Biosynthesis of Yeast Mannan

ISOLATION OF KLUYVEROMYCES LACTIS MANNAN MUTANTS AND A STUDY OF THE INCORPORATION OF N-ACETYL-D-GLUCOSAMINE INTO THE POLYSACCHARIDE SIDE CHAINS*

(Received for publication, November 4, 1974)

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SUMMARY

One side chain in the cell wall mannan of the yeast Kluveromyces lactis has the structure

\[ \alpha\text{Man}(1 \rightarrow 3) \alpha\text{Man}(1 \rightarrow 2) \alpha\text{Man}(1 \rightarrow 2) \text{Man} \mid \alpha\text{GNAc}(1 \rightarrow 2) \]

(RASCHE, W. C., AND BALLOU, C. E. (1972) Biochemistry 11, 3807). This \((\text{Man})_4\text{GNAc}\) unit (the \(N\)-acetyl-\(D\)-glucosamine derivative of mannotetraose) and the \((\text{Man})_4\) side chain, \(\alpha\text{Man}(1 \rightarrow 3) \alpha\text{Man}(1 \rightarrow 2) \alpha\text{Man}(1 \rightarrow 2) \text{Man}\), are the principal immunochemical determinants on the cell surface. Two classes of mutants were obtained which lack the \(N\)-acetyl-\(D\)-glucosamine-containing determinant. The mannan of one class, designated \(\text{mnn}1\), lacks both the \((\text{Man})_4\text{GNAc}\) and \((\text{Man})_4\) side chains. Apparently, it has a defective \(\alpha\)-1-3-mannosyltransferase and the \((\text{Man})_4\) unit must be formed to serve as the acceptor before the \(\alpha\)-1-2-\(N\)-acetylglucosamine transferase can act. The other mutant class, \(\text{mnn}2\), lacks only the \((\text{Man})_4\text{GNAc}\) determinant and must be defective in adding \(N\)-acetylglucosamine to the mannotetraose side chains. Two members of this class were obtained, one which still showed a wild type \(N\)-acetylglucosamine transferase activity in cell-free extracts and the other lacking it. They are allelic or tightly linked, and were designated \(\text{mnn}2\text{-1}\) and \(\text{mnn}2\text{-2}\).

Protoplast particles from the wild type cells catalyzed a \(\text{Mn}^{2+}\)-dependent transfer of \(N\)-acetylglucosamine from UDP-\(N\)-acetylglucosamine to the mannotetraose side chain of endogenous acceptors. Exogenous mannotetraose also served as an acceptor in a \(\text{Mn}^{2+}\)-dependent reaction and yielded \((\text{Man})_4\text{GNAc}\). Related oligosaccharides with terminal \(\alpha(1 \rightarrow 3)\)mannosyl units were also good acceptors. The product from the reaction with \(\alpha\text{Man}(1 \rightarrow 3)\text{Man}\) had the \(N\)-acetylglucosamine attached to the mannosyl unit at the reducing end, which supports the conclusion that the cell-free glucosyltransferase activity is identical with that involved in mannan synthesis. The reaction was inhibited by uridine diphosphate.

*This work was supported by National Science Foundation Grant GB-35229X, and by United States Public Health Service Research Grant AM584 and Postdoctoral Fellowship No. 5 F02 GM52056.
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Cell wall mannan-proteins of the yeasts Saccharomyces cerevisiae and Kluyveromyces lactis have a linear \(\alpha\)-1-6-linked backbone to which oligosaccharide side chains are attached by \(\alpha\)-1-2 and \(\alpha\)-1-3 linkages (1-5) (Fig. 1). The large polymannose chains are attached, through \(N\)-acetyl-\(D\)-glucosamine, to asparagine units in the protein (6-8). Short mannooligosaccharides are also found linked O-glycosidically to the hydroxyl groups of serine and threonine residues (6, 8, 9). These latter oligosaccharides have structures that are identical with the fragments produced by selective chemical cleavage of \(\alpha\)-1-6 linkages in the large mannan chains (6). All of these side chains exhibit polymorphic structures that are strain-specific (10).

\[^{[4C]}\text{Gmannan}\] synthesized endogenously from GDP-\(\text{D-[4C]}\)mannose by Saccharomyces carlsbergensis protoplast particles is a complex product of the action of several different mannosyltransferases (11). Attempts to study any of the transferases separately using cell wall mannan mutants of S. cerevisiae (12, 13) have thus far been unsuccessful. One handicap has been the relatively low activity of the mannosyltransferases involved in side chain synthesis (11, 14).

The presence of \(N\)-acetyl-\(D\)-glucosamine in the side chains of K. lactis mannan (5) offered the possibility to study transfer of a terminal sugar residue to endogenous mannan acceptors in the absence of synthesis of new mannosyl linkages. Using mannan mutants of K. lactis, we have demonstrated that addition of \(N\)-acetylglucosamine to the mannan side chains requires the presence of a precursor mannotetraose unit with a terminal \(\alpha\)-1-3 linkage. Moreover, broken protoplast membranes transfer \(N\)-acetylglucosamine from UDP-\(N\)-acetylglucosamine to existing...
The structural and regulatory gene defects of several K. lactis were labeled GDP-n-mannose from Calbiochem and unlabeled S. cerevisiae X2180 mannan (1, 2, 18). All other chemicals were phenol-sulfuric acid method using D-glucose as the standard (19), and aMan(l+6)aMan(l+S)aMan(l+2)Man that were obtained Mr. P. Lipke donated samples of βMan(li2)χMan(l+2)Man n-glucosamine as the standard. Total phosphate was determined by the procedure of Bartlett (21). Protein was estimated using a monomoph based on the extinction coefficients of enolase and nucleic acid at 260 and 280 nm (22).

Descending paper chromatography was done on Whatman No. 1 filter paper using (in volume ratios) ethyl acetate-pyridine-water (10:3:7) (Solvent A) and ethyl acetate-pyridine-acetic acid (5:5:3:1) (Solvent B). Neutral sugars were detected with alkaline silver nitrate (23). Paper chromatograms were scanned for radioactivity by cutting a 2-cm-wide strip into 1-cm horizontal bands which were counted in 10 ml of Bray's solution (24) using a Packard Tri-Carb scintillation counter.

β-D-Fructofuranosidase activity was assayed by a modification of the procedure of Bernfeld (25, 26). Gas chromatography of partially methylated alditol acetates was carried out at 160°C on a column (2.5 ft 3% OV-210) using a Varian Aerograph 1400 instrument equipped with a DuPont 21-491 mass spectrometer and operating at an ionizing voltage of 70 eV.

Preparation and Assay of Mannan—Kluyveromyces lactis Y-43a his8 (lacking imidazole glycerol phosphate dehydrogenase) and Kluyveromyces lactis Y-58a his4C (lacking histidinol dehydrogenase) (27) were supplied by Dr. James Haber, Brandeis University. These strains, and mutants derived from them, were grown at 30°C to late stationary phase in media containing 5% D-glucose, 0.5% yeast extract, and 0.3% Casamino acids. Mannan was extracted from cells by autoclaving them with 0.02 M sodium citrate, pH 7.0, and the extracted material was purified either by precipitation with Fehling's solution (28) or by Cetavlon (hexadecyltrimethyl ammonium bromide) precipitation (6, 29). Purified mannan was acetylated as described by Raschke and Ballou (5).

Mild Base Hydrolysis of Mannan—Mannan (2 g), purified by the Cetavlon procedure, was dissolved in 150 ml of 0.1 M NaOH and the mixture was allowed to stand at 23°C for 24 hours. The solution was neutralized with 2 M acetic acid and dialyzed against 5 changes of 200 ml of water. The solution outside the dialysis bag, which contained the oligosaccharides released from linkage to seirine and threonine, was lyophilized.

Methylation Procedure—Methylation of reduced oligosaccharide acetylation products was performed by the Hakomi procedure (30). The methylsulfanyl anion was prepared according to Sanford and Conrad (31), whereas the methylation conditions followed those of Hellerqvist et al. (32). The reduced and methylated oligosaccharides were hydrolyzed as described by Raschke and Ballou (5). For analysis by gas chromatography-mass spectrometry, the hydrolysis products were converted to alditol acetates by reduction with sodium borohydride followed by acetylation with acetic anhydride and anhydrous sodium acetate (33).

Antiserum—Antiserum against K. lactis NRRL 1140, S. cerevisiae 2828C and X2180, and Kloeckera brevis 55-45 were prepared as reported earlier (34, 35). Antiserum specific for the N-acetylglucosamine-containing pentasaccharide side chain of K. lactis was prepared by adsorption of anti-K. lactis serum with S. cerevisiae X2180 cells (5, 34).

Isolation of Mannan Mutants—Mutagenesis of K. lactis strains Y-43a his8 and Y-58a his4C with ethyl methanesulphonate was performed as described by Raschke et al. (12). The mutagenized cells were grown at 30°C for 9 days, then harvested and resuspended in 0.9% NaCl. Wild-type cells having the N-acetylglucosamine-containing pentasaccharide side chain were agglutinated with 0.5 ml of antiserum directed against that determinant. After 1 hour, the suspension was shaken and the agglutinated cells were again allowed to settle. A portion, 0.2 ml, of the supernatant was added to 2 ml of fresh medium and grown at 30°C for 48 hours. The agglutination and growth procedures were repeated twice. The resulting suspension, enriched in cells with altered surface determinants, was plated onto a complete agar medium. Following 48-hour growth, single colonies were selected and assayed to complete medium. After an additional 24 hours, the colonies were tested for their ability to agglutinate with antiserum against the N-acetylglucosamine-containing pentasaccharide of K. lactis by the procedure of Antalis et al. (36).

Genetic Studies—K. lactis haploid cells of opposite mating types, with complementing histidine requirements, were mutagenized on Difco malt extract-agar under aerobic conditions for 1 to 3 days. The diploid stage of this yeast is transitory (27) and ascus dissection was performed after approximately 1% of the culture had formed zygotes and then sporulated.

**Fig. 1.** Generalized structures of the polysaccharide components of the wild type mannans of (A) Saccharomyces cerevisiae X2180 and (B) Kluyveromyces lactis NRRL 1140. The latter shows only the outer chain components, and it has not been established that this mannan has an inner core structure similar to that in S. cerevisiae mannan, although it does possess alkali-labile serine and threonine-linked oligosaccharides comparable to those in the outer chain. In this figure, M is n-mannose, P is phosphate, GNAc is N-acetyl-D-glucosamine, whereas Ser, Thr, and Asn are amino acids in a protein chain. All anomer linkages are α except for those in the trisaccharide connecting unit M-GNAc-GNAc-GNAc→Asn which are α,β. The anomeric linkage of mannose to serine and threonine has not been established.

**Experimental Procedures**

Materials—Bio-Gel P-2 (200 to 400 and 400 mesh) and Dowex AG 1-×2 (200 to 400 mesh) were obtained from Bio-Rad Laboratories. Glucomalase was from Enzo Laboratories. GDP-D-[U-14C]n-mannose (151 Ci/mol) and UDP-D-[U-14C]D-glucosamine (36 Ci/mol) were purchased from New England Nuclear. Unlabeled GDP-n-mannose was from Calbiochem and unlabeled UDP-n-mannose was from Sigma. A sample of 3-O-methyl-D-mannose was provided by Dr. S. K. Macrae; and α-Man(1→3)Man,1 produced by mild acid hydrolysis of Saccharomyces cerevisiae X2180 mannan (15), was supplied by Mr. L. Rosenfeld. Mr. P. Lipke donated samples of α-Man(1→2)α-Man(1→2)Man and α-Man(1→2)α-Man(1→2)α-Man(1→2)Man that were obtained by partial acetylation of Hansenula polymorpha mannan (16). The pentasaccharide, α-Man(1→3)α-Man(1→3)α-Man(1→2)α-Man(1→2)Man, was prepared by acetylation of Saccharomyces italicus (17). The pentasaccharide, α-Man(1→6)α-Man(1→6)α-Man(1→4)GNAc, was derived from the inner core portion of S. cerevisiae mannan (8). Other oligosaccharides were obtained by partial acetylation of S. cerevisiae X2180 mannan (1, 2, 18). All other chemicals were reagent grade from commercial sources.

**General Procedures**—Total carbohydrates was measured by the phenol sulfuric acid method using D-glucose as the standard (19), and hexoseamine by the Elson-Morgan Method (20) with N-acetyl-
Preparation of Protoplast Particles—Protoplasts were prepared by a procedure similar to that described by van Rijn et al. (37).

K. lactis cells were grown to early log phase on 5% d-glucose, 0.5% yeast extract, and 0.3% casamino acids, then harvested and washed with 1% KCl. Cells, 1 g wet weight, were incubated for 30 min at 30°C in 10 ml of 0.05 M EDTA, pH 6.9, containing 10 mM dithiothreitol. The treated cells were washed 3 times with 10-ml portions of 12% d-mannitol and suspended in 25 ml of 0.05 M sodium succinate, pH 5.8, containing 12% d-mannitol and 0.25 ml of Glutulase. After the digest had shaken gently at 30°C for 30 to 60 min, only protoplasts were observed under a phase contrast microscope. The protoplasts were washed 3 times with 12% d-mannitol by centrifugation and then were broken by addition of 0.1 M imidazole-HCl, pH 6.5. The resulting particles were collected by centrifugation at 25,000 X g for 20 min, and the pellet was resuspended in 0.07 M imidazole-HCl, pH 6.5, containing 33% glycerol and stored at -10°C usually for no more than 2 weeks before use.

Enzyme Assays with Endogenous Acceptors—Protoplast particles, 0.5 to 2.0 mg of protein, were incubated in assay mixtures containing 0.3 nmol of GDP-α-[U-14C]mannose or 0.8 nmol of UDP-α-[1-14C]glucosamine and 0.4 ml of MnCl₂ in 0.05 M imidazole-HCl, pH 6.5, in a final volume of 0.5 ml. The reactions were stopped by addition of 2 ml of absolute ethanol, the precipitate was collected by centrifugation, washed 4 times with 1 ml of ethanol, and dried. Selective acetylation (1, 28) was performed on the pelleted fraction, using 1 ml of a 1:1 mixture of acetic anhydride and pyridine for the acetylation step and 2 ml of a 10:10:1 mixture of acetic anhydride-acetic acid-concentrated sulfuric acid for the acetylation step. The acetylation products were deacetylated and separated by paper chromatography in Solvent A.

Enzyme Assays with Exogenous Acceptors—Incubations were carried out as described above for endogenous mannan acceptors except that protoplast particles were preincubated 10 min both with and without oligosaccharide before addition of the UDP-N-acetyl-α-[1-14C]glucosamine to initiate the reactions. After a fixed reaction time, the mixture was applied to a column (0.5 X 6 cm) of Dowex AG1-X2 (200 to 400 mesh), prepared in a disposable Pasteur pipette, and allowed to flow into the bed of the column (approximately 2 min). The neutral material on the column was eluted with 1 ml of water into a scintillation vial. A 10-ml portion of Bray’s solution (24) was added to the vial and the sample was counted.

**Table I**

Agglutination of *Kluyveromyces lactis* mutants with mannan antiserum

| *K. lactis* strain | Sera       |
|-------------------|------------|
|                   | Anti-*K. lactis*<sup>a</sup> | Anti-*X2180*<sup>b</sup> | Anti-*K. brevis*<sup>c</sup> |
| Y-43α (wild type) | +          | +                       | -                      |
| Y-43α(3-55)       | -          | +                       | -                      |
| Y-43α(2-31)       | -          | +                       | -                      |
| Y-43α(2-22)       | -          | -                       | -                      |
| Y-43α(3-79)       | -          | -                       | -                      |
| Y-43α(4-45)       | -          | -                       | -                      |
| Y-43α(3-18)       | -          | -                       | -                      |
| Y-58α (wild type) | +          | +                       | -                      |
| Y-58α(6)          | -          | -                       | -                      |
| Y-58α(10)         | -          | -                       | -                      |
| Y-58α(15)         | -          | -                       | -                      |
| Y-58α(21)         | -          | -                       | -                      |
| Y-58α(29)         | -          | -                       | -                      |
| Y-58α(55)         | -          | -                       | -                      |
| Y-58α(54)         | +          | +                       | -                      |

<sup>a</sup> Diluted anti-*K. lactis* serum adsorbed with *Saccharomyces cerevisiae* X2180 cells.

<sup>b</sup> Diluted anti-*S. cerevisiae* X2180 serum adsorbed with *S. cerevisiae* 448-24D-B1 cells.

<sup>c</sup> Diluted anti-*Kloeckera brevis* serum that was capable of agglutinating *S. cerevisiae* X2180-1A mnnl cells which possess the α-1m-mannosylphosphate determinant.

**Selection of *Kluyveromyces lactis* Mannan Mutants—**Two classes of mutants were found that failed to agglutinate with *K. lactis* antiserum directed against the (Man)_nGNAc determinant (Table I). One class agglutinated with *Saccharomyces cerevisiae* X2180 antiserum specific for the tetrasaccharide side chain, αMan(1→3)αMan(1→2)αMan(1→2)Man, indicating that these mutants still made this unit. The other class of mutants failed to agglutinate with anti-X2180 serum, which suggested that they possessed side chains no longer than a trisaccharide (13). Neither the wild type strains nor the mutants reacted with antiserum directed against the α-1m-mannosylphosphate determinant (anti-*Kloeckera brevis* serum) (35). As expected, all strains failed to grow on a minimal medium unless supplemented with histidine.

**Acetylation of *K. lactis* Mutant Mannans—**Acetylation patterns of mannans from the wild type and one from each of the two classes of mutants are shown in Fig. 2. Acetylation patterns of mannans from *K. lactis* strains Y-43α(3-55) and Y-58α(54) lacked only the (Man)_1GNAc and (Man)_2GNAc fragments that are characteristic of mannans from the wild type parents. This suggested that these mutants were defective in the enzyme catalyzing transfer of N-acetylglucosamine onto the mannan side chains. The mutations were designated mnn1-1 for Y-43α(3-55) and mnn-2 for Y-58α(54) after the genetic and biochemical analyses described below. Mannans from the *K. lactis* strains Y-43α(2-22) and Y-58α(10) lacked not only the two above fragments but also the mannitetraose acetylation product (Man)_4. These were designated mnn1 mutants and apparently were defective in an α-1→3-mannosyltransferase analogous to the mnn1 mutation first reported in *S. cerevisiae* (12). This result suggests that the formation of the α-1→3 linkage is required for attachment...
of N-acetylglucosamine to the mannan side chains. The ratios of acetolysis fragments from several representative mutants are given in Table II.

Methylation Analysis of Acetolysis Fragments—Table III summarizes the results of analysis by gas chromatography-mass spectrometry of partially methylated alditol acetates prepared from the reduced acetolysis fragments of several mannans. Both the wild type Y-58a, and the mnnl-2 mutant, Y-58a(10), produce similar mannotriose and mannotetraose fragments. The methylation results for the tetrasaccharides agreed with the expected for a mutant defective in the cr-1+3-mannosyltransferase as well as (Man)4GNAc. Mild base treatment of the mnnl-2 mutant did not yield (Man)4GNAc, whereas mannan from the mnnl mutant lacked this oligosaccharide as well as (Man)4.

β-Fructofuranosidase-Content and Glusulase-Sensitivity of K. lactis Mutants—Cell wall mannan mutants of S. cerevisiae (12, 13) have different capacities to retain external invertase when whole cells are treated with mercaptoethanol (38). In contrast, stationary phase K. lactis wild type and mutant cells released similar amounts (8 to 10%) of their external invertase into the medium on incubation for 150 min with 10 mM dithiothreitol. However, alteration of the cell wall structure of the K. lactis mutants was apparent from the differential sensitivities of the mutant and wild type cells to enzymic lysis in a hypotonic buffer containing Glusulase. Removal of terminal N-acetylglucosamine and mannoside units from the mannan side chains apparently made the cell wall glucan more susceptible to glucanases.

Segregation of Mutant Phenotypes—Evidence that the K. lactis strains with altered mannans were the result of single mutations was obtained by tetrad analysis of crosses between wild type and mutant strains (39, 40). All crosses yielded tetrads with viable spores in which the mutant mannan phenotype, scored by ag-
glutination with antisera directed against the (Man)₄ and (Man)₅GNAc determinants, segregated 2⁺:2⁻. About 8 tetrads were analyzed from each cross.

Various crosses of mnn₁, mnn₂-1, and mnn₂-2 mutants yielded the results summarized in Table IV. Independently derived mnn₁ mutations from the α and α mating types were closely linked as shown by the formation only of parental ditype tetrads when the two identical phenotypes were crossed. The same was true for mnn₂-1 mutants. These data suggest that the non-segregating mutant pairs were allelic (40). The diploid from a cross of Y-43a(3-55) with Y-58a(54) yielded only parental ditype tetrads although these two mutants differ biochemically as shown below. Thus, these two mutants also appear to be allelic, although some difficulties in genetic studies with this yeast make the results somewhat unreliable.

Crosses of mnn₁ with mnn₂-1 and mnn₅-2 mutants gave segregation frequencies characteristic of unlinked genes. In addition, the tetratype frequency was that expected if either the mnn₁ gene or the mnn₂ gene was not centromere linked. As a control, tetrads of all crosses were examined for segregation of the unlinked histidine markers present in the two parents. His₃ by his₅C crosses had a PD : NPD : T ratio of 15:10:62, close to the 1:1:4 ratio expected for these unlinked genes that are not centromere linked (27, 40).

Incorporation of N-Acetylglucosamine into Endogenous Mannan Acceptors—Incubation of broken protoplast preparations from the wild type K. lactis Y-58a with UDP-N-acetyl-[1⁴C]glucosamine led to incorporation of radioactivity into ethanol-insoluble material. Treatment of the ethanol-precipitated product with 0.1 M NaOH for 24 hours did not release any ¹⁴C-labeled oligosaccharide that chromatographed in Solvent A with (Man)₃GNAc (B). Thus, the glucosamine had not been transferred to serine-linked oligosaccharides. In contrast, the mild base-labile products accounted for over 60% of the radioactivity incorporated with GDP[¹⁴C]mannose as the glycosyl donor. Partial acetolysis of the ethanol-insoluble material yielded radioactive products with the mobilities of (Man)₄GNAc and (Man)₅GNAc (Fig. 4). (Man)₅GNAc normally results from degradation of (Man)₄GNAc during acetolysis (5). Fig. 5 shows the gel filtration properties of the radioactive acetolysis fragment which chromatographed with (Man)₅GNAc in Solvent A. The radioactivity was eluted in the same position as (Man)₄GNAc produced by acetolysis of K. lactis Y-58a mannan. Biosynthesis of the endogenous (Man)₅GNAc fragment was linear with time for 60 min and it showed a linear dependence on protein concentration.

Although a substantial portion of radioactivity, presumably as chitin (41), was obtained in the ethanol-insoluble material in the absence of Mn²⁺ (but in the presence of Mg²⁺), no acetolysis product with a mobility of (Man)₅GNAc was detected under these conditions. Optimum synthesis occurred with 10 mM MnCl₂, and half-maximal activity occurred with a Mn²⁺ concentration of 2.5 mM. This Mn²⁺ requirement is similar to that reported for mannan biosynthesis from GDP-mannose (11, 14).

Preincubation of the protoplast particles with GDP-d-mannose did not increase their acceptor activity on addition of UDP-N-acetyl-[¹⁴C]glucosamine, which suggests that new mannan synthesis was not required for incorporation of N-acetylglucosamine.
TABLE V
Formation of (Man)$_4$GNAc from endogenous acceptors by protoplast particles

| Source of protoplast particles | Specific activity pmol/min/mg protein |
|-------------------------------|-------------------------------------|
| Y-43a (wild type)             | 0.40                                |
| Y-43a(2-22) (mnna)            | 0.00                                |
| Y-43a(3-55) (mnna=$\delta$)   | 0.00                                |
| Y-58a (wild type)             | 0.04                                |
| Y-58a(10) (mnna)              | 0.00                                |
| Y-58a(54) (mnna=$\delta$)    | 0.06                                |

FIG. 6. Time course of (Man)$_4$GNAc formation using (Man)$_4$ as the acceptor. Broken protoplast particles from Kluyveromyces lactis Y-58a (1.8 mg of protein) were incubated for the indicated times with 0.8 nmol of UDP-N-acetyl-$\beta$-[U-$^14$C]glucosamine, and 5 pmol of MnCll in 0.1 M imidazole-HCl, pH 6.5, at 22°. Soluble neutral radioactivity was determined as described in the text. With 110 nmol of the tetrasaccharide, $\alpha$Man(1-2)$\alpha$Man(1-2), $\alpha$Man(1-2)$\alpha$Man(1-2)$\alpha$Man(1-2)$\alpha$Man, $\alpha$Man(1-2)$\alpha$Man(1-3)$\alpha$Man(1-2)$\alpha$Man(1-2)$\alpha$Man(1-2)$\alpha$Man, $\alpha$Man(1-3)$\alpha$Man(1-2)$\alpha$Man(1-2)$\alpha$Man, $\alpha$Man(1-3)$\alpha$Man(1-2)$\alpha$Man(1-2)$\alpha$Man, $\alpha$Man(1-3)$\alpha$Man(1-2)$\alpha$Man(1-2)$\alpha$Man, $\alpha$Man(1-3)$\alpha$Man(1-2)$\alpha$Man(1-2)$\alpha$Man, $\alpha$Man(2-1)$\alpha$Man. "O 30 60 INCUBATION TIME (min)

FIG. 7. Paper chromatography of the products resulting from incubation of broken protoplast particles from Kluyveromyces lactis Y-58a with various acceptors. Incubations were performed as described in the text with $\alpha$Man(1-3)$\alpha$Man(1-2)$\alpha$Man(1-2)$\alpha$Man(1-2)$\alpha$Man (top), $\alpha$Man(1-3)$\alpha$Man(1-2)$\alpha$Man(1-2)$\alpha$Man (middle) and $\alpha$Man(1-3)$\alpha$Man(1-2)$\alpha$Man(1-2)$\alpha$Man (bottom). The resulting neutral soluble products were chromatographed in Solvent A. The bars represent the positions of standards as visualized with alkaline silver nitrate, the letters corresponding to A, GNAc; B, Man; C, (Man)$_2$; D, (Man)$_3$; E, (Man)$_4$GNAc; F, (Man)$_5$; G, (Man)$_6$GNAc. The radioactive product from each incubation had the chromatographic properties expected for the N-acetylglucosamine derivative of the acceptor.

neither 3-O-methylmannose nor the pentasaccharide, $\alpha$Man(1-3)$\alpha$Man(1-2)$\alpha$Man(1-2)$\alpha$Man(1-2)$\alpha$Man, served as a substrate for the N-acetylglucosamine transfer. Fig. 7 shows the chromatographic behavior of the radioactive products resulting from incubation of Y-58a particles, UDP-N-acetyl-
that the glucosamine transferase in the mutant occurred in an
unknown form, but was absent in the broken protoplast preparation.
We observed a stimulation of the transferase activity by glycerol
that was added to the resuspended protoplast particles. This effect
was eventually found to result from a stimulation of the trans-
ferase activity of the particulate glucosamine transferase. GDP-
mannosedid not inhibit the transfer of N-acetylglucosamine from UDP-
GMP caused less than a 10% decrease in the rate of N-acetyl-
glucosamine transfer. We did not attempt to prepare lipid-free
protoplast particles to test for a lipid requirement, but we did
find that a lipid extract of yeast cells failed to stimulate the ac-
ceptor activity of the particulate glucosamine transferase. GDP-mannose
did not inhibit the transfer of N-acetylglucosamine from UDP-
N-acetylglucosamine as might be expected were the same lipid
involved in the transfer of mannose to mannan acceptors.

As expected, formation of (Man)₄GNAc from (Man)₄ was
catalyzed by protoplast particles from Y-58a, Y-43α, and both
mnnl mutants (Table VII), but not by particles from mnn2-1
mutants. However, consistent with the results obtained with
endogenous mannan acceptors, the mnn2-2 mutant derived from
Y-58a did form (Man)₄GNAc from exogenous (Man)₄. This
latter reaction showed a Mn²⁺-dependence identical to that ob-
served with the parent strain and the Km for (Man)₄ (13 mM)
was also similar to that observed with Y-58a protoplast particles
(Fig. 8). Furthermore, the rate of (Man)₄GNAc formation by
Y-58a and Y-58a(54) particles increased similarly in response to
increasing UDP-N-acetylglucosamine concentrations over the
range of 10⁻⁴ to 10⁻¹ M.

The failure of the Y-58a(54) mutant to produce (Man)₄GNAc
side chains in the intact cell could have been due to the overpro-
duction of some inhibitor. Glucosamine transferase assays on the
whole cell extract of the mutant did show about one-third of the
total activity of the isolated protoplast particles, but the wild
type extracts showed a similar reduction in activity. This effect
was eventually found to result from a stimulation of the trans-
ferase activity by glycerol that was added to the resuspended
particles but that was absent in the broken protoplast preparation
that contained the whole cell extract. The possibility also existed
that the glucosamine transferase in the mutant occurred in an
inactive form that was activated by nonspecific proteases follow-
ing cell breakage. However, we were not able to isolate such an
inactive zymogen by cell disruption at low temperature or in the
presence of protease inhibitors such as phenylmethylsulfo-
yl fluoride.

Characterization of Product Formed with aMan(1→8)Man as
Acceptor—The disaccharide acceptor, 8 μmol, was incubated
with 1.6 nmol of UDP-N-acetyl-[1-14C]glucosamine, 5 μmol of
MnCl₂, and protoplast particles (2 mg of protein) from K. lactis
Y43α in a final volume of 0.5 ml of 0.05 M imidazole-HCl buffer,
PH 6.5, for 1 hour at 30°C. The reaction mixture was applied di-
rectly to a column (0.5 x 6 cm) of Dowex AG 1-X2 to remove
excess radioactive UDP-N-acetylglucosamine, and the material
eluted from the column with water was applied to a column (1 x 100 cm) of Bio-Gel P-2. The position of elution of the radio-
active product corresponded to that of a tetrasaccharide (Fig.
9) which would be expected for a compound with two mannose
units and one N-acetylglucosamine (42).

The radioactive fractions were pooled and concentrated to 0.2
ml on the rotary evaporator. A 50-μl sample was hydrolyzed in
1 N HCl at 100°C for 3 hours, the acid was removed by evacua-
tion under vacuum, and the residue was reduced with sodium
borotritide at pH 10. The excess reducing agent was destroyed
by adding acetic acid, and the residue was removed by repeat-
ed evaporation of the sample after addition of methanol.
Paper chromatography of this hydrolyzed and reduced product
(Solvent B) revealed the presence of mannitol, reduced N-acetyl-
glucosamine and reduced glucosamine (Fig. 10). The latter was
presumably formed by de-N-acetylation during the acid hy-
dalysis. Thus, the product of the above enzymic reaction con-
tained mannose and N-acetylglucosamine.

A second portion of the NaBT₄-reduced product was partially
hydrolyzed with 0.3 N HCl at 100°C for 2 hours. After neutrali-
zation of the reaction with dilute NaOH, the product was ap-
plicated to a column (1 x 100 cm) of Bio-Gel P-2 and resolved by
electron with water into two radioactive substances, one with the
size of the starting material and the second approximately the
size of a trisaccharide (Fig. 11). This product was presumed to
be a disaccharide composed of one mannose unit and one N

### Table VII

| Source of protoplast particles | Specific activity |
|------------------------------|------------------|
| Y-43α (wild type)            | 1.4              |
| Y-43α(2-52) (mnn1)           | 1.6              |
| Y-43α(3-85) (mnn2-1)         | 0.0              |
| Y-58a (wild type)            | 0.35             |
| Y-58a(10) (mnn1)             | 0.37             |
| Y-58a(6) (mnn5-1)            | 0.00             |
| Y-58a(54) (mnn2-2)           | 0.45             |

* Particles (0.5 to 0.7 mg of protein) were incubated with 0.8
  nmol of UDP-N-acetyl-p-[1-14C]glucosamine, 5 μmoles of MnCl₂,
  and 165 nmol of αMan(1→3)αMan(1→2)αMan(1→2)Man; the rate
  of soluble neutral product formation was determined as de-
scribed in the text. A minimum of 3 time points was used for each rate
determination.

![Fig. 8. The effect of (Man)₄ concentration on the rate of
(Man)₄GNAc synthesis with Y-58a and Y-58a(54) broken
protoplasts. Protoplast particles (1.0 mg of protein) from Y-58a
(○—○) and Y-58a(54) (△—△) were incubated for 20 min with
0.00 μM UDP-N-acetyl-p-[1-14C]glucosamine, 10 mM MnCl₂, and
varying concentrations of (Man)₄. The rate of product formation,
expressed as pmol/min/mg of protoplast protein, was measured
as described in the text. The Km for both reactions was about 13
mM.](http://www.jbc.org/content/259/8/3432/F6.large.jpg)
FIG. 9. Recovery by gel filtration on Bio-Gel P-2 of the radioactive product from incubation of the disaccharide αMan(1→3)Man with UDP-N-acetyl-[14C]glucosamine and protoplast particles prepared from Kluyveromyces lactis Y-43α. Conditions of the experiment are given in the text. The bars numbered 1 to 6 correspond to the elution positions of standard mono- to pentasaccharides. N-Acetylglucosamine has the elution volume of a disaccharide.

FIG. 10. Radioactive monosaccharide components obtained by sodium borotritide reduction of the complete acid hydrolysate of the enzymic product isolated in Fig. 9. The separation was done by paper chromatography with Solvent B. The reduced glucosamine resulted from de-N-acetylation during the hydrolysis. The bars indicate the positions of standards: A, N-acetylglucosamine; B, reduced N-acetylglucosamine; C, mannose; D, mannitol; E, glucosamine; F, reduced glucosamine.

Acetylglucosamine. The substance was labeled both with 14C and 3H, indicating that the N-acetylglucosamine had been added to the mannose at the reducing end of the disaccharide acceptor. To confirm this conclusion, the product from partial acid hydrolysis was subjected to complete acid hydrolysis in 1 N HCl at 100° for 3 hours and the hydrolysate was chromatographed on paper with Solvent B. Radioactive peaks corresponding to mannitol, N-acetylglucosamine and glucosamine were obtained. All of the above results demonstrate that N-acetylglucosamine was transferred to the mannose at the reducing end of the disaccharide αMan(1→3)Man to give αMan(1→3)Man. This is the result expected if the cell-free reaction had the same specificity as the transferase of the intact cell that adds N-acetylglucosamine to the corresponding position of the mannotetrasaccharide side chain.

DISCUSSION

Yeast mannan biosynthesis appears to involve at least four levels of glycosylation; namely, those reactions for synthesis of the serine- and threonine-linked units (6, 9), for synthesis of the inner core (8), for synthesis of the outer chain (11), and for addition of the substituents such as mannosylphosphate and N-acetylglucosamine that modify the outer chain (1-5). In this study we have dealt with the last kind of reaction in which N-acetyl-D-glucosamine is added in α-1→2 linkage to mannotetraose side chains during the maturation of Kluyveromyces lactis mannan. This reaction occurred with both endogenous and exogenous acceptors, using protoplast particles from the wild type strain of K. lactis, with UDP-N-acetyl-[14C]glucosamine as the donor. The endogenous reaction product yielded (Man)nGNAc on acetolysis, whereas the exogenous reaction with (Man), as the acceptor yielded (Man),GNAc directly. α1→3 linkages was inactive. In this reaction, the N-acetylglucosamine was added to the mannose unit at the reducing end of the disaccharide acceptor αMan(1→3)Man, a result consistent with the location of this amino sugar in the tetrasaccharide side chains of the mannan (5). Thus, the N-acetylglucosamine transferase is quite specific even though it appears to be involved in adding this sugar to those mannotetrasaccharides both in the outer chain and on serine and threonine. The reaction was inhibited by uridine diphosphate in a manner suggesting that a lipid-P-GNAc intermediate could be involved, but alternative explanations for this inhibition are possible.

Because the N-acetylglucosamine was added to endogenous mannan side chains in the absence of synthesis of new mannosyl linkages, the reaction probably proceeds by a stepwise addition and not by polymerization of preformed oligosaccharide side chains linked to lipid. The α1→2 N-acetylglucosamine transferase activity is similar in its Mn**-dependence (11, 14) and nucleotide diphosphate inhibition (43) to the mannosyltransferase studied previously (43-47). Lehle and Tanner (48) report that...
the transfer of mannos from GDP-mannose to free mannos and to endogenous acceptors does not appear to involve a lipid-bound intermediate. They suggest that only the initial mannos units added to the serine and threonine in the protein comes from such a derivative. Although exogenous oligosaccharides are good acceptors for the glycosyltransferases that are involved in polysaccharide biosynthesis in other fungi, such as Cryptococcus laurertii (49, 60), the oligosaccharides previously tested in yeast mannan biosynthetic systems did not function well as mannos acceptors (11, 14), possibly owing to a high $K_m$ (48). In contrast, we find that (Man),GNAc is readily synthesized from $\alpha$Man(1--3)$\alpha$Man(1--2)$\alpha$Man(1--2)Man and UDP-N-acetylglucosamine in the K. lactis system.

Yeast mutants were isolated that lacked the (Man),GNAc determinant. The mnn1 class apparently has a defective $\alpha$1--3-mannosyltransferase because it failed to make the mannnterotase units as well. Consequently, the mannan lacked acceptors for the N-acetylglucosamine. However, protoplast particles of this mutant had full N-acetylglucosamine transferase activity with exogenous acceptors. Two classes of mnn2 mutants were obtained that made mannan with the mannnterotase side chains, but they failed to add N-acetylglucosamine. One of these lacked N-acetylglucosamine transferase activity in the protoplast particles, but the other showed a normal wild type activity. Genetic analysis suggested that they were allelic or very tightly linked. Therefore, we considered that the mutant that showed transferase activity in the isolated protoplast particles, even though it did not add this sugar to the endogenous mannan, might make an altered transferase with a high $K_m$ for one of the substrates. However, we failed to observe any difference in the $K_m$ for UDP-N-acetylglucosamine, Mn$^{2+}$ or mannotetraose acceptor. The possibility remains that the defect may concern the presumed lipid intermediate, but lacking a suitable assay we were unable to test this point.

Because of the uncertainty that these two mutants are in fact allelic, we have considered that the mnn2-2 strain may be a regulatory mutant. One possibility is that the wild type cells produce a glucosamine transferase inhibitor that normally regulates the activity of the enzyme, and that in the mutant this inhibitor was overproduced. However, mixing experiments with the supernatant extracts of the mutant did not reveal any such inhibitor. We also considered that the mutant might make an inactive transferase that was activated by nonspecific proteases after the protoplasts were broken. However, we could find no method of preparing protoplast particles that failed to show activity, whether it was done at low temperature or in the presence of protease inhibitors.

Six classes of mannan mutants have now been obtained from yeast strains that have the $\alpha$1--6-backbone structure (Saccharomyces cerevisiae, K. lactis) (12, 13). Each of these mutations affects a different step in the synthesis of the oligosaccharide side chains emanating from the $\alpha$1--6-linked mannan backbone. Four of these mutations (mnn1 and mnn2 of S. cerevisiae; mnn1 and mnn2-2 of K. lactis) also cause corresponding alterations in the structure of those oligosaccharides that are linked to serine and threonine (6). Thus, several of the enzymes appear to be polyfunctional, or else the mutations are of a regulatory nature. Under normal growth conditions none of the mutants loses the capacity to secrete mannan proteins (12, 13, 26) or to retain extracellular enzymes within the cell wall (26). However, the alteration of side chains does enhance the release of the external invertases of S. cerevisiae cells during dithiothreitol treatment (38). This suggests that the side chains may be important in mobilizing proteins in the cell wall. In addition, cell wall mannan mutants of both S. cerevisiae and K. lactis are more susceptible than their wild type parents to digestion with Glucon $\alpha$-glucosidase. Thus, the side chains of the mannan could protect cell wall components against extracellular hydrolyases of competing organisms.

Acknowledgments—We thank Lun Ballou and Dr. William Whelan for help in dissecting the ascI for genetic experiments.

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*J. Biol. Chem.* 1975, 250:3426-3435.

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