PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Genetic and Biochemical Studies of Asparagine-Linked Oligosaccharide Assembly [and Discussion]

M. D. Snider, T. C. Huffaker, J. R. Couto, P. W. Robbins and T. Feizi

Phil. Trans. R. Soc. Lond. B 1982 **300**, 207-223
doi: 10.1098/rstb.1982.0167

Phil. Trans. R. Soc. Lond. B **300**, 207–223 (1982)
Printed in Great Britain

Genetic and biochemical studies of asparagine-linked oligosaccharide assembly

By M. D. Snider, T. C. Huffaker, J. R. Couto and P. W. Robbins

Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, *U.S.A.*

The formation of *N*-glycosidic linkages of eukaryotic glycoproteins involves the assembly of a specific lipid-linked precursor oligosaccharide in the endoplasmic reticulum. This oligosaccharide is transferred from the lipid carrier to appropriate asparagine residues during protein synthesis. The protein-linked oligosaccharide then undergoes processing reactions that include both removal and addition of carbohydrate residues.

In this paper we report recent studies from our laboratory on the synthesis of asparagine-linked oligosaccharides. In the first part we describe the isolation and characterization of temperature-sensitive mutants of yeast blocked at specific stages in the assembly of the lipid-linked oligosaccharide. In addition, we are using these mutants to clone the genes for the enzymes in this pathway by complementation of the temperature-sensitive phenotype. The second part deals with the topography of asparagine-linked oligosaccharide assembly. Our studies on the transmembrane movement of sugar residues during the assembly of secreted glycoproteins from cytoplasmic precursors are presented. Finally, experiments on the control of protein-linked oligosaccharide processing are described. Recent data are presented on the problem of how specific oligosaccharides are assembled from the common precursors at individual sites on glycoproteins.

Introduction

The asparagine-linked oligosaccharides of eukaryotic glycoproteins are assembled in two distinct stages (reviewed in Hubbard & Ivatt 1981; Struck & Lennarz 1980). The first step involves the assembly of a large lipid-linked precursor oligosaccharide containing *N*-acetylglucosamine (GlcNAc), mannose (Man) and glucose (Glc) (figure 1). This species then acts as the donor in the transfer of the oligosaccharide from the lipid carrier to nascent polypeptide chains. In the second stage, the initial common protein-linked oligosaccharide is extensively modified to yield the diverse array of final products.

The precursor oligosaccharide-lipid is assembled in the membrane of the endoplasmic reticulum (e.r.) from the sugar nucleotides UDP-GlcNAc, GDP-Man, and UDP-Glc (figure 2). The first sugar is added as GlcNAc-P, to the polyisoprenoid lipid, dolichyl phosphate (Dol-P), with the formation of the pyrophosphate lipid–sugar linkage. Then six more sugars are added direct from the sugar nucleotides to yield the key intermediate $Man_5GlcNAc_2$-PP-Dol. The seven outer sugars are added via the phosphate-linked intermediates, Dol-P-Man and Dol-P-Glc. These two compounds are made from Dol-P and the respective sugar nucleotides (figure 2). Upon completion, the $Glc_3Man_9GlcNAc_2$ oligosaccharide can be transferred to growing polypeptide chains in the membrane of the e.r.

The second stage of asparagine-linked oligosaccharide synthesis begins with the removal of

the three glucose residues from the newly made glycopeptide in the e.r. Then, in the Golgi apparatus, Man residues are removed, and terminal sugars, phosphate and sulphate residues are added to yield mature structures. While the reactions of the first stage of the glycosylation pathway appear to be shared by nearly all eukaryotes, the reactions in the second stage are specific for individual species, cell types and glycoproteins. These reactions are responsible for the synthesis of the diverse array of final products from a single common precursor.

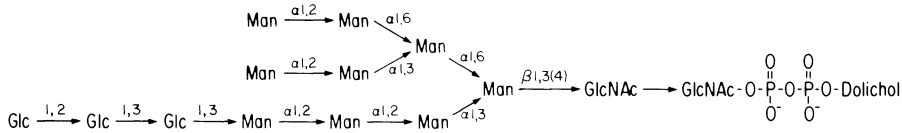


FIGURE 1. Structure of the dolichol-linked precursor oligosaccharide-lipid.

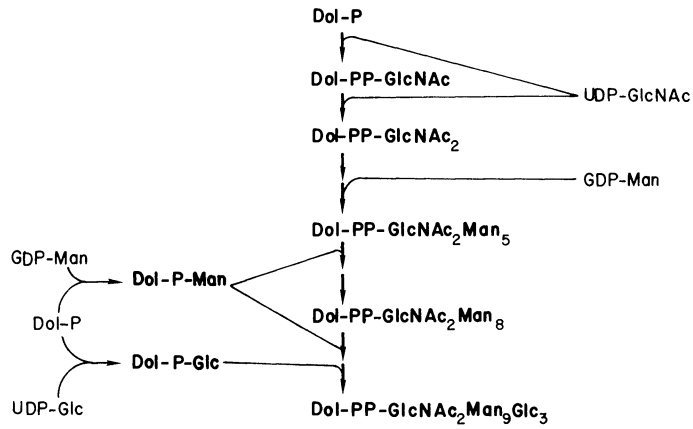


FIGURE 2. Pathway of oligosaccharide-lipid synthesis.

In this paper, we report recent studies from our laboratory on the synthesis of asparagine-linked oligosaccharides. The first section deals with the isolation of mutants in yeast that are defective in lipid-linked oligosaccharide assembly and the use of these mutants to clone the affected genes. The second section examines the topography of asparagine-linked oligosaccharide assembly and the transmembrane movement of sugar residues during glycoprotein synthesis. Finally, experiments on the control of the second stage of glycosylation are described.

YEAST MUTANTS DEFICIENT IN PROTEIN GLYCOSYLATION

We believe that the characterization of mutants will be useful in unravelling the pathway of asparagine-linked glycosylation and have been working with the yeast *Saccharomyces cerevisiae*. Although recent studies with mammalian cell mutants have made valuable contributions to the understanding of protein glycosylation (Chapman *et al.* 1979; Hunt 1980), the relative ease of obtaining mutants in yeast has allowed us to use a genetic and biochemical approach that has been less feasible in animal cell systems. Like higher eukaryotic cells, yeast cells make lipid-linked $Glc_3Man_9GlcNAc_2$ (Trimble *et al.* 1980; Lehle 1980), which is transferred *en bloc* to protein and then modified by excision of the glucose residues (Parodi 1979; Lehle 1980).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Whereas further processing in animal cells can involve the removal of up to six mannose residues and the addition of sugars such as *N*-acetylglucosamine, galactose, fucose and sialic acid, yeast cells add mannose residues and produce oligosaccharides ranging in size from 13 mannose residues, in carboxypeptidase Y (Trimble & Maley 1977; Hasilik & Tanner 1978), to over 50, in invertase (Tarentino *et al.* 1974; Lehle *et al.* 1979). The addition of these outer mannose residues has been studied extensively by Ballou and coworkers, and mutants that fail to synthesize the outer chain, the *mnn* mutants, have been isolated (Ballou 1980).

We have been interested in obtaining mutants affected in the early steps of the glycosylation pathway, which yeast cells have in common with higher eukaryotic cells. These include mutants blocked in the synthesis of the lipid-linked oligosaccharide precursor, its co-translational transfer to protein, and the first steps of post-translational processing. A commonly used method for obtaining protein glycosylation mutants in yeast and animal cells is the selection of cells unable to bind either lectins or antibodies that recognize cell–surface components (Stanley 1980; Ballou 1980). Although this approach has produced many mutants with defects in the late stages of oligosaccharide processing, only a few have been obtained that are affected in the earlier steps of protein glycosylation. This suggests that many of these early steps may be necessary for cell viability. We have therefore developed procedures for isolating mutants in yeast that contain temperature-sensitive defects in asparagine-linked glycosylation. A [^{3}H]mannose suicide selection was used to enrich for these mutants. The surviving cells were screened at the non-permissive temperature for a decreased ability to incorporate [^{3}H]mannose and for defects in glycosylation of the secreted protein invertase. These methods have allowed us to identify a number of mutants that are blocked at various stages in the assembly of the lipid-linked oligosaccharide precursor. In addition we are cloning the genes for the enzymes in this pathway by complementation of the temperature-sensitive mutant phenotype.

[^{3}H]*Mannose suicide selection*

To obtain mutants in yeast that are temperature-sensitive for the ability to glycosylate proteins, we employed a [^{3}H]mannose suicide selection. It was predicted that cells blocked in protein glycosylation would incorporate less mannose than wild-type cells and would therefore be less likely to sustain lethal radiation damage. To ensure that the selection was specific for mannose incorporation, mannose tritiated in the 2-position was used. Catabolism of [2-^{3}H]-mannose results in removal of the label as tritiated water when mannose phosphate isomerase converts mannose 6-phosphate to fructose 6-phosphate.

The suicide selection involved labelling ethyl methanesulphonate mutagenized yeast cells with [^{3}H]mannose for 30 min at the non-permissive temperature. Washed cells were frozen in 25% glycerol and stored at -80 °C to allow accumulation of radiation damage. Periodically, aliquots were thawed and analysed for cell survival. Figure 3 shows the time course of cell killing for a culture labelled with a radioactive count of approximately 50 min^{-1} per cell of [^{3}H]mannose. During the first 27 days, the rate of cell killing followed first-order kinetics. After 35 days, the survival was about 0.2%.

Screening for mutants

To screen for mutants blocked in protein glycosylation, colony fluorography was used to identify colonies that failed to incorporate wild-type levels of [^{3}H]mannose after 30 min at the non-permissive temperature. Since any temperature-sensitive defect that lowers the rate

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

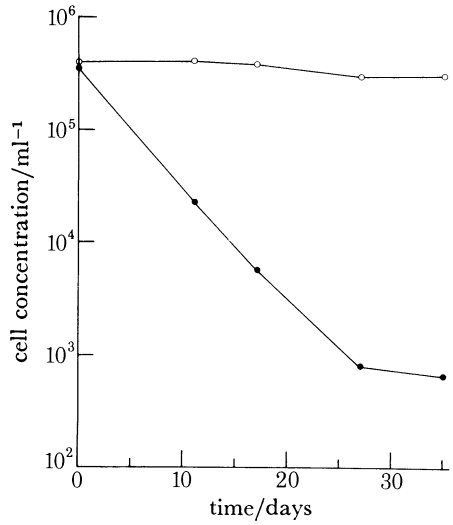


FIGURE 3. Cell survival during [^{3}H]mannose suicide selection. Mutagenized yeast cells were labelled with [^{3}H]-mannose at the non-permissive temperature and stored at − 80 °C (Huffaker & Robbins 1982). The number of viable cells per millilitre were determined during the course of the suicide by scoring for growth on plates at the permissive temperature. ●, [^{3}H]Mannose-labelled cells; ○, unlabelled cells included as a control.

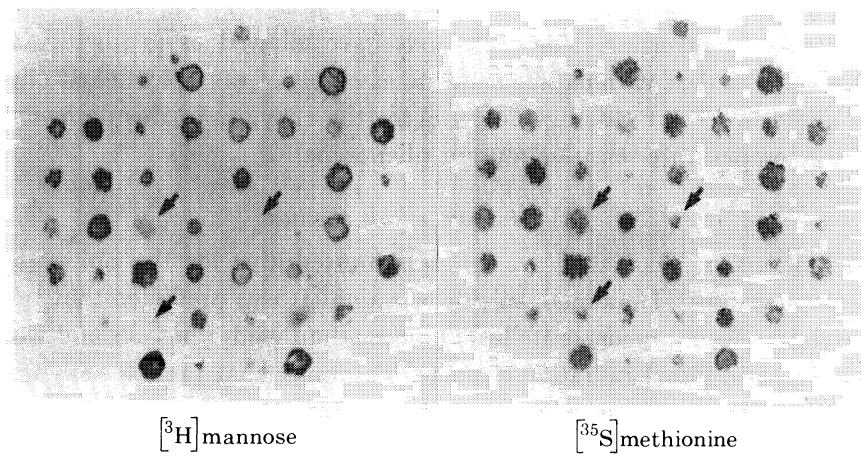


FIGURE 4. Colony screen for incorporation of [^{3}H]mannose and [^{35}S]methionine. Colonies were replica-plated onto two filter papers and assayed for their ability to incorporate [^{3}H]mannose and [^{35}S]methionine at the non-permissive temperature (Huffaker & Robbins 1982). Fluorograms of [^{3}H]mannose and [^{35}S]methionine-labelled replicas from a single plate are shown. Colonies that incorporated [^{35}S]methionine but failed to incorporate [^{3}H]mannose are indicated by arrows.

of protein synthesis could indirectly affect the rate of protein glycosylation, colonies were also screened for their ability to incorporate [^{35}S]methionine. Cells that survived 35 days of the [^{3}H]mannose suicide were grown on plates and the colonies were replica-plated onto two filter papers. One filter was incubated with [^{3}H]mannose and the other with [^{35}S]methionine in medium at the non-permissive temperature. After fixing the cells with glutaraldehyde, the filters were rinsed and impregnated with 2,5-diphenyloxazole. Fluorography of the filters was used to determine the amount of incorporation of the labelled compounds (figure 4). Approximately 5–10 % of the colonies screened incorporated substantially decreased levels of [^{3}H]-

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

mannose, while [^{35}S]methionine incorporation was nearly normal. These were chosen as the most likely to be mutants with glycosylation defects.

Cells that incorporated low amounts of [^{3}H]mannose in the colony screen were next assayed for their ability to glycosylate the secreted form of invertase. The synthesis of secreted invertase is repressed when cells are grown in the presence of 2 % glucose, but is derepressed when cells are grown in low-glucose medium (Perlman & Halvorson 1981). Cultures of mutants identified by the colony screen were derepressed for invertase production at the non-permissive temperature. Cell extracts were prepared and run on polyacrylamide gels. After the gels had been stained for invertase activity, the extent of invertase glycosylation was estimated by observing its electrophoretic mobility (figure 5). Wild-type cells (lane *o*) synthesized secreted invertase, which migrated as a broad band at the top of the gel. The non-glycosylated cytoplasmic form

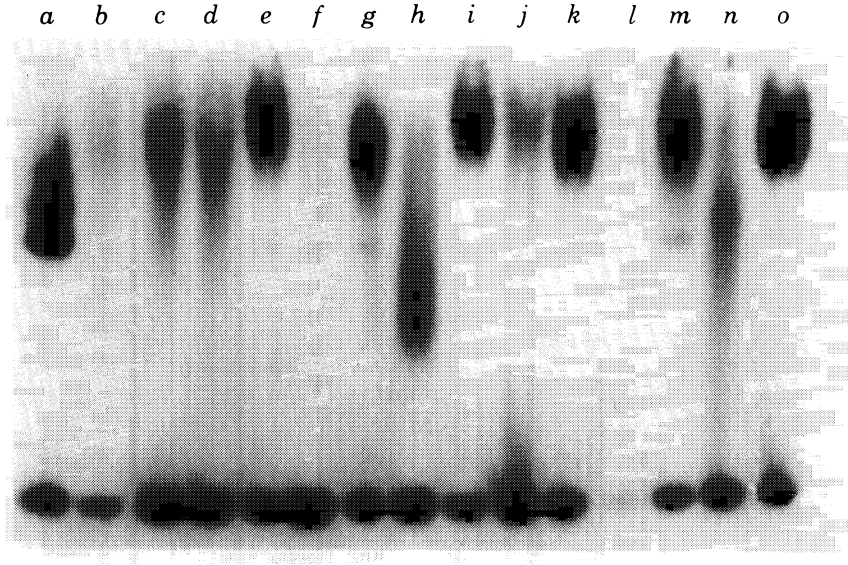


FIGURE 5. Assay for glycosylation of invertase. Cultures were derepressed for invertase synthesis at the non-permissive temperature. Cell extracts were run on a polyacrylamide gel and the gel was stained for invertase activity (Huffaker & Robbins 1982). Lane *o*, wild type; lanes *a*–*n*, mutants identified by the colony screen.

of invertase ran near the bottom (Meyer & Matile 1974). The mutants can be divided into three classes. Some produced normal amounts of fully glycosylated invertase (lanes *e*, *g*, *i*, *k* and *m*). However, a few synthesized much less of the fully glycosylated invertase than wild-type cells (lanes *b*, *f*, *j* and *l*), and others appeared to make incompletely glycosylated invertase based on the altered mobilities observed (lanes *a*, *c*, *d*, *h* and *n*).

Mutants blocked in the synthesis of the precursor lipid-linked oligosaccharide

Mutants that failed to glycosylate invertase normally are likely to contain general protein glycosylation defects. To determine whether any of these mutants are affected in the synthesis of the precursor lipid-linked oligosaccharide, cultures were labelled with [^{3}H]mannose at the non-permissive temperature for 10 min. The lipid-linked oligosaccharides were extracted and subjected to mild acid hydrolysis. The released oligosaccharides were then analysed by gel

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

filtration chromatography on Bio-Gel P-4 (minus 400 mesh). In wild-type cells $Glc_3Man_9GlcNAc_2$ was the major lipid-linked oligosaccharide labelled, with smaller amounts of $Man_8GlcNAc_2$, $Man_7GlcNAc_2$ and $Man_5GlcNAc_2$ (figure 6*a*). Of the 50 mutants examined, 22 failed to synthesize detectable amounts of $Glc_3Man_9GlcNAc_2$ or accumulated other intermediates in the synthesis of the lipid-linked precursor, or both. Figure 6 shows gel filtration profiles of representative mutants that accumulate as major peaks $Man_9GlcNAc_2$, $Man_8GlcNAc_2$,

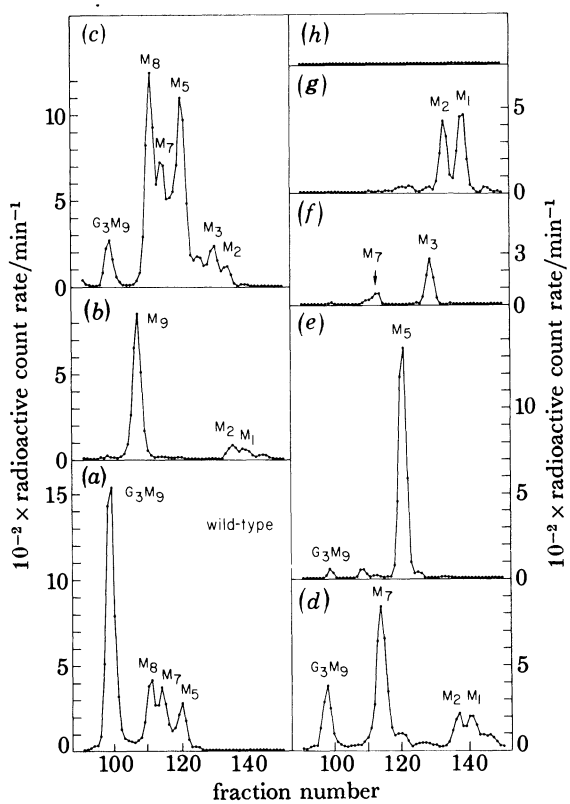


FIGURE 6. Gel filtration chromatography of lipid-linked oligosaccharides labelled *in vivo*. Wild-type (*a*) and mutant cells (*b–h*) were labelled with [^{3}H]mannose at the non-permissive temperature and the lipid-linked oligosaccharides were extracted, hydrolysed and analysed by gel filtration on Bio-Gel P4 (minus 400 mesh) (Huffaker & Robbins 1982). G, Glc; M, Man. All species contain two GlcNAc residues at the reducing end.

$Man_7GlcNAc_2$, $Man_5GlcNAc_2$, $Man_3GlcNAc_2$ or $Man_2GlcNAc_2$. One mutant fails to incorporate any detectable amount of [^{3}H]mannose into lipid-linked oligosaccharides (figure 6*h*). This mutant (*alg1-1*) has been shown to be blocked in the addition of the first mannose residue to the oligosaccharide-lipid (Huffaker & Robbins 1982).

Complementation analysis can be readily performed in yeast by mating two haploid mutant cells and examining the phenotype of the resultant diploid. For those mutations that are lethal at the non-permissive temperature, complementation analysis has been done by assaying diploids for temperature-sensitive growth. We have found one complementation group that contains nine members and includes mutants that accumulate predominantly $Man_8GlcNAc_2$ and the one mutant that accumulates $Man_7GlcNAc_2$. A second complementation group contains three members, each of which accumulates $Man_2GlcNAc_2$ and $Man_1GlcNAc_2$. For those

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

mutations that are not temperature-sensitive lethal, complementation analysis is being performed by examining the lipid-linked oligosaccharides synthesized by the relevant diploids at the non-permissive temperature.

The [^{3}H]mannose suicide and the screens described have proved to be useful in isolating mutants blocked in lipid-linked oligosaccharide assembly, and it seems reasonable to expect that this pathway can be saturated with mutations. Approximately half of the mutants that fail to glycosylate invertase normally synthesize the complete lipid-linked oligosaccharide $Glc_3Man_9GlcNAc_2$. Some of these mutants may contain a defective oligosaccharide transferase, the enzyme that transfers the precursor oligosaccharide from lipid to protein. Alternatively, these mutants may be blocked in processing the protein-linked oligosaccharide, preventing its elongation by the enzymes that add the mannose residues of the outer chain. While we have not yet identified mutants of this type, procedures similar to those used here should allow their isolation in the future.

Cloning of genes involved in oligosaccharide synthesis

We are currently attempting to isolate the genes in the asparagine-linked glycosylation pathway. Our experimental approach is as follows. (1) Isolation and characterization of temperature-sensitive lethal mutations in different steps of the pathway. This has been described in detail

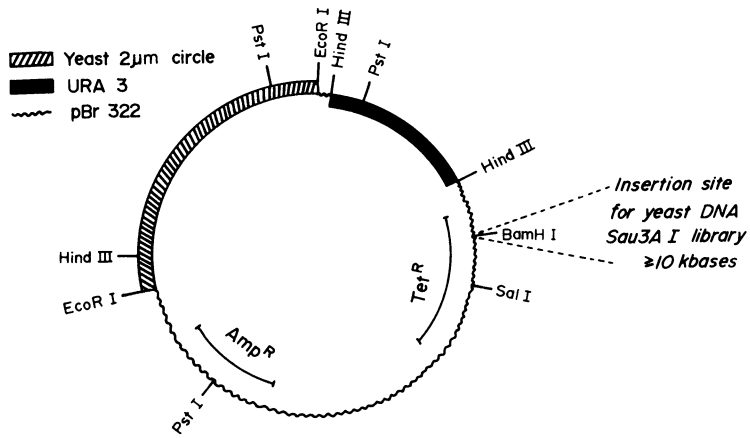


FIGURE 7. YEp24 (Botstein *et al.* 1979) transforms both yeast and *E. coli*. It contains part of the 2 μ yeast plasmid with its origin of replication, the yeast URA3 gene, and pBR322. It complements both the *ura3* mutation in yeast and the *pyr* mutation in *E. coli*, and contains the *Amp*^{r} and *Tet*^{r} markers for drug selection in *E. coli*. It has two unique restriction sites, *Bam*HI and *Sal*I. The *Bam*HI site was used for the insertion of *Sau*3AI partial restriction digests for the construction of the yeast library (Carlson & Botstein 1982).

above. (2) Transformation of these mutants with libraries (Maniatis *et al.* 1978) of wild-type yeast DNA. A library is a collection of plasmids each containing a different fragment of DNA randomly excised from a whole genome. Thus a library should in principle contain all genes of a given strain represented. (3) Selection of transformants in which the temperature-sensitivity has been complemented by a fragment of the DNA library. It is then reasonable to conclude that such transformants acquired a plasmid containing the gene in question, and that its expression is now enabling the transformants to grow at the non-permissive temperature. (4) Subcloning and characterization of the gene. Using this approach we have isolated a plasmid, pJC1, which complements the *alg1-1* mutation (Huffaker & Robbins 1982).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The library we used was constructed by Carlson & Botstein (1982) and consists of YEp24 plasmids (Botstein *et al.* 1979) into which random fragments of strain DBY939 yeast genomic DNA of size 10 kilobases or larger were inserted. YEp24 (figure 7) transforms both yeast and *Escherichia coli*, replicates episomally, and allows for the expression of any gene in the insert. It contains the complete yeast *URA3* gene, which is used for the selection of transformants in yeast *ura3*$^-$ strains. *E. coli* transformants can be selected by using the *tet*^{r} and *amp*^{r} drug resistance markers or alternatively by complementing the *pyrF* mutation with the *URA3* gene.

TABLE 1. PLASMID SEGREGATION IN YEAST TRANSFORMANTS

	platings from Alg1 selective growth (36 °C, +U)		platings from non-selective growth (26 °C, +U)	
	growth ratio in plates with U +U, 36 °C/26 °C	growth ratio in plates at 26 °C 26 °C, $^-$U/+U	growth ratio in plates with U +U, 36 °C/26 °C	growth ratio in plates at 26 °C 26 °C, $^-$U/+U
1	0.61	0.56	0.05	0.05
2	0.61	0.59	0.06	0.05
3	0.58	0.52	0.08	0.09
4	0.81	0.71	0.36	0.21
5	0.79	0.72	0.40	0.40
alg$^+$ *ura*$^-$	—	0.00	—	—

Transformants were grown for 2 days in complete liquid medium either with selective pressure for Alg1 (36 °C) but not for Ura3, or non-selectively for both markers. They were then plated either at 26 °C or at 36 °C, with or without uracil. The ratios from these platings were calculated after averaging from three plates in each particular case. The last line in the table shows the results of a control experiment in which the strain *ALG1*$^+$*ura3*$^+$, transformed with YEp24, was grown non-selectively in complete medium at 36 °C.

We prepared library plasmid DNA and used it to transform the yeast double mutant *alg1-1*, *ura3-52*. Selection of Alg1$^+$Ura3$^+$-complemented transformants was done in two stages. In the first stage we recovered yeast transformants growing in medium without uracil at 26 °C, the permissive temperature for *alg1-1*. In the second stage the Ura3$^+$ transformants were screened for growth at 36 °C, the non-permissive temperature for *alg1-1*. The transformants that could grow at 36 °C in medium lacking uracil were then subjected to plasmid segregation experiments to eliminate the possibility that either the *ura3-52* or the *alg1-1* mutation had reverted, and to show that both mutations were being complemented by the same plasmid. We showed that when we removed the selective pressure for both markers, by growing at 26 °C in complete medium, the transformants lost both the Alg1$^+$ and the Ura3$^+$ phenotypes (table 1). However, when we maintained selective pressure on Alg1 but not on Ura3, by growing at 36 °C in complete medium, the transformants retained both phenotypes (table 1). We therefore concluded that the yeast transformants contained a plasmid capable of complementing the *alg1-1* and the *ura3-52* mutations simultaneously.

We transformed *E. coli* HB101 to ampicillin resistance with a total DNA extract of yeast transformants. We then isolated the plasmid pJC1. Restriction analysis showed that pJC1 was YEp24 ligated to an 11 kilobase insert at the *Bam*HI site. This plasmid could transform the yeast double mutant *alg1-1ura3-52* and complement both mutations.

We are now subcloning the yeast DNA insert in pJC1 and are preparing to clone other *alg* mutations. After we prove that we have a series of genes in the asparagine-linked glycosylation pathway, we plan to study structural features of the gene products, their regulation, synthesis and mode of action. In addition, increased gene copy number should lead to an overproduction of the enzyme and thus will facilitate its isolation and characterization.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Topography of lipid-linked oligosaccharide synthesis

The assembly of the lipid-linked oligosaccharide and its transfer to protein involve the transmembrane movement of sugar residues. Oligosaccharides of newly made glycoproteins are first found segregated within the lumen of the e.r. This segregation within the e.r. is the first step in the secretion process. Because these oligosaccharides are assembled from cytoplasmic sugar nucleotides (Coates *et al.* 1980), it is clear that the synthesis of the oligosaccharide-lipid and its transfer to protein are coupled to the transport of sugar residues across the e.r. membrane. Over the last several years, we have been studying how this transmembrane movement occurs.

Our experiments have used microsomal vesicles *in vitro*. These are sealed vesicles derived from rough and smooth e.r. Microsomes have a unique orientation, with the cytoplasmic side of the membrane facing the medium. Thus non-penetrating reagents will act only on the cytoplasmic side of the membrane. We and others have used this system to examine the transmembrane orientation of oligosaccharide-lipids, and the enzymes responsible for their synthesis.

Studies of enzymes of oligosaccharide–lipid synthesis

We have used proteases as probes to study the orientation of enzymes of oligosaccharide assembly (Snider *et al.* 1980). Microsomes from rat liver were used. Figure 8 shows an experiment in which intact vesicles and vesicles made leaky with a non-ionic detergent were treated with pronase. Enzymes of oligosaccharide-lipid synthesis and the microsomal enzyme glucose-6-phosphatase were then assayed. As previously observed, glucose-6-phosphatase, an enzyme of the microsomal lumen, was not inactivated by pronase treatment of intact vesicles. However, in leaky vesicles, most of this activity was lost. Thus pronase did not penetrate intact microsomal vesicles under our conditions of treatment.

In contrast, all the activities of oligosaccharide-lipid synthesis that we assayed were inactivated by pronase treatment of intact vesicles. Moreover, the protease sensitivity of the enzymes was the same in intact and leaky vesicles, suggesting that the protease-sensitive sites of these enzymes are on the cytoplasmic side of the microsomal membrane.

We found that the synthesis of Dol-P-Man and Dol-P-Glc were largely inactivated by pronase treatment of intact microsomes (figure 8). Activity in the other two assays was less sensitive to pronase. This can be explained by the fact that these latter assays measure groups of reactions; one reaction in each group was not protease sensitive under our conditions of treatment. This was demonstrated by fractionating the products of these reactions. In the assay measuring the incorporation of GlcNAc residues from UDP-GlcNAc into lipid, we showed that the synthesis of $GlcNAc_2$-lipid is protease sensitive in both intact and leaky vesicles. However, GlcNAc-lipid synthesis is not protease sensitive under either condition. Similarly, in the assay measuring the incorporation of Glc residues from Dol-P-Glc into oligosaccharide-lipid, protease treatment of intact microsomes resulted in large decreases in the synthesis of $Glc_3Man_9GlcNAc_2$-lipid and $Glc_2Man_9GlcNAc_2$-lipid, whereas the synthesis of the monoglucosyl species was largely unaffected.

These results show that at least four reactions of oligosaccharide-lipid synthesis have protease-sensitive sites on the cytoplasmic side of the microsomal membrane. In addition, none of the enzymes we examined were protease-resistant in intact vesicles but protease-sensitive in leaky vesicles. Thus no enzymes had protease-sensitive sites exclusively on the luminal side of the membrane. How are these enzymes oriented in the microsomal membrane? These results are

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

consistent with two possible arrangements. The first is that of enzymes with catalytic sites facing the cytoplasm. In the second, transmembrane enzymes have luminal catalytic sites, but cytoplasmic protease-sensitive sites. If this second possibility is correct, it is likely that the cytoplasmic segments of these enzymes play an important role in the function of these enzymes. This argument is based on the finding that five other enzymes of the e.r. and Golgi apparatus that have luminal active sites are protease resistant in intact vesicles, but sensitive in leaky vesicles (DePierre & Dallner 1975; Grinna & Robbins 1979; Carey & Hirschberg 1981; Fleischer 1981). No enzymes showed the behaviour that we found for the enzymes of oligosaccharide-lipid synthesis.

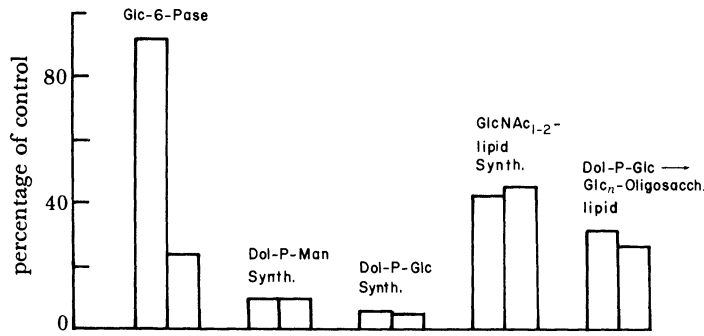


FIGURE 8. Enzymes of oligosaccharide-lipid synthesis have protease-sensitive sites on the cytoplasmic side of the microsomal membrane. Rat liver microsomal vesicles were treated with pronase, and enzymes of oligosaccharide-lipid synthesis and glucose-6-phosphatase were assayed as previously described (Snider *et al.* 1980). Results from treatment of intact vesicles (left-hand bars) and leaky vesicles (right-hand bars) are expressed as a percentage of control samples that were not treated with protease.

Both of these possible arrangements of the synthetic enzymes argue strongly that sugar nucleotides are utilized directly at the cytoplasmic face of the e.r. during oligosaccharide-lipid synthesis. Supporting this conclusion is the recent demonstration by Hanover & Lennarz (1982) that UDP-GlcNAc, GDP-Man and UDP-Glc are not able to penetrate microsomal vesicles *in vitro*. This finding is significant in light of the fact that sugar nucleotide transport *has* been observed in Golgi vesicles by using similar methods (Carey *et al.* 1980).

Orientation of mature oligosaccharide-lipid

Although sugar nucleotides are utilized at the cytoplasmic face of the e.r. membrane, it is clear that sugars must cross this membrane during the assembly of oligosaccharide-lipid. This conclusion is based on our recent demonstration that the largest oligosaccharide-lipid is found on the luminal side of the membrane (Snider & Robbins 1982). These studies used concanavalin A (Con A), a plant lectin that binds tightly to the precursor oligosaccharide-lipid. In order to use this lectin as a probe for the orientation of oligosaccharide-lipid, we developed an assay for the binding of lectin to this compound. The assay is based on the solubility of the Con A–oligosaccharide-lipid complex. Although oligosaccharide-lipid is extracted with chloroform–methanol–water (10:10:3), the Con A–oligosaccharide-lipid complex is not. Thus Con A binding is measured as a loss of extractable oligosaccharide-lipid.

We have examined the binding of Con A to oligosaccharide-lipid in microsomes prepared from [^{3}H]mannose-labelled cultured fibroblasts (figure 9). In intact vesicles, a small fraction of the oligosaccharide-lipid was bound by Con A. In contrast, most of this material was bound by

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Con A in leaky vesicles. In addition, much of the binding seen in intact vesicles was probably due to the fact that roughly 20 % of the vesicles in the original preparation were leaky. This approach was extended by fractionating the lipid-linked oligosaccharides in these samples. In control vesicles, the major lipid-linked species was the full-sized oligosaccharide $Glc_3Man_9GlcNAc_2$. In intact vesicles, only 12 % of this compound was bound by Con A, while in leaky vesicles 75 % was bound by Con A (not shown).

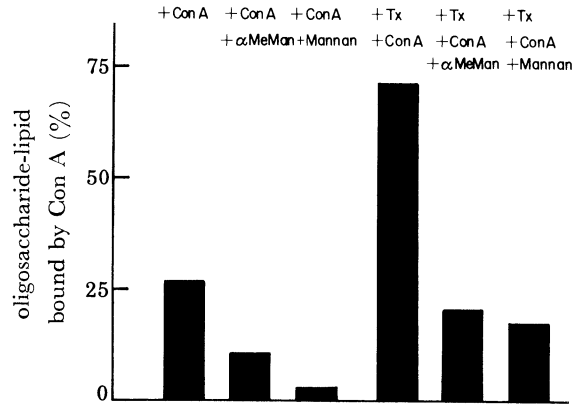


FIGURE 9. Oligosaccharide-lipid is located in the luminal side of microsomal vesicles. Membranes prepared from [^{3}H]mannose-labelled Chinese hamster ovary cell cultures were incubated with Con A and, where indicated, Triton X-100 (Tx), α-methyl mannoside (αMeMan) or yeast mannan. The amount of oligosaccharide-lipid bound by Con A was determined as described in the text (Snider & Robbins 1982). The amount of oligosaccharide-lipid bound by Con A is much greater in the presence of detergent, suggesting that the compound faces the luminal side of the vesicles. The specificity of Con A binding oligosaccharide-lipid is shown by the fact that it is inhibited by the competitors α-methyl mannoside and mannan.

These results suggest that the largest lipid-linked oligosaccharide, the donor in protein glycosylation, faces the microsomal lumen. Thus, sugar residues must cross the microsomal membrane during the assembly of this compound. Moreover, this orientation argues that oligosaccharide is transferred from lipid to protein on this side of the membrane. This conclusion is supported by several lines of evidence. First, oligosaccharide transferred from lipid to peptide acceptors was found within the lumen of hen oviduct microsomes *in vitro*, even though peptide translocation probably did not occur (Hanover & Lennarz 1980). Moreover, glycoprotein polypeptides translated *in vitro* were glycosylated only if they were cotranslationally inserted into microsomal membranes (Katz *et al.* 1977; Lingappa *et al.* 1978). Second, the precursor to adrenocorticotrophic hormone is glycosylated post-translationally in adrenal tumour cells *in vivo*, presumably while the polypeptide is sequestered in the e.r. lumen (Roberts *et al.* 1978). Finally, a study on the glycosylation of ovalbumin nascent chains suggested that glycosylation could occur only after there was a peptide segment between the ribosome and the acceptor site on the peptide chain long enough to span the bilayer (Glabe *et al.* 1981).

How then do sugar residues derived from cytoplasmic sugar nucleotides move across the e.r. membrane during the assembly of luminal oligosaccharide-lipid? One possibility is the coupling of glycosylation to transport: glycosyltransferases using cytoplasmic sugar nucleotides would carry residues across the membrane and add them to growing oligosaccharide chains on the luminal side of the membrane. Such a mechanism is likely for the synthesis of the second intermediate in the pathway, Dol-PP-$GlcNAc_2$. Hanover & Lennarz (1979, 1982) have localized

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

this compound to the luminal side of microsomal vesicles. In addition, those workers could find no evidence for the appearance of this compound on the cytoplasmic side of the membrane during its synthesis. However, the assembly of this compound was sensitive to proteolysis from the cytoplasmic side of the membrane (Snider *et al.* 1980; Hanover & Lennarz 1982). Because neither UDP-GlcNAc nor the intermediate itself can cross the membrane, it is likely that the transport of GlcNAc residues occurs as an integral part of the glycosylation process.

A second possibility is that sugar residues are transported by the transmembrane movement of the lipid-linked intermediates themselves. This possibility has been ruled out for two dolichyl-pyrophosphate-linked oligosaccharides, $GlcNAc_2$-lipid (Hanover & Lennarz 1979, 1982) and $Glc_3Man_9GlcNAc_2$-lipid (Snider & Robbins 1982). However, the recent studies of Haselbeck & Tanner (1982) suggest that Dol-P-Man, an intermediate that donates the outer four mannose residues to growing oligosaccharide-lipids, may serve to transport mannose residues into the lumen. Those workers have presented evidence for the transmembrane movement of Dol-P-Man in reconstituted phospholipid vesicles containing purified Dol-P-Man synthetase from yeast. This translocation is almost certainly mediated by protein because similar compounds do not flip spontaneously in model membranes (McCloskey & Troy 1980). If the same translocation occurs *in vivo*, Dol-P-Man might be made on the cytoplasmic side of the e.r. membrane, and then flip so that it can serve as a mannose donor in the assembly of luminal oligosaccharide-lipid. However, confirmation of this model will require the direct probing of the orientation of this compound in the e.r. membrane.

These studies have greatly increased our understanding of the transport of sugar residues that occurs during the synthesis of asparagine-linked oligosaccharides. The demonstration that nucleotide sugars are utilized from the cytoplasmic side of the membrane allows the possibility of uptake into the lumen to be ruled out. Similarly, the luminal location of mature oligosaccharide-lipid and $GlcNAc_2$-lipid rules out the possibility of assembly of the oligosaccharide on the cytoplasmic face of the membrane. These studies have also suggested two possible transport mechanisms. The first, which suggests the coupling of glycosylation and transport, could be active in the addition of the first seven sugar residues of the oligosaccharide-lipid, which are added directly from sugar nucleotides. The second possible transport mechanism, involving the flipping of Dol-P-Man, could function in the addition of the outer sugars of the oligosaccharide-lipid. The confirmation of these mechanisms, as well as the details of the transport processes, remain a subject for future experiments.

Control of asparagine-linked oligosaccharide processing

Immediately after transfer to proteins, the initially homogeneous population of precursor oligosaccharides (compound *a*, figure 10) begins to undergo a series of modifications that will eventually produce the diverse array of *N*-linked glycans found on mature glycoproteins. We have previously shown that in rat liver, two enzymes of the e.r. lumen, glucosidases I and II, sequentially remove the three Glc residues to produce $Man_9GlcNAc_2$ (compound *b*, figure 10) (Grinna & Robbins 1979). Two Golgi α-mannosidases (mannosidases I–A and I–B) can then remove up to four of the nine Man residues to produce $Man_5GlcNAc_2$ (compound *c*, figure 10) (Tabas & Kornfeld 1979; Tulsiani *et al.* 1982). These reactions yield $Man_{5-9}GlcNAc_2$ structures, termed high-mannose.

The synthesis of complex-type oligosaccharides, a second major class of structures, begins

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

with the action of GlcNAc transferase I on $Man_5GlcNAc_2$ to yield compound *d* in figure 10. After the removal of two more mannose residues, mature complex structures (compounds *f* and *g*) are formed by the addition of a variety of peripheral sugar residues.

Although the outlines of this processing pathway are now clear, a key question remains: what factors determine whether a given glycosylation site will carry an extensively processed (i.e. complex) or partly processed (i.e. high-mannose) oligosaccharide? We have been investigating this problem by using the two membrane glycoproteins of Sindbis virus, E1 and E2. Because these glycoproteins have both high-mannose and complex oligosaccharides, they represent an excellent system for investigating this problem.

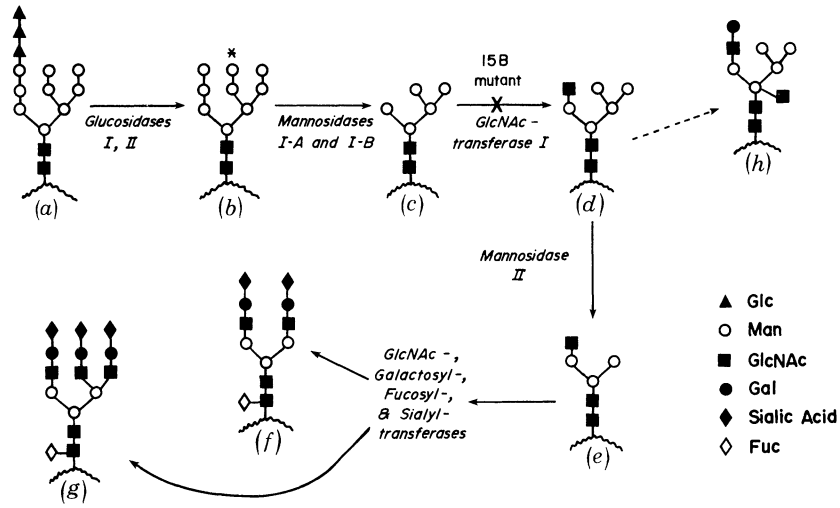


FIGURE 10. Processing of asparagine-linked oligosaccharides. The conversion of the $Glc_3Man_9GlcNAc_2$ precursor oligosaccharide (*a*) to high-mannose (*b*, *c*) and complex (*f*, *g*) oligosaccharides is shown.

Correlation of extent of processing with 'exposure' of oligosaccharide site measured with glycosidase probes

Although the requirement for an -Asn-X-Ser/Thr- sequence in *N*-glycosylation is well established, no specific amino acid sequence directing oligosaccharide processing to high-mannose or complex-type structures has been found. This has led us to the hypothesis that oligosaccharide processing enzymes are relatively insensitive to detailed protein structure but act on any appropriate oligosaccharide structure sterically available to their active sites. Thus, the *N*-linked glycans occupying the most 'exposed' sites on the surface of a native glycoprotein would be expected to have fully processed (complex-type) structures, whereas those obscured by polypeptide chain folding or other interactions would retain high-mannose structures.

The evaluation of this hypothesis has necessitated the development of techniques to separate individual glycosylation sites to allow the examination of their oligosaccharide structures. This is accomplished by high-performance liquid chromatography (h.p.l.c.) of tryptic glycopeptides. Using this technique we have characterized four sites on E1 and E2 as either complex, high-mannose or variable (see below). The relative 'exposure' of glycosylation sites on the surface of a native glycoprotein was determined by using the enzyme endo-β-*N*-acetylglucosaminidase H (endo H), which cleaves between the two GlcNAc residues in high-mannose oligosaccharides.

To use this enzyme as a probe for the exposure of complex sites as well as high-mannose sites, we performed these experiments on the glycoproteins of Sindbis virions grown in a mutant cell line (15B) in which sites normally carrying complex oligosaccharides carry $Man_5GlcNAc_2$ instead.

The experiment is presented in schematic form and results are shown in figure 11 (Hsieh & Robbins 1982). In excellent agreement with the hypothesis, endo H released oligosaccharides from the two complex sites more rapidly than from the two high-mannose sites. All four sites were equally susceptible to endo H after denaturation or pronase digestion.

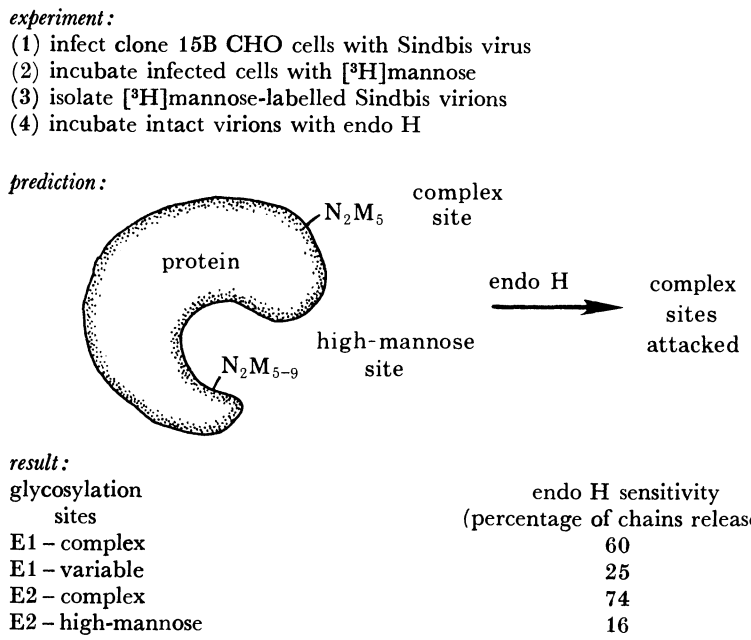


FIGURE 11. Oligosaccharides at complex glycosylation sites are more exposed than oligosaccharides at high-mannose or variable sites in Sindbis virus glycoproteins. Sindbis virions were isolated from [^{3}H]mannose-labelled infected CHO clone 15B cells. Intact virions were then treated with endo H under native conditions. The degree of cleavage by endo H at each site was analysed by h.p.l.c. of tryptic glycopeptides from treated and untreated samples (Hsieh & Robbins 1982). The sites that normally bear complex oligosaccharides are much more susceptible to endo H cleavage than the high-mannose site or the variable site (see text).

Related experiments with endo H as a probe have been carried out with yeast invertase by R. B. Trimble, and with mouse β-glucuronidase by J. U. Baenziger (personal communication). Trimble has found that the large mannan-type oligosaccharide chains are much more susceptible to endo H cleavage in native invertase than 'core' glycosylation sites, which carry smaller high-mannose chains of about 12–16 sugar residues. Baenziger has demonstrated endo H sensitivity for the phosphorylated (processed) oligosaccharides of β-glucuronidase and endo H resistance for unphosphorylated residues. In each case both types of oligosaccharide were sensitive to endo H action after protein denaturation.

In addition to the general correlation between processing and 'exposure' discovered above, we have recently observed a striking variability in the ability of different host cells to process particular glycosylation sites. The gel filtration profile of glycopeptides and oligosaccharides attached to one of the glycosylation sites of E1 after growth of the virus in three separate hosts

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

is shown in figure 12. Clearly, in chick embryo fibroblasts this site is left as a high-mannose structure (figure 12*a*). In contrast, the same site is extensively processed to complex oligosaccharides in BHK cells (figure 12*b*) and becomes a mixture of complex and high-mannose oligosaccharides when the virus is grown in CHO cells (figure 12*c*).

It is conceivable that oligosaccharides at a particular site may be oriented such that processing enzymes of one cell can act extensively at the site whereas enzymes from another cell are less

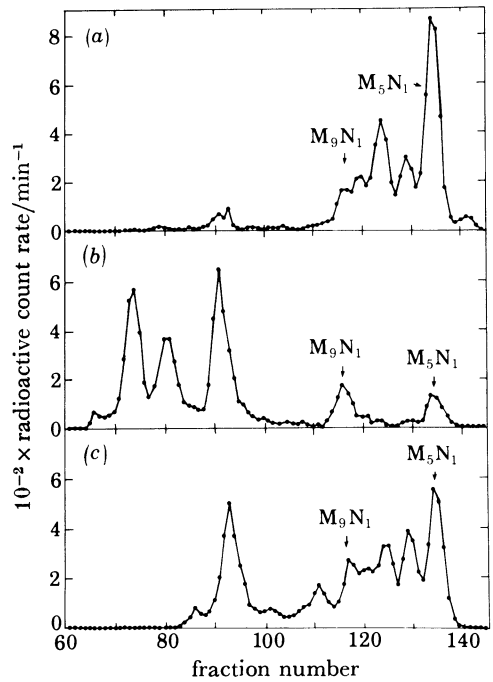


FIGURE 12. Variability with host cell type of the oligosaccharides at one of the glycosylation sites of glycoprotein E1. Labelled Sindbis virions were prepared from [^{3}H]mannose-labelled infected cells, and the tryptic glycopeptides representing one of the glycosylation sites of E1 prepared by h.p.l.c. Samples were then treated with pronase and endo H. The resulting glycopeptides and oligosaccharides were analysed by gel filtration chromatography on Bio-Gel P-4 (minus 400 mesh) (Hsieh & Robbins 1982). Complex glycopeptides elute in the left half of each profile, while high-mannose oligosaccharides are found in the right half. The elution positions of $Man_9GlcNAc$ (M_9N_1) and $Man_5GlcNAc$ (M_5N_1) are indicated. Profiles for this E1 site from virions grown in (*a*) primary chick embryo fibroblasts, (*b*) BHK-21 cells and (*c*) CHO cells are shown.

able to do so. Alternatively, different cells may have varying effects on the nature or duration of physical availability of oligosaccharides at a single site to processing enzymes. Either of these possibilities would account for host-dependent variation seen in this site on E1. It remains to be determined whether this same phenomenon also occurs with other glycoproteins.

We thank R. Das, P. Hsieh, S. C. Hubbard, A. Mercurio and K. Runge for helpful discussions. This work was supported by grants no. CA14142 and CA14051 (to P.W.R. and S.E.L.) from the National Cancer Institute, Department of Health, Education and Welfare.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

References

Ballou, C. E. 1980 In *Fungal polysaccharides* (ed. P. A. Sanford & K. Matsuda), pp. 1–14. Washington, D.C.: American Chemical Society.

Botstein, D., Falco, S. C., Stewart, S. E., Brennan, M., Scherer, S., Stincomb, D. T., Struhl, K. & Davis, R. W. 1979 Sterile host yeasts (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. *Gene* **8**, 17–23.

Carey, D. J. & Hirschberg, C. B. 1981 Topography of sialoglycoproteins and sialyltransferases in mouse and rat liver Golgi. *J. biol. Chem.* **256**, 989–993.

Carey, D. J., Sommers, L. W. & Hirschberg, C. B. 1980 CMP-*N*-acetylneuraminic acid: isolation from and penetration into mouse liver microsomes. *Cell* **19**, 597–605.

Carlson, M. & Botstein, D. 1982 Two differentially regulated mRNAs with different 5′ ends encode secreted and intracellular forms of yeast invertase. *Cell* **28**, 145–154.

Chapman, A., Trowbridge, I. S., Hyman, R. & Kornfeld, S. 1979 Structure of the lipid-linked oligosaccharides that accumulate in Class E Thy-1-negative mutant lymphomas. *Cell* **17**, 509–515.

Coates, S. W., Gurney, T. Jr, Sommers, L. W., Yeh, M. & Hirschberg, C. B. 1980 Subcellular localization of sugar nucleotide synthetases. *J. biol. Chem.* **255**, 9225–9229.

DePierre, J. W. & Dallner, G. 1975 Structural aspects of the membrane of the endoplasmic reticulum. *Biochim. biophys. Acta* **415**, 411–472.

Fleischer, B. 1981 Orientation of glycoprotein galactosyltransferase and sialyltransferase enzymes in vesicles derived from rat liver Golgi apparatus. *J. Cell Biol.* **89**, 246–255.

Glabe, C. G., Hanover, J. A. & Lennarz, W. J. 1980 Glycosylation of ovalbumin nascent chains. The spatial relationship between translation and glycosylation. *J. biol. Chem.* **255**, 9236–9242.

Grinna, L. S. & Robbins, P. W. 1979 Glycoprotein biosynthesis. Rat liver microsomal glucosidases which process oligosaccharides. *J. biol. Chem.* **254**, 8814–8818.

Hanover, J. A. & Lennarz, W. J. 1979 The topological orientation of *N,N′*-diacetylchitobiosylpyrophosphoryldolichol in artificial and natural membranes. *J. biol. Chem.* **254**, 9237–9246.

Hanover, J. A. & Lennarz, W. J. 1980 *N*-linked glycoprotein assembly. Evidence that oligosaccharide attachment occurs within the lumen of the endoplasmic reticulum. *J. biol. Chem.* **255**, 3600–3604.

Hanover, J. A. & Lennarz, W. J. 1982 Transmembrane assembly of *N*-linked glycoproteins. Studies on the topology of saccharide-lipid synthesis. *J. biol. Chem.* **257**, 2787–2794.

Haselbeck, A. & Tanner, W. 1982 Dolichyl phosphate-mediated mannosyl transfer through liposomal membranes. *Proc. natn. Acad. Sci. U.S.A.* **79**, 1520–1524.

Hasilik, A. & Tanner, W. 1978 Carbohydrate moiety of carboxypeptidase Y and perturbation of its biosynthesis. *Eur. J. Biochem.* **91**, 567–575.

Hsieh, P. & Robbins, P. W. 1982 (In preparation.)

Hubbard, S. C. & Ivatt, R. J. 1978 Synthesis and processing of asparagine-linked oligosaccharides. *A. Rev. Biochem.* **50**, 555–583.

Huffaker, T. C. & Robbins, P. W. 1982 Temperature-sensitive yeast mutants deficient in asparagine-linked glycosylation. *J. biol. Chem.* **257**, 3203–3210.

Hunt, L. A. 1980 CHO cells selected for phytohemagglutinin and Con A resistance are defective in both early and late stages of protein glycosylation. *Cell* **21**, 407–415.

Katz, F. N., Rothman, J. E., Lingappa, V. R., Blobel, G. & Lodish, H. F. 1977 Membrane assembly *in vitro*: synthesis, glycosylation, and asymmetric insertion of a transmembrane protein. *Proc. natn. Acad. Sci. U.S.A.* **74**, 3278–3282.

Lehle, L. 1980 Biosynthesis of the core region of yeast mannoproteins. *Eur. J. Biochem.* **109**, 589–601.

Lehle, L., Cohen, R. E. & Ballou, C. E. 1979 Carbohydrate structure of yeast invertase. *J. biol. Chem.* **254**, 12209–12218.

Lingappa, V. R., Lingappa, J. R., Prasad, R., Ebner, K. E. & Blobel, G. 1978 Coupled cell-free synthesis, segregation and core glycosylation of a secretory protein. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2338–2342.

McCloskey, M. A. & Troy, F. A. 1980 Paramagnetic isoprenoid carrier lipids. 2. Dispersion and dynamics in lipid membranes. *Biochemistry, Wash.* **19**, 2061–2066.

Maniatis, T., Ross, C. H. & Lacy, E. 1978 The isolation of structural genes from libraries of eucaryotic DNA. *Cell* **15**, 687–701.

Meyer, J. & Matile, P. 1974 Regulation of isoenzymes and secretion of invertase in bakers' yeast. *Biochem. Physiol. Pflanzen* **166**, 377–385.

Parodi, A. J. 1979 Biosynthesis of yeast mannoproteins. *J. biol. Chem.* **254**, 10051–10060.

Perlman, D. & Halvorson, H. O. 1981 Distinct repressible mRNAs for cytoplasmic and secreted yeast invertase are encoded by a single gene. *Cell* **25**, 525–536.

Roberts, J. L., Phillips, M., Rosa, P. A. & Herbert, E. 1978 Steps involved in the processing of common precursor forms of adrenocorticotropin and endorphin in cultures of mouse pituitary cells. *Biochemistry, Wash.* **17**, 3609–3618.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Snider, M. D. & Robbins, P. W. 1982 Transmembrane organization of protein glycosylation. Mature oligosaccharide-lipid is located on the luminal side of microsomes from Chinese hamster ovary cells. *J. biol. Chem.* **257**, 6796–6801.

Snider, M. D., Sultzman, L. A. & Robbins, P. W. 1980 Transmembrane location of oligosaccharide-lipid synthesis in microsomal vesicles. *Cell* **21**, 385–392.

Stanley, P. 1980 In *The biochemistry of glycoproteins and proteoglycans* (ed. W. Lennarz), pp. 161–189. New York: Plenum Press.

Struck, D. K. & Lennarz, W. J. 1980 In *The biochemistry of glycoproteins and proteoglycans* (ed. W. Lennarz), pp. 35–83. New York: Plenum Press.

Tabas, I. & Kornfeld, S. 1979 Purification of a rat liver golgi α-mannosidase capable of processing asparagine-linked oligosaccharides. *J. biol. Chem.* **254**, 11655–11663.

Tarentino, A. L., Plummer, T. H. Jr & Maley, F. 1974 The release of intact oligosaccharides from specific glycoproteins by endo-β-*N*-acetylglucosaminidase H. *J. biol. Chem.* **249**, 818–824.

Trimble, R. B. & Maley, F. 1977 The use of endo-β-*N*-acetylglucosaminidase H in characterizing the structure and function of glycoproteins. *Biochem. biophys. Res. Commun.* **78**, 935–944.

Trimble, R. B., Maley, F. & Tarentino, A. L. 1980 Characterization of large oligosaccharide-lipids synthesized *in vitro* by microsomes from *Saccharomyces cerevisiae*. *J. biol. Chem.* **255**, 10232–10238.

Tulsiani, D. R. P., Hubbard, S. C., Robbins, P. W. & Touster, O. 1982 α-D-Mannosidases of rat liver Golgi membranes. Mannosidase II is the GlcNAcMan$_5$-cleaving enzyme in glycoprotein biosynthesis and mannosidases IA and IB are the enzymes converting Man$_9$ precursors to Man$_5$ intermediates. *J. biol. Chem.* **257**, 3660–3668.

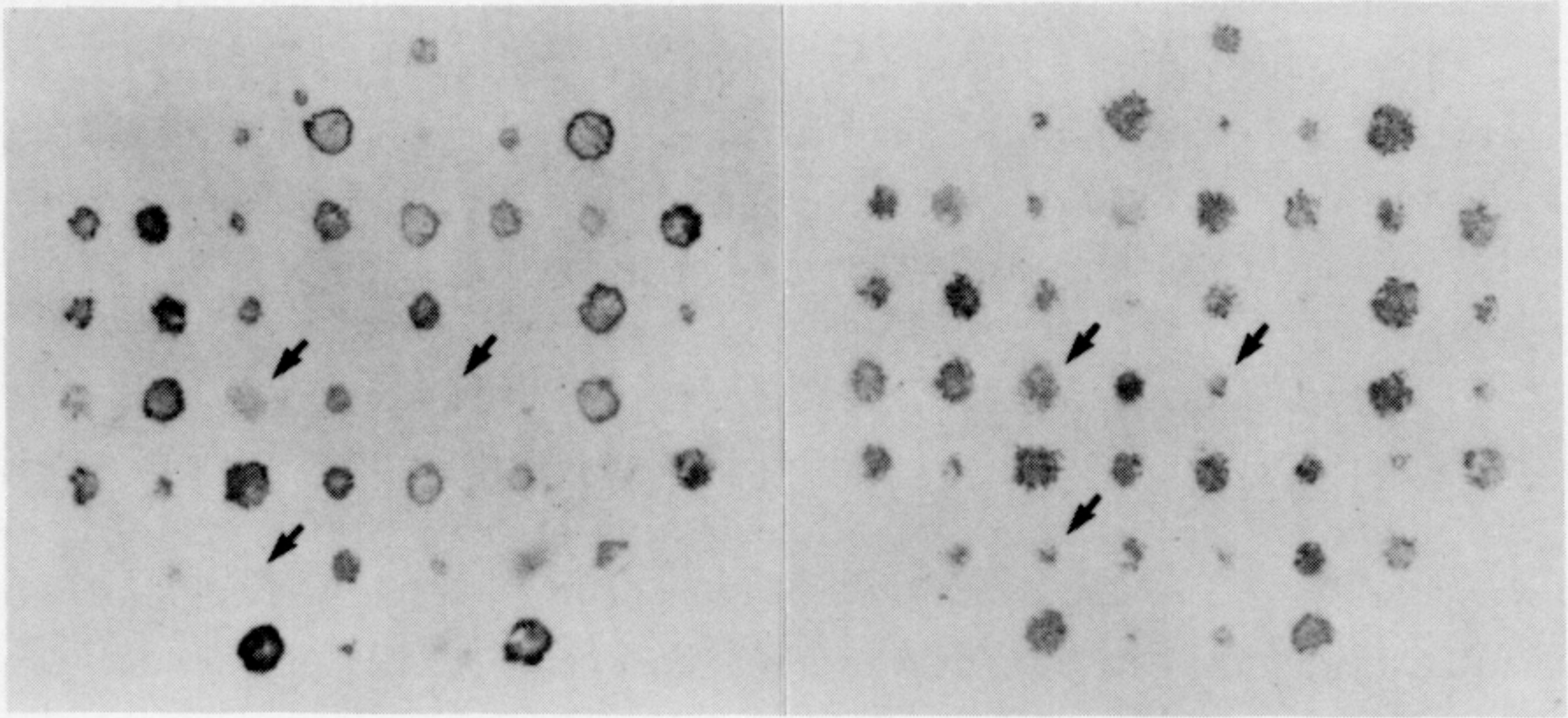
Discussion

T. Feizi (*Division of Communicable Diseases, Clinical Research Centre, Harrow, U.K.*). One possible explanation for differences in degree of processing of oligosaccharides at the same glycosylation site is that the cells in a given cell line are not absolutely uniform and the glycoproteins are a mixture of products derived from cells that are subtly different. In that case (and if these differences are stable) it might be possible to clone the cells and obtain glycoproteins with uniform glycosylation. We often find heterogeneity of the expression of carbohydrate antigens on cultured cell lines (Childs *et al.* 1980).

Reference

Childs, R. A., Kapadia, A. & Feizi, T. 1980 Expression of blood group I and i active carbohydrate sequences on cultured human and animal cell lines assessed by radioimmunoassays with monoclonal cold agglutinins. *Eur. J. Immunol.* **10**, 379–384.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



[^{3}H]mannose [^{35}S]methionine

FIGURE 4. Colony screen for incorporation of [^{3}H]mannose and [^{35}S]methionine. Colonies were replica-plated onto two filter papers and assayed for their ability to incorporate [^{3}H]mannose and [^{35}S]methionine at the non-permissive temperature (Huffaker & Robbins 1982). Fluorograms of [^{3}H]mannose and [^{35}S]methionine-labelled replicas from a single plate are shown. Colonies that incorporated [^{35}S]methionine but failed to incorporate [^{3}H]mannose are indicated by arrows.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

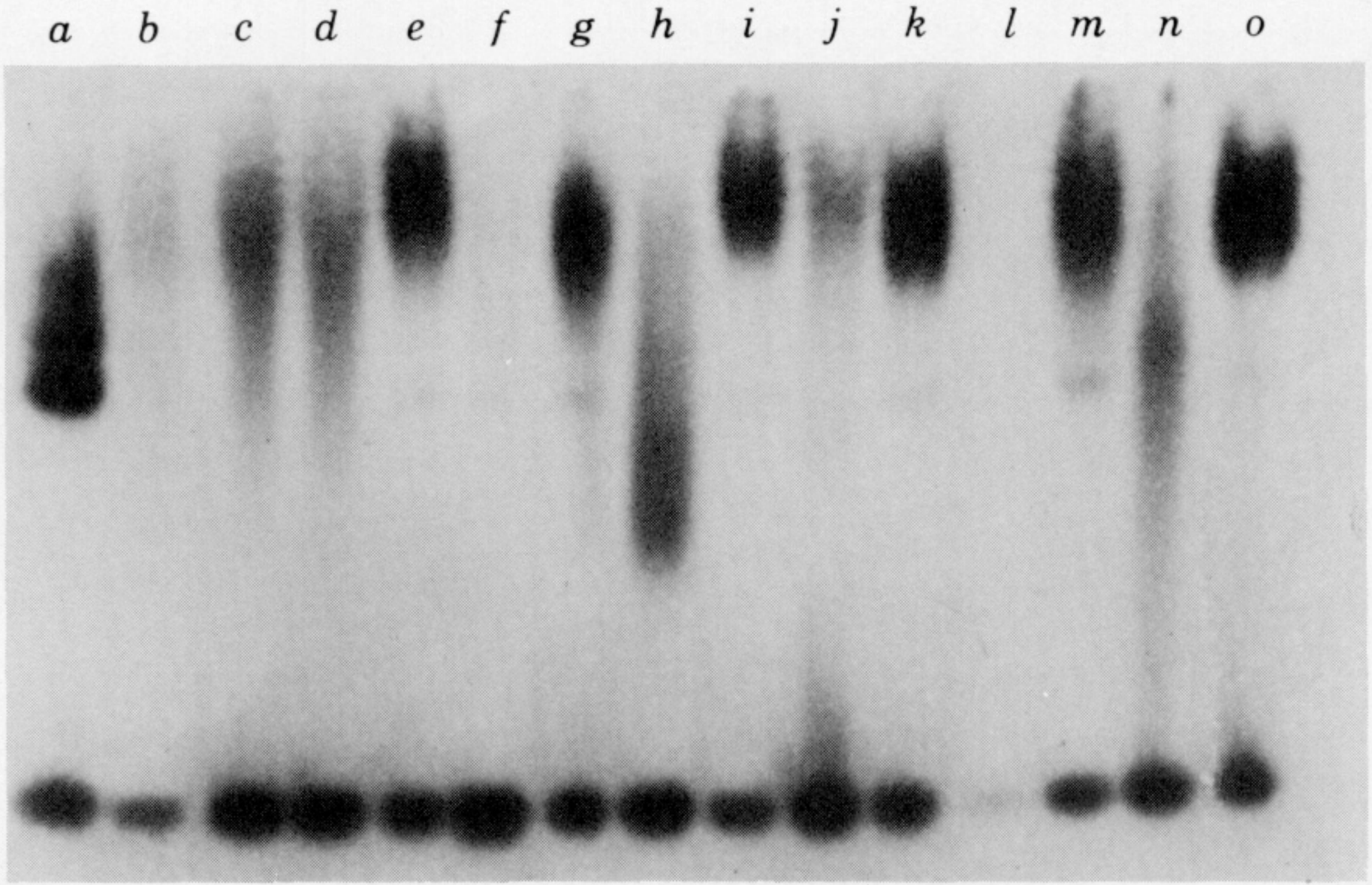


FIGURE 5. Assay for glycosylation of invertase. Cultures were derepressed for invertase synthesis at the non-permissive temperature. Cell extracts were run on a polyacrylamide gel and the gel was stained for invertase activity (Huffaker & Robbins 1982). Lane *o*, wild type; lanes *a*–*n*, mutants identified by the colony screen.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES