 of 43 tetrads giving rise to four viable spore clones, each spore clone growing at the same rate. These results imply that the double mutant is still perfectly capable of transporting glucosamine. However, if *gcn1* and *tun1* were linked, the double mutant may not have been formed among the segregants. The segregation of *tun1* and *gcn1* was determined to test this hypothesis. In each of 38 tetrads, *gcn1* segregated 2+:2− on rich medium without glucosamine. All spore clones grew on YPD supplemented with glucosamine. However, on supplemented minimal medium with glucosamine, the *gcn1* mutants were still unable to grow for reasons that remain unclear. On YPD with glucosamine plus tunicamycin, there were two resistant and two sensitive segregants in each tetrad, and the ratio of parental asci to tetratype and nonparental ditype asci (6:25:6) clearly demonstrates nonlinkage of *gcn1* and *tun1-1*. Since many *gcn1*

TABLE 3. Enzyme assays of *ALG7* mutants

Strain	nmol of NAG incorporated (h/mg)	Relative activity
a *ALG7* (JRY22)	0.53	1.0
a *ALG7-3*	1.48	2.6
a *ALG7-4*	1.10	2.0
a *ALG7-5*	1.55	2.7

tun1-1 double mutant segregants were capable of growth in the presence of exogenous glucosamine, we conclude that the *TUN1* gene has no essential role in glucosamine transport.

Analysis of dominant mutants. Dominant mutants selected as being resistant to an inhibitor can be expected to fall into two major classes: those that lead to overproduction of the product (thus titrating the inhibitor) and those that lead to an altered product that is no longer affected by the inhibitor. We analyzed three of the dominant mutants to determine whether the mutations resulted in a quantitative effect on the level of transferase activity in the mutants relative to their isogenic parent. In these mutants the activity of the enzyme increased from 2.0- to 2.7- (±0.15) fold (Table 3). Although the difference in enzyme activity levels was modest, the difference in growth rate in the presence of tunicamycin between mutant and wild type was dramatic.

Since the selection for tunicamycin resistance could be fulfilled by increased production of the transferase, we expected that gene amplification of the structural gene for transferase might be found among dominant mutants. Alternatively, mutations that lead to overproduction of an enzyme in *S. cerevisiae* frequently occur through insertion of a transposable element next to the gene encoding that enzyme. Either transposition or amplification should result in a change in the arrangement of restriction sites adjacent to the gene. We tested four independent dominant mutations by gel transfer hybridizations using the insert in pJR39 as a probe. pJR39 contains a gene that may be the structural gene for transferase (see below). The results shown in Fig. 2 demonstrate no change in the relative position of restriction sites flanking the gene. In a second experiment, no rearrangement was found within 10 kilobases of adjacent DNA. We conclude that the dominant mutations that lead to an increase in transferase activity do not result in a gross change in the chromosomal region near the chromosomal copy of the gene carried by pJR39. If the junction between the amplified sequences and nonamplified sequences were far from the sequences homologous to our probe, we would be unable to detect a rearrangement. However, since the intensity of the hybridization signal was comparable in the lanes from mutant and wild-type cells, we find no evidence for gene amplification as the basis for tunicamycin resistance. Since the size of the bands in the autoradiogram corresponds to the position of restriction sites in the pJR39 clone, we conclude that there is a single copy of this gene in the yeast genome.

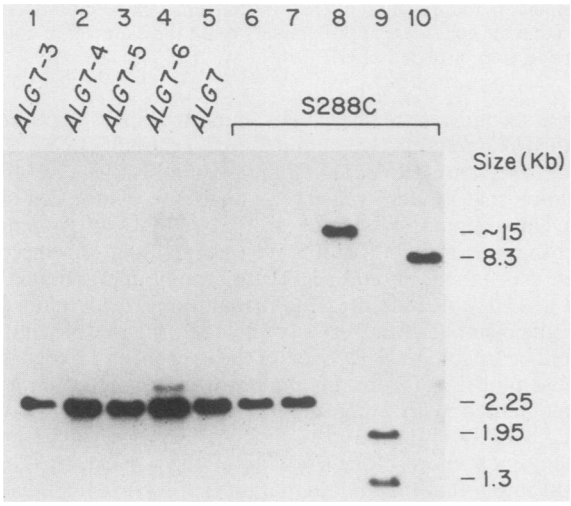

FIG. 2. In this gel transfer hybridization experiment, radiolabeled probe was prepared by using putative *ALG7* gene from pJR39. Lanes 1 through 5 contain DNA from four independent dominant *ALG7*⁻ mutants and their parent strain. Lanes 7 through 10 contain DNA from a common wild-type laboratory strain. Lanes 1 through 6 were digested with *Bam*HI, lane 7 with *Bam*HI and *Xho*I, lane 8 with *Xho*I, lane 9 with *Eco*RI, and lane 10 with *Pvu*II. Lanes 1 through 5 contain DNA prepared from a yeast DNA mini-preparation procedure, whereas S288C DNA was purified by CsCl equilibrium gradient centrifugation (3). The difference in signal intensity between different lanes is a function of the amount of DNA in each lane as judged by ethidium bromide staining and fluorescence photography. The faint bands in lane 4 are due to incomplete *Bam*HI digestion. Kb, Kilobases.

TABLE 4. Sporulation deficiency of *ALG7* mutants

Diploid	% Sporulation[a]
α ALG7 (JRY9) × a *ALG7* (JRY22)	10.4
α ALG7 (JRY9) × a *ALG7-3*	<0.1
α ALG7 (JRY9) × a *ALG7-4*	<0.1
α ALG7 (JRY9) × a *ALG7-5*	<0.1
α ALG7 (JRY9) × a *ALG7-6*	<0.1
α ALG7 (JRY9) × a *ALG7* with pJR39	5.0
α ALG7 (JRY9) × a *ALG7-6* with pJR39	4.2

[a] Percent sporulation was determined as described in the text. Both types of sporulation media were used, with similar results obtained from each.

Since these mutations affect an enzyme known to be involved in one of the first steps of glycoprotein biosynthesis, we have adopted the nomenclature of Huffaker and Robbins (9) and refer to the gene(s) affected by the dominant mutations as *ALG7* for asparagine-linked glycosylation. The dominant mutations have an additional phenotype that is unexpected. Diploids formed between a number of different *ALG7* mutants and a wild-type strain fail to sporulate, whereas the isogenic diploid lacking the *ALG* mutation does sporulate. Each of four independent *ALG7* mutations resulted in a drop in sporulation efficiency of at least two orders of magnitude (Table 4). As the effect on sporulation was unexpected, we determined whether the effect was due to overproduction of transferase activity per se or to some other qualitative change in the enzyme caused by the dominant mutations. Previously, we isolated from a multicopy plasmid library a number of recombinant plasmids that overproduce transferase activity by approximately eightfold (20). If simply overproduction of the transferase activity per se is responsible for the sporulation defect, then the presence of one of these plasmids should also block the sporulation of a diploid. We transformed plasmid pJR39, which overproduces the transferase activity, into JRY22 (the parent of the *ALG7* mutants) and into *ALG7-6*. These transformants were mated to the same strain used above for the sporulation tests (JRY9), and the resulting diploids were tested for their efficiency of sporulation on a sporulation medium lacking uracil to select for the maintenance of the plasmid. Overproduction of the transferase alone had no

TABLE 5. Chromosome loss mapping of *TUN1* and *ALG7*[a]

Chromosome	*tun1-1*			*tun1-2*			*ALG7-1*			*ALG7-2*		
	No. of violations	No. of losses	No. of colonies	No. of violations	No. of losses	No. of colonies	No. of violations	No. of losses	No. of colonies	No. of violations	No. of losses	No. of colonies
1	10	16	120	4	16	120	5	3	120	30	7	122
2	18	30	119	34	54	213	14	13	175	60	46	243
3												
4	46	91	239	52	113	333	22	23	375	82	56	243
5	6	19	117	12	34	125	12	12	120	27	14	122
6	7	25	117	10	24	120	7	6	120	20	21	183
7	0	48	240	0	55	456	0	27	401	1	35	303
8	10	29	240	53	82	576	49	40	601	82	57	425
9	6	11	117	12	27	243	31	23	226	20	21	182
10	28	38	240	41	98	456	46	41	401	43	36	303
11	6	10	117	7	27	243	24	11	226	12	15	182
12												
13	5	17	119	17	37	213	33	15	401	27	5	121
14	20	28	117	9	20	243	32	16	226	15	11	182
15	12	28	117	9	76	243	11	26	226	6	23	182
16	3	12	117	12	35	120	8	5	120	31	14	122

[a] This mitotic mapping technique employs diploids heterozygous for the mutation of interest and for additional mutations marking various chromosomes. Chromosome loss events are measured in which particular marked chromosomes and the mutation of interest are lost. We refer to violations as loss events that are inconsistent with the mutation of interest being on the chromosome monitored. Specifically, violations refer to the sum of the losses of the mutation of interest but not the marked chromosome plus the losses of the marked chromosome but not the mutation of interest. Losses refer to the number of colonies in which the chromosome was lost, and colonies refer to the total number of colonies that were screened.

significant effect on sporulation efficiency (Table 4). Therefore, we infer that the dominant mutations, which increase the enzyme activity, must also result in a qualitatively altered enzyme. Furthermore, the presence of pJR39 in a diploid cell containing the *ALG7-6* mutation suppresses the sporulation defect of *ALG7-6*.

The reason for the sporulation deficiency in the *ALG7* mutants is not clear. If the deficiency is simply due to overproduction of glycoproteins used in the ascal case or spore wall, then the sporulation defect should occur rather late in sporulation, after the nuclei have divided. We tested this hypothesis with four independent diploids, each heterozygous for a dominant *ALG7* mutation (*ALG7-3*, *ALG7-4*, *ALG7-5*, and *ALG7-6*), by staining the sporulating cultures with mithramycin, a fluorescent stain that labels nuclei (25). In asci from mithramycin-treated wild-type diploids, all four nuclei are easily visualized. For each of the four mutant diploids, greater than 200 cells were examined, and in no case were there four nuclei in a cell. In one case three nuclei were observed, several cells had two nuclei, and the remaining 200 cells had one nucleus. From these results we conclude that the sporulation block occurs early in sporulation before the first meiotic division. This sporulation defect, although potentially interesting, presents a serious limitation to genetic analysis of these mutants.

Effect of *ALG7* mutations on the synthesis of a glycoprotein. We know that the dominant mutations in *ALG7* affect the activity level of the transferase, an enzyme essential for the synthesis of *N*-linked glycoproteins. To determine whether the effect of the mutation can be monitored at the level of a specific glycoprotein, we examined the effect of these mutations on the production of invertase. Invertase is a secreted protein that hydrolyzes sucrose, which cannot be efficiently transported into the cell, into glucose and fructose that can be efficiently transported. The secreted form of invertase is a glycoprotein with as many as nine oligosaccharide side chains attached to it. In the presence of tunicamycin, invertase is synthesized and secreted as an inactive nonglycosylated protein (6). Since the parent strains of most of the tunicamycin-resistant mutants contain the *SUC* gene, they are capable of synthesizing invertase. Therefore, we were able to determine whether active secreted invertase is made by the mutants in the presence of tunicamycin in solid BCB SUC medium. The parent strains JRY22 and JRY311 were capable of secreting a low amount of active invertase on this medium. This low level probably reflects the low pool of *N*-acetylglucosamine-P-P-dolichol and invertase that had already received core glycosylation before tunicamycin treatment. Alternatively, the slight activity could reflect a small amount of cytoplasmic invertase made in an active form in the presence of tunicamycin. However, both the dominant and recessive mutants were able to synthesize and secrete abundant active invertase as judged by the high levels of sucrose fermentation causing large yellow zones around the colonies. Therefore, the mutations that confer resistance to the inhibitor of glycosylation allow secretion of an active and therefore glycosylated product in the presence of the inhibitor.

Genetic mapping. Since the *ALG7* mutations failed to sporulate in the initial crosses, it was difficult to determine whether each of the dominant mutations is defective in the same gene. However, we were able to use mitotic mapping to assign the mutations to a chromosome. Since genes of related function are seldom clustered in *S. cerevisiae*, if the dominant mutations can be isolated in a number of different genes, then it is unlikely that all will be on the same chromosome. We employed the *rad52* motitic mapping procedure developed by D. Schild to map two independent dominant mutations and two independent recessive mutations. The results of these mapping experiments are presented in Table 5. The data indicate that both of the *tun1* mutations and both of the *ALG7* mutations mapped to chromosome VII.

Since all four mutations mapped to the same chromosome, we determined whether *TUN1* and *ALG7* in fact represent different types of mutations in the same gene. By screening a number of different strains marked with chromosome VII mutations, we were able to find a strain that would form diploids capable of sporulating when crossed to both the dominant and recessive mutants. Meiotic analysis indicated

TABLE 6. Genetic mapping[a] of *TUN1* and *ALG7*[b]

Interval	No. of:			Map distance (centimorgans)	Published value[c]
	Parental ditype	Tetra-type	Non-parental ditype		
CLY8 vs *TUN1*	4	13	1	53	
CYH2 vs *TUN1*	1	11	1	>100	
ADE5 vs *TUN1*	2	12	13	>100	
TRP5 vs *TUN1*	2	11	5	>100	
TRP5 vs *CYH2*	4	13	0	38	42
CLY8 vs *ALG7*	8	12	0	30	
CYH2 vs *ALG7*	8	8	10	>100	
ADE5 vs *ALG7*	2	13	4	>100	
TRP5 vs *ALG7*	17	5	1	24	
TRP5 vs *CLY8*	7	13	0	32	90

[a] Mapping was performed by crossing JRY22 *tun1-1* and JRY22 *ALG7-1* to JRY475.

[b] Gene order on chromosome VIII: *ADE5 CYH2 TRP5 ADE6 ALG7 CLY8 TUN1*.

[c] From reference 16.

that both the dominant and recessive mutations are linked to chromosome VII genes but lie in distinctly different locations (Table 6). In addition, a cross between a *tun1-1* and an *ALG7-1* strain yielded wild-type tunicamycin-sensitive recombinants, indicating that *TUN1* and *ALG7* are distinct loci separable by recombinantion. We are unable to accurately determine the frequency of recombination due to poor viability. We estimate the frequency of recombinants to be between 5 and 30% of the segregants.

Analysis of recombinant clones. To investigate the nature of the tunicamycin resistance conferred by plasmid pJR39, a multicopy yeast *E. coli* shuttle vector, we isolated the insert DNA from pJR39 that was responsible for overproduction of transferase activity and cloned it into the plasmid vector YCp50. YCp50 is a yeast-*E. coli* shuttle vector consisting of pBR322, the *URA3* gene of yeast, a chromosomal ARS element, and the centromere of chromosome IV (26, 27). DNA fragments inserted into YCp50 are propagated in *S. cerevisiae* at a single copy per cell. This CEN plasmid, pJR116, was transformed into JRY22 and into three independent *tun1* mutants isogenic to JRY22. JRY22 containing this plasmid is much less resistant to tunicamycin than is the same strain containing pJR39, the multicopy plasmid. The insert in pJR116 was isolated and cloned into YEp24 regenerating pJR39. This plasmid, when transformed into *S. cerevisiae*, conferred tunicamycin resistance to the same extent as the original pJR39 plasmid. This result demonstrates that the resistance associated with pJR39 is a function of copy number.

Formally, it is possible that the gene carried on pJR39 and pJR116 is the *TUN1* gene. The *tun1* mutants containing pJR116 offer a direct test of this hypothesis. If the gene in pJR116 is the *TUN1* gene, then it should complement the recessive mutations in the chromosomal copy of *TUN1* and cause the cells to be sensitive to tunicamycin. However, we observe that these mutants with the plasmid are no less resistant that the same mutants lacking the plasmid.

A more reasonable hypothesis for the identity of the gene on pJR39 and pJR116 is that it is *ALG7*. If this hypothesis is correct, then it should be possible to isolate dominant mutations in pJR116 that confer resistance at one copy per cell that are indistinguishable from chromosomal *ALG7* mutations. Tunicamycin-resistant mutants were selected from three independent cultures of JRY22 containing pJR116. Several mutants were picked from each culture and analyzed to determine whether any of the mutations mapped to the plasmid (by definition these would be dominant). Two independent tunicamycin resistance mutations were mapped to the plasmid, suggesting that the plasmid-borne gene may be *ALG7*.

DISCUSSION

The ubiquitous nature of *N*-glycosylation in eucaryotic organisms argues that this modification serves an essential role in cells. However, definitive evidence for this view has been scarce since tunicamycin, the most widely used inhibitor of glycosylation, is a mixture of homologs with different effects. Consequently, it has been difficult to interpret which effects of tunicamycin treatment are a direct consequence of the lack of *N*-glycosylation and which are due to other effects. A genetic approach offers a clear resolution of these ambiguities. We have demonstrated that mutations in the nuclear gene *ALG7*, which may be the structural gene for the enzyme inhibited by tunicamycin, confer resistance to cell division inhibition caused by tunicamycin. This gene segregates as a single Medelian locus. The fact that these mutations, selected by their effect on cell division, simultaneously result in an alteration in the transferase enzyme and the synthesis of a known glycoprotein establishes a causal connection between the transferase, *N*-glycosylation, and cell division. Recent results of others (10) have shown that a temperature-sensitive mutation in the enzyme that adds the first mannose residue to the oligosaccharide chain results in a temperature-sensitive cell cycle arrest phenotype. These results, together with our data on *tun1* and *ALG7* mutations, provide independent evidence that *N*-glycosylation is an essential function. Furthermore, both sets of genetic experiments confirm earlier conclusions regarding cell cycle arrest in tunicamycin-treated cells. The *tun1* and *ALG7* mutations will continue to be useful in distinguishing between effects due to inhibition of glycosylation and secondary effects caused by the other homologs of tunicamycin.

We favor the hypothesis that *ALG7* is the structural gene for transferase due to two findings. First, mutations in *ALG7* result in an increase in the specific activity of transferase in cell extracts. Second, these same mutations result in a dominant sporulation defect. We determined that an increase in enzyme activity per se does not affect sporulation since a plasmid that results in a dramatic elevation of enzyme activity has no effect on sporulation. We therefore consider the sporulation defect to reflect a qualitative change in enzyme activity. Since the *ALG7* mutations result in both quantitative and qualitative effects on the activity of transferase, it seems likely that these mutations define the structural gene for the transferase. The two- to threefold increase in activity of the transferase may not be the entire explanation for tunicamycin resistance. For example, the same alteration that results in a modest increase in activity may result in a pronounced increase in the ability of the enzyme to discriminate between tunicamycin and either UDP-*N*-acetylglucosamine or dolichol phosphate. Although we have not demonstrated that all of the dominant mutations reside at the same locus, the fact that two independent mutations map to the same chromosome and that all dominant mutations have the same effect on sporulation argues strongly that the *ALG7* locus is at least a major locus of dominant tunicamycin-resistant mutations.

We previously reported the isolation of recombinant clones, such as pJR39, that lead to overproduction of transferase activity (20). Regarding the identity of the gene carried on this plasmid, several facts are consistent with the

gene contained on pJR39 being the *ALG7* gene. First, the level of tunicamycin resistance is a function of the copy number of the insert in pJR39. Second, the presence of the plasmid pJR39 in cells harboring an *ALG7* mutation suppresses the sporulation defect of *ALG7* mutations. Third, when the insert in pJR39 is cloned into a centromere vector, thus reducing the copy number of the gene(s), dominant tunicamycin-resistant mutations can be isolated that map to the plasmid. These mutations are phenotypically indistinguishable from the *ALG7* mutations. The sporulation defect of *ALG7* mutations has prevented us from testing whether an integrated pJR39 is allelic with *ALG7*. Our current working hypothesis is that pJR39 contains a copy of a wild-type *ALG7* gene which is the structural gene for the transferase. The tunicamycin resistance conferred by pJR39 according to this model is a consequence of increased gene dosage of the transferase gene in a multicopy vector. Alternatively, pJR39 may encode a regulator of the transferase, but there is no evidence in any organism for the existence of this hypothetical regulator. However, we emphasize that the identities of both the *ALG7* gene product and the gene on pJR39 have yet to be conclusively established.

Nine independent recessive mutations that confer tunicamycin resistance all fall into one complementation group that we designate *TUN1*. The simple view of how loss of function mutations can confer drug resistance is by loss of the transport system that brings the drug into the cell. In contrast to the data from CHO cells, we find no evidence that *TUN1* is involved in the transport of glucosamine; glucosamine does not compete with tunicamycin to offer protection against tunicamycin, and glucosamine auxotrophs with a mutation in the *TUN1* gene can still be supplemented with glucosamine from the medium. As yet we do not know the basis of resistance in the recessive mutants, but enzyme assays have detected no change in the transferase activity in recessive mutants.

The sporulation defect associated with *ALG7* mutations is at least partly suppressed in certain diploids for reasons that remain unclear. Thus we were able to map *ALG7*. How can the sporulation defect associated with the *ALG7* mutations be dominant and still be suppressed by pJR39 if the plasmid actually has the *ALG7* gene and this gene actually encodes the transferase? This dilemma can be resolved if sporulation requires that the transferase associates into multimers to respond to some sporulation signal and the presence of mutant subunits blocks the function of wild-type subunits in the multimer. If plasmid pJR39 contains the structural gene for the transferase, then correspondingly greater quantities of the wild-type enzyme will be produced, allowing sufficient functional multimers to form and carry out the sporulation-specific function.

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