A Galactosyltransferase from the Fission Yeast *Schizosaccharomyces pombe*

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Abstract. A membrane-associated galactosyltransferase has been purified to homogeneity from the fission yeast, *Schizosaccharomyces pombe*. The enzyme has a molecular weight of 61,000 and is capable of transferring galactose from UDP-galactose (UDP-Gal) to a variety of mannose-based acceptors to form an α-1,2 galactosyl mannoside linkage. Immunofluorescence localization of the protein is consistent with the presence of the enzyme in the Golgi apparatus of *S. pombe*. This, together with the presence of terminal, α-linked galactose on the N-linked oligosaccharides of *S. pombe* secretory proteins, suggests that the galactosyltransferase is an enzyme involved in the processing of glycoproteins transported through the Golgi apparatus in fission yeast.

In recent years, two complementary approaches have been taken to identify components involved in protein transport from the endoplasmic reticulum, through the Golgi apparatus, to the cell surface. The genetic approach, exemplified by Schekman and his co-workers, has identified several classes of gene products involved at different stages of protein secretion in *Saccharomyces cerevisiae*, from initial translocation and glycosylation to final delivery to the cell surface (for review, see Schekman, 1985). The use of cell-free systems, initiated by Rothman, has increased our understanding of the biochemical mechanisms involved in vesicular traffic, primarily through the Golgi apparatus of mammalian cells (for review see Pfeffer and Rothman, 1987). The demonstration that yeast contains all the cytosolic components required for mammalian intra-Golgi transport (Dunphy et al., 1986) and the recent finding that the *sec18* gene product of *S. cerevisiae* is functionally equivalent to one of the purified components of the Golgi transport system (*N*-ethylmaleimide-sensitive factor) (Wilson et al., 1989) demonstrate the efficacy of a combined genetic and biochemical approach to the questions of vesicular trafficking.

The primary method for tracing the progress of a protein through the transport pathway is to follow the processing of N-linked oligosaccharides. In the endoplasmic reticulum of mammalian cells, N-linked $Glc_3Man_9GlcNAc_2$ oligosaccharides are first trimmed to a $Man_8GlcNAc_2$ core, and then, as the glycoprotein is transported through the Golgi apparatus, processed by a series of mannosidases and sugar transferases to a final complex structure containing mannose, additional GlcNAc, galactose (Gal)[1] and sialic acid (for review, see Kornfeld and Kornfeld, 1985). Elucidation of the pathway of N-linked oligosaccharide processing and immunolocalization of some of the enzymes involved have demonstrated that the Golgi apparatus in mammalian cells consists of distinct biochemical compartments through which secretory, lysosomal, and plasma membrane proteins pass in a vectorial fashion (for review, see Dunphy and Rothman, 1985). The relatively simple processing of N-linked oligosaccharides in *S. cerevisiae*, coupled with its poor Golgi morphology, precludes many of the techniques that have proved useful in the analysis of the mammalian Golgi complex. We have therefore turned to the fission yeast *Schizosaccharomyces pombe* as a model system that is amenable to many of the genetic techniques available in *S. cerevisiae* and possesses a Golgi complex that is, morphologically, as good as, if not better than, the Golgi apparatus found in cultured mammalian cells.

In budding yeast, elongation of the N-linked core oligosaccharide occurs by the extension of a 1,6 α-linked Man backbone onto which are built side chains containing 1,2 and 1,3 α-linked Man (for review, see Ballou, 1982). While the N-linked oligosaccharides of *S. pombe* are less well characterized than those of *S. cerevisiae*, the increases in molecular weight seen during oligosaccharide processing of secretory proteins such as acid phosphatase and invertase are similar in the two yeasts. Work on *S. pombe* acid phosphatase by Dibenedetto and Cozzani (1975) and Schweingruber et al. (1986) is consistent with the initial addition of 5–10 $Glc_3Man_9GlcNAc_2$ core structures, each of which is subsequently elongated by the addition of 50–100 hexose units as the protein is transported to the cell surface. Fission and budding yeasts differ, however, in that the core oligosaccharide of *S. pombe* is extended by the addition of both Man and Gal before the protein arrives at the cell surface. Because *S. pombe* secretory proteins such as invertase (Moreno et al., 1985) and acid phosphatase (T. Chappell, unpublished results) bind to *Bandeiraea simplicifolia* lectin I agarose and can be eluted using α-methyl galactoside, Gal must be terminally attached to oligosaccharides through an α linkage. We have used this observation as the basis to look for galactosyltransferase activities in membrane fractions of *S. pombe*, as prob-

1. *Abbreviations used in this paper*: Gal, galactose; Glc, glucose; GlcNAc, *N*-acetyl-glucosamine; Man, mannose.

able marker activities for a Golgi compartment or compartments in this fission yeast.

Materials and Methods

S. pombe strain 972 h^- (*leu1-32, ura4-D18, his3*$^-$) (kindly provided by P. Nurse, Oxford University) was grown at 30°C in 3% Glc, 0.5% yeast extract (Difco Laboratories Inc., Detroit, MI) supplemented with 100 μg/ml adenine hemisulfate (YEA). UDP-[^{3}H]Gal (sp act, 40.4 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Unless otherwise noted, all reagents were obtained from Sigma Chemical Co. (Poole, UK).

Buffers

TM: 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$; TMD(x): TM + x% (wt/vol) Triton X-100; TMS: TM + 0.25 M sucrose; PEM: 100 mM Pipes-NaOH (pH 7.0), 1 mM EGTA, 1 mM $MgSO_4$; PEMS: PEM + 1.2 M sorbitol; PEMF: PEM + 0.5% (wt/vol) fish skin gelatin.

Cytosol and Microsome Extract Preparation

Five grams of fresh *S. pombe* cells were washed with water, resuspended in 5 ml of TMS and broken by six, 1-min bursts in a Bead Beater (Biospec Products, Bartlesville, OK) containing 0.5 mm glass beads. Glass beads, unbroken cells, and large cell debris were removed by a 15-min 10,000 *g* centrifugation. The low speed supernatant was fractionated by a 60-min 100,000 *g* centrifugation into a microsomal pellet and a cytosol supernatant. The microsomal pellet was resuspended in TMS to a protein concentration of ~20 μg/ml and the membranes disrupted by the addition of Triton X-100 to a final concentration of 2%. After a further 60-min 100,000 *g* centrifugation, the detergent solubilized material in the supernatant was retained as the microsome extract. Galactosyltransferase activity in the microsome extract was stable at 4°C over a period of several days in the absence of any protease inhibitors.

Galactosyltransferase Assays

Each 50-μl assay contained 0.1 M Hepes-NaOH (pH 7.0), 1 mM $MnCl_2$ with 25 nmol UDP-[^{3}H]Gal (sp act, 1–2 mCi/mmol) and 5 μmol of a given sugar acceptor. Incubation mixtures contained 0.05–0.2% Triton X-100, depending on the detergent concentration of the *S. pombe* protein fractions. Activity of the purified enzyme was independent of Triton X-100 over this concentration range. After 60 min at 37°C, each incubation mixture was diluted to 200 μl with water and loaded onto an 800-μl Dowex-1 (Cl^- form, 200–400 dry mesh) anion exchange column packed in a 2-ml syringe. The column was washed twice with 1 ml of water and the combined eluants counted after the addition of 2.5 vol Ready-Sol CP scintillation cocktail (Beckman Instruments, Inc., Palo Alto, CA). The Dowex columns were regenerated by washing with 2 ml 5 M NaCl, 2 ml 0.1 M HCl, and 5 ml of water.

Protein Purification

S. pombe was grown in 16-liter batches to near saturation. Cells were collected and washed with water on a filtration apparatus (Pellicon; Millipore Continental Water Systems, Bedford, MA), frozen in liquid nitrogen and stored at −80°C. Unless otherwise noted, all further procedures were carried out at 4°C. Thawed pellets (100 *g* wet weight) were washed with TMS and resuspended in 100 ml of the same buffer. Cells were broken by six, 1-min bursts in a Bead Beater. The homogenate was centrifuged at 10,000 *g* for 15 min and the pellet rebroken and centrifuged. The combined low speed supernatants were centrifuged at 100,000 *g* for 60 min. The 100,000 *g* microsomal pellet was resuspended in 100 ml TM using a teflon dounce homogenizer and an equal volume of TMD(5) was added to disrupt the membranes. The microsomal extract was centrifuged at 100,000 *g* for 60 min, and the pellets were resuspended in 100 ml TMD(2.5) and recentrifuged.

The combined 100,000 *g* supernatants were adjusted to 500 ml total volume with TMD(2) and loaded onto a 50-ml DEAE Fast Flow column. The anion exchange column was washed with 100 ml of TMD(0.5) followed by 200 ml of TMD(0.5) + 50 mM NaCl. The galactosyltransferase activity was eluted with TMD(0.5) + 300 mM NaCl. Fractions containing activity (~80 ml) were pooled and dialyzed against 2 × 4 liters of TMD(0.1). The dialysate was adjusted to 100 ml total volume with TMD(0.5), brought to 0.1 mM $MnCl_2$, and loaded onto a 10-ml UDP-hexanolamine agarose column that had been preequilibrated with TMD(0.1) + 0.1 mM $MnCl_2$. The column was washed with 100 ml TMD(0.1) + 0.1 mM $MnCl_2$ and the galactosyltransferase activity eluted with TMD(0.1) + 500 mM NaCl. After reequilibration, the UDP-hexanolamine agarose column was reloaded with the flow through and wash fractions, rewashed, and again eluted with TMD(0.1) + 500 mM NaCl. Fractions from each elution that contained galactosyltransferase activity (~25 ml) were pooled and dialyzed against 4 liters TMD(0.1).

The eluant from the UDP-hexanolamine column was adjusted to 0.1 mM $MnCl_2$ and loaded onto a 2-ml Con A Sepharose column. The column was washed with 50 ml TMD(0.1) + 0.1 mM $MnCl_2$, 150 mM NaCl and the galactosyltransferase activity eluted with 100 mM α-methyl mannoside in TMD(0.1) + 150 mM NaCl. Fractions containing activity were dialyzed against TMD(0.1) and loaded onto a Mono Q FPLC anion exchange column (Pharmacia Fine Chemicals, Piscataway, NJ). The column was washed with TM + 0.1% octyl β-D-thioglucoside until the absorption at 280 nm reached baseline. Bound protein was eluted from the column with a gradient of NaCl (17.5 mM/ml) in TM + 0.1% octyl-β-D-thioglucoside.

Product Analysis

Radiolabeled reaction products were size fractionated at room temperature on a BioGel P2 (minus 400 mesh; Bio-Rad Laboratories, Cambridge, MA) column (1.6 × 88 cm; Pharmacia Fine Chemicals) with a total bed volume of ~175 ml. The column was run in water at 0.25 ml/min using an FPLC pump (Pharmacia Fine Chemicals) for pressure and flow control. Glucose size standards for the sizing column were generated by hydrolyzing [^{3}H] dextran (70,000 M_r) (Amersham Corp., Arlington Heights, IL) in 0.1 M HCl for 4 h at 95°C (Yamashita et al., 1982). After hydrolysis, the material was lyophilized and resuspended in water. By collecting 3-ml fractions, size standards up to 5 glucose units were resolved.

Antibody Production and Affinity Purification

Polyclonal antibodies against purified *S. pombe* galactosyltransferase were raised in a rabbit according to Goudie et al. (1966). 50 μg of protein in complete Freund's adjuvant was injected into the popliteal lymph nodes and intradermally along the back. The rabbit was boosted subscapularly at regular intervals with 50 μg protein in incomplete Freund's adjuvant and then bled 7 d after each boost. Antiserum was diluted 1/50 in PEMF and incubated with paraformaldehyde fixed cells (~2 × 10^8 cells/ml) to remove antibodies against cell surface oligosaccharides. Typically, three sequential incubations depleted the antiserum of all cell surface staining IgG. The depleted antiserum was then affinity purified against galactosyltransferase according to the procedure of Burke et al. (1982). Briefly, 50–100 μg of the purified protein was run on a 10% polyacrylamide gel and electro-blotted onto nitrocellulose. The protein band was visualized on the nitrocellulose with 0.2% Ponceau S in 3% TCA, excised, and used as an affinity matrix for antibody purification. Galactosyltransferase bound Ig was eluted with glycine HCl (pH 2.8) containing 0.2% (wt/vol) fish skin gelatin and neutralized with Tris base. The affinity-purified antibodies were diluted twofold with PEMF for immunofluorescence staining.

Microscopy

For EM, cells were grown to mid-log phase in YEA, washed with water and fixed for 60 min at room temperature in freshly prepared 2% potassium permanganate (wt/vol in water). After extensive washing with water, the cells were dehydrated in a graded ethanol series followed by propylene oxide. The fixed cells were embedded in medium 812 resin (Taab Laboratories, Reading, UK) according to the manufacturer's instructions and sectioned with a diamond knife. Sections were stained with lead citrate according to Reynolds (1963) and viewed in an electron microscope (1,200× JEOL USA, Cranford, NJ).

A modified procedure of Hagan and Hyams (1988) was used for immunofluorescence. Except where noted, washing and incubations were performed at room temperature with gentle agitation. Cells were grown to mid-log phase in YEA, washed with PEM, and fixed for 60 min in freshly prepared 4% paraformaldehyde (wt/vol in PEM). After fixation the cells were washed twice with PEM, once with PEMS, and resuspended at 5 × 10^7 cells/ml in PEMS. The cell walls were digested with 0.5 mg/ml Zymolyase-100T (Seikagaku Kogyo Co., Tokyo, Japan) and 2.5 mg/ml Novozym 234 (Novo Enzyme Products, Basingstoke, UK) for 60 min at 37°C. After 3 washes with PEMS, the cells were permeabilized with 1% Triton X-100 in PEMS for 30 s, washed once with PEMS and twice with PEM. After incubation with PEMF for 1 h to block nonspecific binding sites, the cells

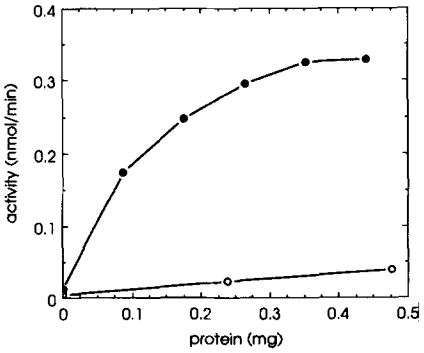

Figure 1. Microsomal localization of a Man dependent activity that releases Gal from UDP-Gal. Assays containing 25 nmol UDP-[^{3}H]Gal (100,000 dpm), 5 μmol Man and the indicated amount of either microsomal (●) or cytosolic (○) protein in a total volume of 50 μl were incubated for 60 min at 37°C. Gal released from UDP-Gal was quantitated by passing the reaction mixture over a Dowex anion exchange column and counting the flow through. The release of Gal in the absence of Man was equivalent to the release in the absence of protein, neither of which was subtracted from the raw data. Each data point is the average of three incubations, with an SEM of <10%.

were labeled overnight with affinity-purified antigalactosyltransferase antibodies. Labeled cells were washed three times with PEMF and incubated 4 h with FITC-conjugated donkey anti-rabbit IgG antibody (Amersham Corp.) diluted 1/100 (vol/vol) in PEMF. The labeled cells were washed three times with PEMF and resuspended in a minimum volume of PEMF. Cells were dried onto polylysine-coated cover slips and mounted in PBS/glycerol antifade (Citifluor Ltd., London, UK).

Confocal immunofluorescence images were generated with a Lasersharp apparatus (Bio-Rad Laboratories) attached to a Microphot FX microscope (Nikon Inc., Garden City, NY). Single images were generated by summing 50 scans with a Kalman algorithm. Optical sectioning was performed with 0.2-μm vertical steps of the microscope stage, accumulating 25 scans at each step. For sectioning, scans were summed with an accumulative algorithm. The digital images were scaled, but not subjected to either high or low pass filtering.

Other Methods

SDS-PAGE was performed according to the procedure of Laemmli (1970), and gels stained with Coomassie R-250 dye. Protein concentrations were determined either by Coomassie dye binding (Bio-Rad Laboratories) or bicinchoninic acid (Pierce Chemical Co., Rockford, IL) assays using BSA as the standard. The Coomassie dye binding assay standards were normalized against the bicinchoninic acid assay standards using microsomal proteins from *S. pombe*.

Table I. Sugar Specificity of the Transferase Reaction

α-Methyl D-mannoside	[1.00]
D-Mannose	0.25
L-Mannose	0.00
Galactose	0.00
Glucose	0.00
Sucrose	0.00
6-Deoxy-L-mannose	0.00
D-Mannosamine	0.00
N-Acetyl-D-mannosamine	0.00

Reaction mixtures (50 μl) containing 25 μg of microsomal protein, 25 nmol UDP-[^{3}H]Gal (240,000 dpm), 50 nmol $MnCl_2$, and 5 μmol of the indicated sugar acceptor were incubated for 60 min at 37°C. Values are given as fractions of the activity obtained with α-methyl D-mannoside as the acceptor.

Table II. Manganese Dependence of the Transferase Reaction

50 mM Mn^{2+}	0.49
10 mM Mn^{2+}	0.99
1 mM Mn^{2+}	[1.00]
0.1 mM Mn^{2+}	0.48
0 mM Mn^{2+}	0.06
10 mM EDTA	−0.01

Reaction mixtures (50 μl) containing 25 μg of microsomal protein, 25 nmol UDP-[^{3}H]Gal (240,000 dpm) and 5 μmol α-methyl D-mannoside were incubated for 60 min at 37°C. $MnCl_2$ concentration was varied as indicated. Values are given as fractions of the activity obtained with 1 mM $MnCl_2$ present in the reaction mixture.

Results

Galactosyltransferase Assay

Since the elongation of *S. pombe* high mannose core oligosaccharides by the addition of both mannose and galactose requires, at some stage, the addition of galactose to an underlying mannose substrate, we started by looking for an activity capable of transferring tritiated galactose from UDP-Gal to mannose. This allowed for a relatively simple assay, in which radiolabeled nucleotide sugar substrate was removed from nonionic reaction products by washing the reaction mixture through an anion exchange column with water. UDP-Gal was retained quantitatively on the anion exchange resin, and the radiolabel in the flowthrough of the column represented Gal released from the nucleotide sugar by either a hydrolysis or transferase reaction.

Our initial criteria for an enzyme activity being involved in oligosaccharide processing was that it should be Man dependent, present in the membrane pellet of broken cells and absent from the cytosol. Fig. 1 shows that *S. pombe* detergent solubilized microsomes, and not the cytosol, contains an activity capable of generating uncharged galactose from UDP-Gal in the presence of 100 mM mannose. The transfer of galactose from nucleotide sugar was linearly dependent on mannose concentration over the range of 0–100 mM. By fractionation on BioGel P2, the reaction product or products were shown to be equivalent in size to a disaccharide, indicating that the activity represented a transferase reaction rather than simple hydrolysis (data not shown).

The specificity for the sugar acceptor is shown in Table I. Use of α-methyl mannoside as the acceptor resulted in a fourfold increase in enzyme specific activity relative to Man. We found no other monosaccharides that showed significant acceptor activity; in particular, galactose was unable to substitute for mannose in the reaction. Based on these specificities, further work was carried out using α-methyl mannoside as the sugar acceptor.

Based on previous work on mammalian transferases (Schachter et al., 1983), Mn^{2+} was included in the reaction incubations. Table II demonstrates that the *S. pombe* activity is also dependent on Mn^{2+}, with a broad optimum between 1 and 10 mM.

Purification

Based on our initial characterization of the galactosyltransferase activity, it was unclear whether we were looking at a single step in oligosaccharide processing or at multiple en-

Table III. Purification of Galactosyltransferase

Purification step	Total protein (*mg*)	Specific activity (*nmol/min/mg*)	Total activity (*nmol/min*)	Purification (*fold*)	Yield (%)
1. Triton X-100 solubilized microsomes	2,135	6.4	1.37×10^4	[1]	[100]
2. DEAE Fast Flow Sepharose	322	32.7	1.05×10^4	5	77
3. UDP-hexanolamine agarose	6.7	1,060	7.17×10^3	165	52
4. Con A agarose	2.5	2,120	5.22×10^3	330	38
5. Mono Q	0.12	31,600	3.80×10^3	4900	28

Sequentially diluted aliquots of the pooled active fractions from each purification step were incubated with 25 nmol UDP-[^{3}H]Gal (240,000 dpm) and 5 μmol α-methyl mannoside for 60 min at 37°C in a total reaction volume of 50 μl. Incubations that incorporated <20% of the UDP-Gal into product over the time course of the reaction were shown to be in the linear range. Parallel incubations were carried out in the absence of α-methyl mannoside and used to correct for sugar dependence. Incubations were done in triplicate and averaged to obtain the total activity in each pooled fraction.

zymes that generated a variety of Gal-Man linkages. We therefore attempted to fractionate and purify the activity or activities involved. Since UDP-hexanolamine affinity has been an important step in purification of mammalian enzymes that use UDP sugars as substrates (see for example, Burchell and Weatherill, 1981; Schachter et al., 1983), we used this as the first approach to purification of the yeast activity. With Mn^{2+} in the buffer, a portion of the activity from detergent solubilized microsomes bound to UDP-hexanolamine in the absence of NaCl, but slowly leached off the resin when washed. To load and wash the UDP-hexanolamine column in a minimum volume, the microsomal Triton X-100 extract was first loaded onto a DEAE Sepharose column and the activity eluted in a small volume with a NaCl step gradient. This initial concentration and purification increased the proportion of the activity that bound to the UDP-hexanolamine agarose and decreased the wash volume needed to effect a reasonable purification with the affinity column. Even with this preliminary step, ~50% of the galactosyltransferase activity was found in the flow through and wash fractions. After the bound activity was eluted from the UDP-hexanolamine column, the pooled flow through and wash fractions were reapplied. In this second loading, ~35% of the activity was retained on the column after washing. The behavior of the activity in the flow through and wash fractions on repetitive passes through UDP-hexanolamine agarose indicated that we were not fractionating two separate activities by UDP affinity. By the third loading, the remaining activity had been diluted sufficiently such that little was retained on the column after washing. We therefore compromised with a 67% yield from two passes.

By fractionating the eluant from the UDP-hexanolamine column by gradient anion exchange on a FPLC Mono Q column, we were able to obtain a substantially enriched preparation of galactosyltransferase activity, consisting of two major proteins by SDS-PAGE analysis. In preliminary experiments, we had found that the galactosyltransferase activity would bind to either *Bandeiraea simplicifolia* or Con

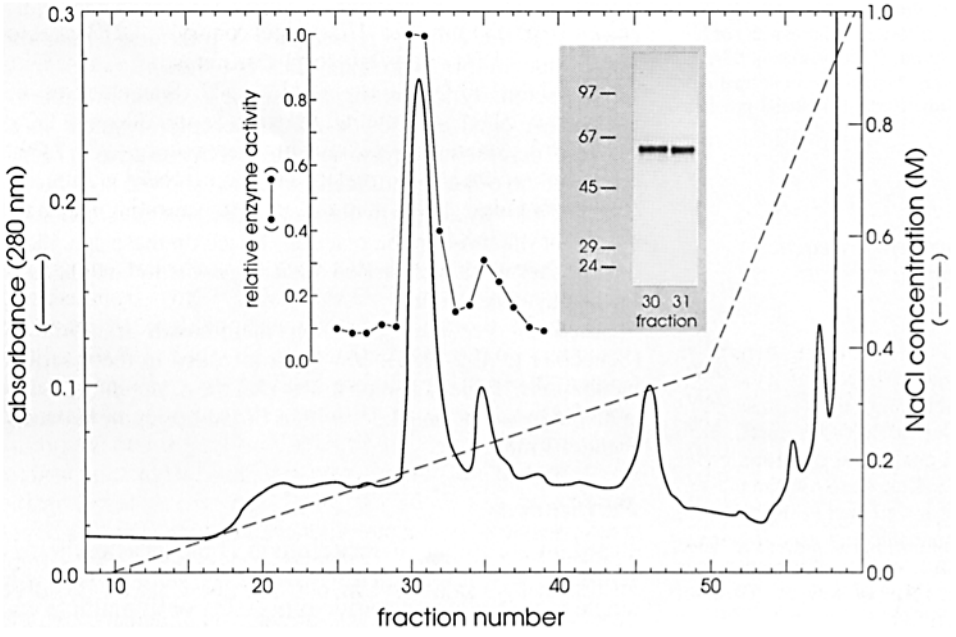

Figure 2. Final step in the purification of the galactosyltransferase–Mono Q fractionation of the eluant from the Con A affinity column. A total of 2.5 mg of protein from the Con A affinity column was loaded onto a 1-ml Mono Q column and eluted with a NaCl gradient at a flow rate of 1 ml/min. Fractions of 0.5 ml were collected. Aliquots of 0.5 μl were assayed for galactosyltransferase activity using the standard conditions. The activities are plotted relative to the activity in fraction 30, and are uncorrected for either Man or protein independent background. The major peak of activity eluted between 175 and 200 mM NaCl. The inset shows the two peak fractions (30 and 31) electrophoresed on a 10% polyacrylamide gel and stained with Coomassie R-250. The migration positions of the reference proteins (molecular weight × 10^{-3}) are indicated: phosphorylase b (97); BSA (67); ovalbumin (45); carbonic anhydrase (29); trypsinogen (24).

A lectin agaroses. This binding indicated that the transferase was itself a glycoprotein modified by the addition of terminal galactose residues. We were able to use the binding to Con A agarose as a final purification step, giving a homogeneous preparation of galactosyltransferase, consisting of a single protein of 61 kD.

Table III outlines the final purification procedure, in which the yield (and final concentration of protein) was increased by performing the Con A agarose affinity step before the Mono Q anion exchange fractionation. All steps in the purification required the presence of detergent to obtain a reasonable recovery; the galactosyltransferase presumably undergoes hydrophobic aggregation in the absence of detergent. Triton X-100 was used for the initial three steps of the purification and then replaced by octyl β-D-thioglucoside during the final step. The latter detergent does not absorb at 280 nm, making it easy to detect the proteins eluted from the Mono Q column.

Fig. 2 shows the 280 nm absorbance and galactosyltransferase activity elution profiles for the Mono Q anion exchange column used in the final step of the purification. There are two peaks of transferase activity, both of which have similar specific activities. The minor peak that elutes at higher salt concentration is slightly lower in molecular weight than the major peak by SDS-PAGE analysis and shows immunoblot cross-reactivity with the major peak (data not shown). Since only the protein of 61 kD is present in immunoblots of freshly prepared *S. pombe* microsomes, the minor peak is probably a proteolytic fragment of the 61-kD protein formed during the purification procedure rather than a distinct transferase activity.

Enzyme Characterization

In the presence of 100 mM α-methyl mannoside, the K_m for UDP-Gal of the purified enzyme was determined to be 1.7 mM (Fig. 3); somewhat higher than the sugar nucleotide

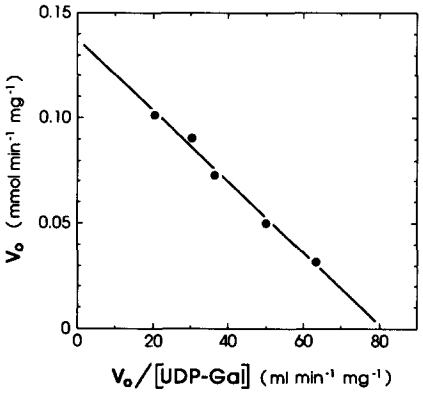

Figure 3. Eadie-Hofstee plot of the initial rate of the transferase reaction versus UDP-Gal concentration. Aliquots of galactosyltransferase were incubated for 60 min at 37°C in the presence of either 0.5, 1, 2, 3, or 5 mM UDP-[^{3}H]Gal (100,000 dpm). Each 50-μl assay contained 3 ng of purified enzyme, a condition under which the assay remained linear over the time course of the incubation. For each data point, parallel assays were run in the presence and absence of 100 mM α-methyl mannoside and used to correct for sugar dependence. A least squares line fit of the five data points gave a slope of −1.67 mM and y intercept of 1.37 × 10^5 nmol min^{-1} mg^{-1} for $-K_m$ and V_{max}, respectively.

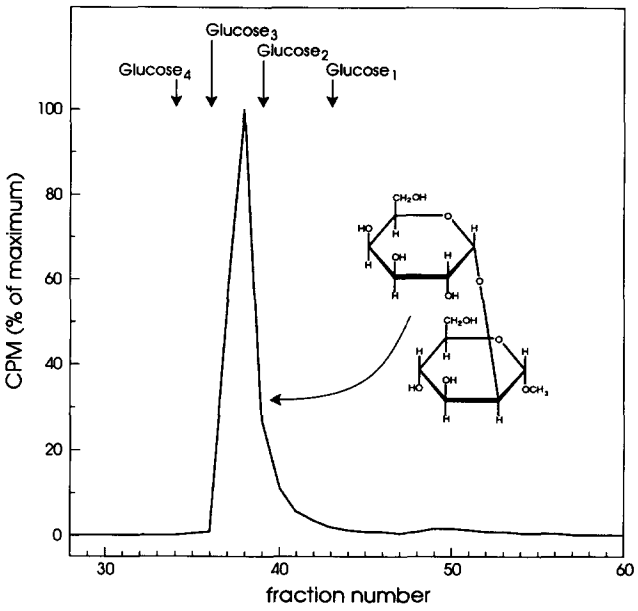

Figure 4. BioGel P2 gel filtration of the product of the galactosyltransferase reaction using α-methyl mannoside as the acceptor. A 500-μl reaction mixture containing 1 μmol of UDP-[^{3}H]Gal (240,000 dpm), 50 μmol α-methyl mannoside and 1 μg of purified galactosyltransferase was incubated at 37°C for 120 min. After passing the reaction mixture over a Dowex anion exchange column to remove unused UDP-[^{3}H]Gal, the reaction product was applied to a precalibrated BioGel P2 gel filtration column and eluted with water at a flow rate of 0.25 ml/min. Fractions of 3 ml were collected, and 100 μl aliquots were counted. The elution profile is shown, plotted as a percentage of the counts in fraction 38. The elution positions of glucose oligomers generated by dextran hydrolysis are indicated, the position of the glucose monomer in the hydrolysate was confirmed with radiolabeled glucose. Fractions 37 and 38 contained ~600 nmol of reaction product as judged by incorporation of [^{3}H]Gal. Fraction 37 was used for both nuclear magnetic resonance and mass spectrometric structural analysis, since it eluted furthest away from the unlabeled α-methyl mannoside present near the Glc monomer. The structure derived for fraction 37 is shown.

K_ms of the mammalian UDP-glucuronosyl-, UDP-galactosyl-, and UDP-*N*-acetylglucosyl-transferases (K_ms ranging from 0.1–1.1 mM) (Falany et al., 1987; Schachter et al., 1983). At 100 mM α-methyl mannoside and 500 μM UDP-Gal, the *S. pombe* enzyme has a turnover number of ~2,000/min, two- to threefold greater than the turnover number for the mammalian galactosyltransferase at saturating sugar nucleotide concentrations.

The purified enzyme was used to synthesize enough reaction product from UDP-Gal and α-methyl mannoside to analyze the product by two-dimensional nuclear magnetic resonance and mass spectrometry. To remove UDP-Gal and α-methyl mannoside before analysis, the reaction mixture was first run through a Dowex anion exchange column and then size fractionated on BioGel P2. Fig. 4 shows the profile of the BioGel P2 column, indicating that the reaction product behaves as a disaccharide. All 13 unique, nonexchangable protons (one or two protons attached to each carbon atom of Gal and Man and three identical protons on the methyl group) of the methyl galactosyl mannoside disaccharide could be resolved and assigned by two-dimensional nuclear magnetic

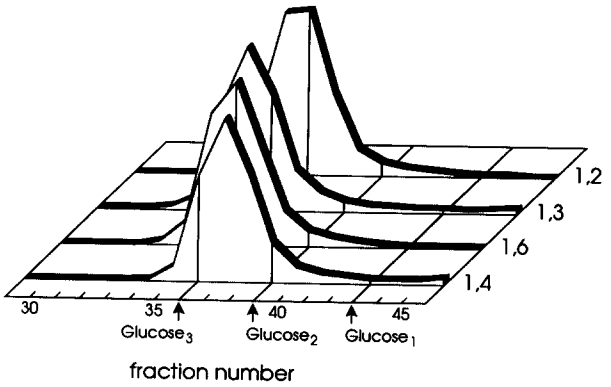

Figure 5. BioGel P2 gel filtration of the products using the four α-methyl α-mannosyl mannosides as acceptors for the transferase reaction. Four 50-μl reaction mixtures, each containing 250 nmol of one of the four possible α-methyl α-mannosyl mannosides (1,2; 1,3; 1,4; and 1,6 α linkages between the two mannose rings), 250 nmol of UDP-[^{3}H]Gal (240,000 dpm) and 50 ng of purified galactosyltransferase were incubated at 37°C for 60 min. Each reaction mixture was analyzed by gel filtration as described for Fig. 4.

resonance. The coupling constant between the protons in positions 1 and 2 of the galactose ring was consistent with an α configuration at carbon-1 of Gal. The chemical shift of the proton at position 2 of the Man ring was suggestive of linkage at this position. This linkage was confirmed by methylation, followed by mass spectrometric analysis. The derived structure of the disaccharide is shown in Fig. 4.

While the enzyme produced only the α 1,2 linked product when run in molar excess of α-methyl mannoside, it was unclear whether it could add Gal at other positions. When the purified disaccharide was incubated with enzyme in the presence of UDP-Gal, we could observe no further addition of Gal, as evidenced by a shift in molecular weight on a BioGel P2 sizing column. The enzyme therefore appears to be specific for the 2 position of Man.

All four possible α-methyl α-mannosyl mannosides (1,2; 1,3; 1,4; and 1,6 α linkages between the two Man rings) act as acceptors to approximately the same extent. When present at 5 mM in an incubation with purified galactosyltransferase, all four behave similarly to α-methyl mannoside at the same concentration. Fig. 5 shows the BioGel P2 column analyses of the reaction products for the four disaccharide acceptors (5 mM acceptor, 5 mM UDP-Gal, reactions run to >50% completion), yielding a single, trisaccharide product in each case. The simplest interpretation of these results is that the enzyme recognizes only the unsubstituted, terminal Man of each disaccharide, adding a single galactose at the 2 position.

Morphology of the Pombe Golgi

Electron Microscopy. Morphological studies of septum formation in *S. pombe* at the electron microscopic level by a number of workers have demonstrated the existence of stacked cisternal Golgi bodies or dictyosomes using either thin section (Johnson et al., 1982) or freeze-etching techniques (Streiblová et al., 1984). From the published micrographs, it appears that the Golgi bodies are present as multiple, discrete structures within the *S. pombe* cell. The exact morphology of the Golgi varies from study to study (possibly in a strain and growth dependent manner), but the maximal cisternal length is consistently in the region of 1 μm.

Fig. 6 (*A* and *B*) shows electron micrographs of longitudinal and transverse thin sections through cells of the *S. pombe* strain used in this work. The cells contain several structures that one would interpret morphologically as Golgi. Potassium permanganate fixation resulted in the best contrast between membranes and cytoplasmic content, although similar Golgi morphology was observed with the more common glutaraldehyde/osmium tetroxide fixation techniques. The Golgi shown at higher magnification in Fig. 6 *C* is typical of those seen in our electron micrographs in that it contains a single distended cisterna, a feature common to many, but not all, of the Golgi profiles observed. This distended cisterna appears to be independent of fixation technique, in that it is observed with both Epon embedding and in frozen thin sections after aldehyde fixation (L. Page, unpublished results). Consistent with the impression given in previous published work, serial sections of the *S. pombe* strain used in this study showed multiple, discrete Golgi structures within a single cell (*data not shown*).

Immunofluorescence Localization

Polyclonal rabbit antiserum raised against the purified galactosyltransferase gave strong cell surface labelling and recognized a large number of proteins in microsomal fractions when used for immunoblotting. Even after affinity purification against galactosyltransferase, the antibodies still recognized the cell surface and other microsomal proteins. Given the strong antigenic nature of the oligosaccharides on *S. cerevisiae* glycoproteins, we assumed that an immune response had been elicited against the carbohydrate portion of the *S. pombe* galactosyltransferase, giving rise to cross-reactivity with other glycosylated proteins. By preadsorbing the antiserum against the cell surface and then affinity purifying against the 61-kD gel band of galactosyltransferase, antibodies that recognized only the 61-kD band by immunoblotting could be generated.

The affinity-purified antibodies were used to localize the galactosyltransferase in *S. pombe* cells by immunofluorescence. The antibodies recognized discrete, multiple structures in the cytoplasm of each cell. At the magnification required to observe the stained structures by conventional immunofluorescence microscopy, the focal plane was too shallow to allow all the structures to be visualized without focussing through the cell. We therefore used laser scanning confocal microscopy to perform optical sectioning. Fig. 7 *A* is a confocal section through a cluster of cells. Individual cells, 10–15 μm in length, can be seen to contain multiple organelles stained with the antigalactosyltransferase antibodies. There appears to be no orientation or localization of the organelles to specific regions of the cytoplasm, either in relationship to each other, or to the nucleus. Fig. 7 *B* is a series of nine optical sections, taken at 0.2-μm steps, showing the stained structures within a single cell. The cell centered in each frame contains at least three and possibly four organelles that appear and then disappear in the sections. By performing the optical sectioning, one can see that the structures are indeed discrete, rather than interconnected.

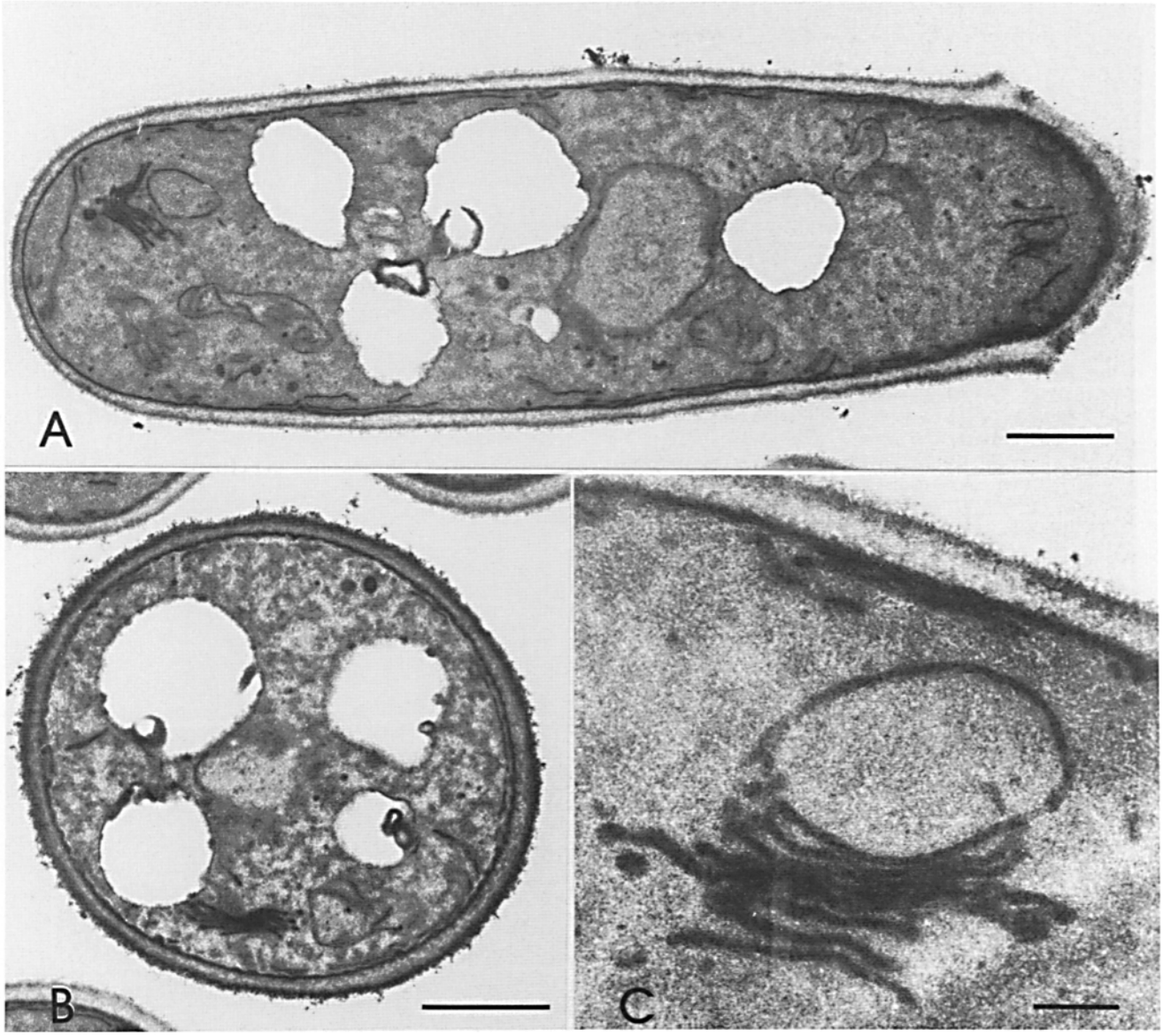

Figure 6. Electron micrographs of *S. pombe* Golgi. *S. pombe* cells were fixed with potassium permanganate and embedded in epoxy resin as described in Materials and Methods. The micrographs show Golgi profiles at several different magnifications. The cell in *A* contains two organelles with distinct Golgi morphology, and the cell in *B* a single convincing profile. Bars, (*A* and *B*) 1 μm; (*C*) 250 nm.

Discussion

A galactosyltransferase activity from *S. pombe* microsomes has been characterized and purified to homogeneity. The enzyme is capable of transferring galactose from UDP-Gal to a variety of Man acceptors, generating an α linkage at the 2 position of the Man ring. This specificity is consistent with the α-galactose linkage present on the N-linked oligosaccharides of the secreted *S. pombe* protein, acid phosphatase. We would therefore propose that the purified enzyme is involved in the processing of N-linked oligosaccharides in the intracellular transport pathway of fission yeast.

Is the purified galactosyltransferase kinetically capable of adding the galactose found on the cell surface of *S. pombe*? The N-linked oligosaccharides of acid phosphatase contribute to the bulk cell surface galactomannan, which accounts for 10–15% of the total cell wall carbohydrate and contains all the cell surface galactose (Bush et al., 1974). In derepressed cells, acid phosphatase is present at ~4 × 10^6 molecules/cell (calculated from Dibenedetto and Cozzani, 1975) and, as each molecule contains several hundred galactose units, accounts for a major portion of the galactomannan, which can be estimated to contain 10^9 molecules of galactose/cell.[2] We purified (with a yield of 28%) 120 μg of galactosyltransferase from 100 g of cell paste. Given that a gram of cell paste contains 5 × 10^9 cells, this corresponds to an initial concentration of ~5,000, 61-kD galactosyltransferase monomers/cell. To resynthesize the 10^9 molecules of Gal on the surface of each cell during a 100-min cell cycle,

2. The analysis of the *S. pombe* cell wall carbohydrate by Bush et al. (1974) found that it contained 3–4% galactose. If 5% of the wet cell mass is cell wall, each cell will contain 10 pg of cell surface carbohydrate. This corresponds to 10^9 molecules of galactose on the cell surface.

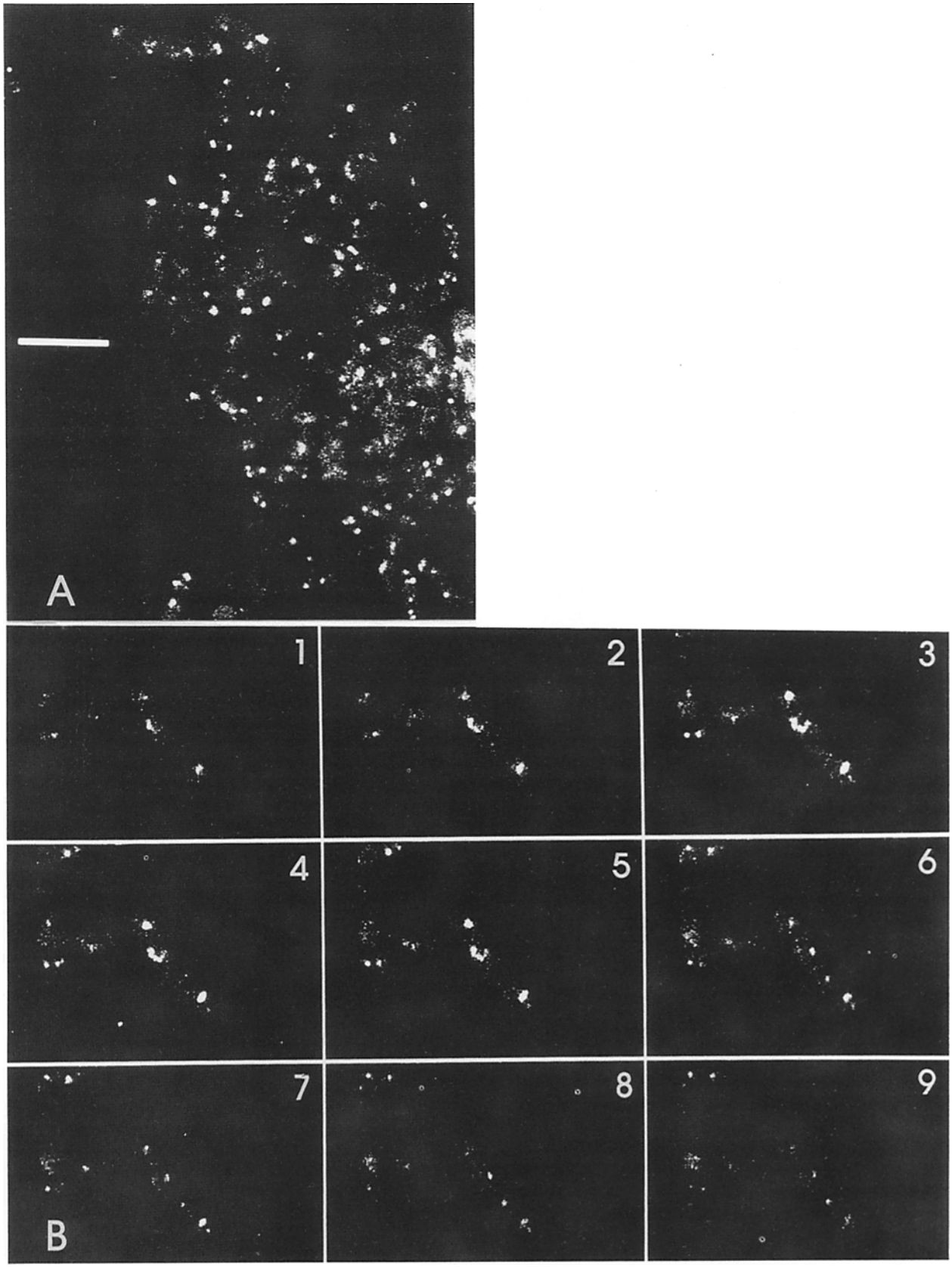

Figure 7. Indirect immunofluorescence localization of the galactosyltransferase in *S. pombe* cells. *S. pombe* cells were fixed with paraformaldehyde and processed for immunofluorescence staining as described in Materials and Methods. The cells were stained with affinity-purified antigalactosyltransferase, followed by fluorescein-conjugated anti-rabbit IgG. *A* is a confocal optical section through a cluster of cells, showing a representative sample of the shapes and sizes of intracellular structures that were stained with the antibody. The outlines of individual cells are discernable near the edges of the cluster. Centered in each frame of *B* is a single cell ~12 μm in length. The nine frames show a series of optical sections taken at 200-nm steps through the cell, and demonstrate that the stained organelles are discrete, rather than interconnected. Bar, 10 μm.

a transferase activity of 10^7 events min^{-1} $cell^{-1}$ is required. This corresponds to a turnover number of 2,000 for each molecule of the purified galactosyltransferase. The kinetics of the enzyme demonstrated in Fig. 3 show that, given a Man substrate concentration of 100 mM, this turnover number is achieved at UDP-Gal concentrations well below the K_m for the enzyme. A rod-shaped *S. pombe* cell, 15 μm in length and 2.5 μm in diameter, with a cell wall thickness of 200 nm (see Fig. 6), will have a total cell wall volume of 20 μm^3. The 10^9 molecules of Gal present in the cell wall would constitute a concentration of ~100 mM. If we assume that the substrate is present internally at a concentration equal to or greater than that of the product at the cell surface, the activity we have purified is capable of accounting for all the cell surface Gal of *S. pombe*.

Morphologically, direct demonstration of the intracellular localization of the galactosyltransferase of *S. pombe* can only be achieved by immunolabeling at the electron microscopic level. While we have been able to demonstrate specific intracellular binding of *B. simplicifolia* lectin I to frozen thin sections of *S. pombe* (L. Page, unpublished results), we have been unable to localize the galactosyltransferase by EM with our present antiserum, because it is unclear whether it will react with glutaraldehyde fixed samples. Several lines of evidence suggest, however, that the enzyme is, in fact, localized in the *S. pombe* Golgi apparatus. First, the immunofluorescence localization of the purified galactosyltransferase shows the enzyme to be present in discrete organelles within the cytoplasm of fission yeast. These organelles are consistent in size and number with the stacked, cisternal Golgi seen in electron micrographs. The single distended cisterna seen in the electron micrographs is visible if one focuses through the immunofluorescence samples, but cannot quite be resolved with confocal imaging. Second, since >80% of the galactose is attached to mannose in *S. pombe* galactomannan (Bush et al., 1974), the specificity and kinetics of the purified enzyme are adequate to account for the addition of galactose to the N-linked oligosaccharides that account for some or all of this galactomannan. By analogy with the known posttranslational processing pathways in mammalian cells and *S. cerevisiae*, we can presume that this addition occurs in the Golgi apparatus of *S. pombe*. Third, while we have not yet been able to isolate unprocessed precursors from *S. pombe* to demonstrate directly N-linked processing by the galactosyltransferase, the purified enzyme adds galactose to the N-linked $Man_5GlcNAc_2$ core structure present on mammalian glycoproteins, and thus is capable of processing N-linked oligosaccharides from a divergent source (data not shown). Direct demonstration of the involvement of the enzyme in N-linked oligosaccharide processing awaits the deletion of the gene from *S. pombe*; fortunately, fission yeast is a system in which such an experiment can easily be done.

It is tempting to speculate from the distinct stacked structure of the Golgi in *S. pombe* that it contains biochemically distinct compartments similar to those found in mammalian Golgi. While we have no direct evidence that the galactosyltransferase is localized to a discrete compartment within the Golgi, the ability of the enzyme to add Gal to Man irrespective of the underlying structure and the percentage of Gal present as terminal units on cell surface carbohydrates suggests that the transferase acts late in the secretory pathway, "decorating" a substantial Man structure that has already been synthesized. This is further reinforced by the presence of a mannosyltransferase activity in *S. pombe* microsomes that shows similar substrate specificity (data not shown), suggesting that the two activities will be present in different compartments. If so, *S. pombe* may provide a system in which to dissect the signals involved in the localization of processing enzymes along the secretory pathway.

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