Neurosci. **10**, 1299 (1990); H. Yaginuma *et al.*, *Development* **108**, 705 (1990).
10. H. W. M. van Straaten, J. W. M. Hekking, F. Thors, E. L. J. M. Wiertz, J. Drukker, *Acta Morphol. Neerl. Scand.* **23**, 91 (1985); H. W. M. van Straaten, J. W. M. Hekking, E. L. J. M. Wiertz-Hoessels, F. Thors, J. Drukker, *Anat. Embryol.* **177**, 317 (1988); J. L. Smith and G. C. Schoenwolf, *J. Exp. Zool.* **250**, 49 (1989).
11. H. Bautzmann, *Arch. Entwicklungsmech. Organ (Wilhelm Roux)* **128**, 665 (1933); J. Oppenheimer, *J. Exp. Zool.* **72**, 409 (1936); R. L. Watterson *et al.*, *Anat. Rec.* **122**, 539 (1955); J. van Limborgh, *Acta Morphol. Neerl. Scand.* **1**, 155 (1956).
12. W. H. Lewis, *Anat. Rec.* **97**, 139 (1947); M. Martins-Green, *Development* **103**, 687 (1988); G. C. Schoenwolf, D. Folsom, A. Moe, *Anat. Rec.* **270**, 87 (1988); J. D. Moury and A. G. Jacobson, *Dev. Biol.* **133**, 44 (1989); G. C. Schoenwolf and J. L. Smith, *Development* **109**, 243 (1990).
13. Dissections and chemotropic assay were performed as follows. Tissues from Hamburger-Hamilton (*15*) stage 6 to 24 chick embryos and E11 rat embryos were dissected after treatment with Dispase (Boehringer Mannheim; 1 mg/ml in L15 air) at room temperature for 10 to 15 min (stages 6 to 10 chick) and 30 min (stages 19 to 24 chick and E11 rat embryos). After isolation, 50- to 200-μm-long pieces of chick tissues were embedded, together with two-segment pieces of the dorsal spinal cords of rat embryos (24 to 27 stage somites), in a collagen gel (*8*). Explants were cultured for 40 to 44 hours in serum-free supplemented Ham's F12 (Gibco) or with 5% heat-inactivated horse serum added, at 37°C in a 5% CO_2 environment.
14. In the presence of floor plate explants or induced neural epithelium, the vast majority of axons grew in fascicles that emerged perpendicular to the ventralmost edge of the dorsal explant and were directed toward the chick target tissue. Although there was some variability in bundle thickness, each bundle was scored as a single unit. Short single axons that were not target-directed were excluded from the analysis, because they were observed only rarely and with similar frequency whether dorsal explants were cultured alone or with targets. Commissural axons were identified by antibodies to TAG-1 (*8*). All axons that emerged in response to floor plate or induced neural epithelium expressed TAG-1. The ability of the chick floor plate or induced lateral tissue to orient commissural axons was determined by placing them adjacent to one end of a long dorsal explant (*8*). Floor plate and induced lateral neural epithelium, but not noninduced lateral neural epithelium, were able to reorient the growth of commissural axons.
15. V. Hamburger and H. Hamilton, *J. Morphol.* **88**, 49 (1951).
16. Notochords were removed from stage 10 chick donor embryos. The vitelline membrane of host chicks above the area to be operated was removed, an incision was made through ectoderm and mesoderm, and the endoderm was left intact. In stage 10 hosts, grafts were positioned adjacent to the closing neural tube at nonsegmented levels. In stages 11 to 14 hosts, grafts were positioned between the spinal cord and prospective somites 10 to 18. Eggshells were sealed and embryos allowed to develop a further 40 to 48 hours at 38°C. The approximate location of the grafted notochord was indicated by displacement of somites from the spinal cord. The grafted notochord often elongated in ovo. The grafted notochord and appropriate regions of the spinal cord were dissected and tested for expression of the chemoattractant.
17. Stage 7 notochord alone occasionally induced axon outgrowth (Fig. 3) (16% of explants, $n = 44$; 4 ± 1 bundles per positive explants, SEM; $P < 0.16$ when compared with dorsal explant cultured alone; Student's t test), although the amount is not sufficient to account for the chemotropic activity detected in neural plate-notochord cocultures. Stage 10 notochord, which can induce chemotropic activity in neural tissue in vitro (*20*), never evoked axon outgrowth ($n = 23$). The low amount of activity detected in stage 7 notochord may have been due to production of chemoattractant by the notochord or to leaching of chemoattractant previously released by the floor plate and bound to the notochord before its removal from the embryo.
18. The dorsal spinal cord explant may provide a cofactor that acts in concert with the notochord-derived signal to induce chemotropic activity in the lateral neural plate. However, we cannot test this directly because the assay of axon outgrowth requires the presence of spinal cord tissue.
19. J. L. Smith and G. C. Schoenwolf, *Anat. Rec.* **218**, 196 (1987); *Cell Tissue Res.* **252**, 491 (1988).
20. Stages 10 to 12 caudal ventral midline cultured alone with a dorsal explant did not evoke axon outgrowth [6% of explants, 2 bundles per positive explant (mean), $n = 16$]. In contrast, stages 10 to 12 caudal ventral midline cultured with stage 10 notochord opposite a dorsal explant evoked extensive outgrowth (88% of explants, 11 ± 3.7 bundles per positive explant SEM; $n = 8$). Axon outgrowth was also observed when stage 10 lateral neural tube was cultured together with stage 10 notochord (55% of explants; 7 ± 1.4 bundles per positive explant SEM; $n = 20$). This suggests that induction of chemotropic activity in the lateral neural epithelium is not dependent on the presence of a host floor plate within the neural tube.
21. Chemotropic activity can be detected at stages 6 to 7 in the midline of the neural plate at the axial level of the second somite. Ventral midline neural tube tissue from stages 10 to 12 that does not exhibit chemotropic activity was taken from the level of unsegmented mesoderm that will give rise to somites 11 to 20.
22. Notochord removal was as follows. Embryos were exposed and treated with in 0.15% trypsin. After incisions were made through ectoderm and mesoderm adjacent to the spinal cord, the notochord was freed from overlying neural tube and underlying endoderm. The notochord was removed, the spinal cord was placed back in its original position, and embryos were allowed to develop for a further 40 to 48 hours at 38°C. In sham operations, the notochord and spinal cord were displaced and then repositioned.
23. S. Homma and R. N. Oppenheim, *Soc. Neurosci. Abstr.* **16**, 347.4 (1990).
24. The detection of chemotropic activity in rostral midline neural plate cells in vitro (*21*) shows that induction of the floor plate is an early step in neural development. Induction may start before gastrulation, when prospective notochord and floor plate cells are located in and around Hensen's node [*26*; G. C. Rosenquist, *Carn. Contrib. Embryol.* **262**, 71 (1966); G. C. Rosenquist, *Anat. Rec.* **207**, 349 (1983); G. Nicolet, *J. Embryol. Exp. Morphol.* **23**, 79 (1970); K. Hara, in *Organizer—A Milestone of a Half-Century from Spemann*, O. Nakamura and S. Toivonen, Eds. (Elsevier/North-Holland, Amsterdam, 1978), p. 221; G. C. Schoenwolf, H. Bortier, L. Vakaet, *J. Exp. Zool.* **249**, 271 (1989)].
25. R. L. Gimlich, *Dev. Biol.* **115**, 340 (1986); L. Dale and J. M. W. Slack, *Development* **99**, 527 (1987); C. B. Kimmel and R. M. Warga, *Trends Genet.* **4**, 68 (1988).
26. G. C. Schoenwolf and P. Sheard, *J. Exp. Zool.*, in press.
27. S. E. Fraser, R. Keynes, A. G. S. Lumsden, *Nature* **344**, 431 (1990).
28. We thank C. Stern for demonstration of chick notochord removal; G. Schoenwolf and R. Oppenheim for discussions and unpublished results; R. Axel, G. Struhl, and A. Furley for comments on the manuscript; I. Schieren and E. Hubel for art; and K. Liebert for secretarial assistance. Supported by grants from the Irma T. Hirschl Foundation, NSF, The Esther A. and Joseph Klingenstein Fund (J.D.), a European Molecular Biology Organization fellowship (M.P.), and the Lucille P. Markey Charitable Trust (M.T.-L.). T.J. is an Investigator of the Howard Hughes Medical Institute.

12 July 1990; accepted 13 September 1990

Correction of a Defect in Mammalian GPI Anchor Biosynthesis by a Transfected Yeast Gene

R. DEGASPERI, L. J. THOMAS, E. SUGIYAMA, H. M. CHANG, P. J. BECK, P. ORLEAN, C. ALBRIGHT, G. WANECK, J. F. SAMBROOK, C. D. WARREN, E. T. H. YEH*

Glycosylphosphatidylinositol (GPI) serves as a membrane anchor for a large number of eukaryotic proteins. A genetic approach was used to investigate the biosynthesis of GPI anchor precursors in mammalian cells. T cell hybridoma mutants that cannot synthesize dolichol-phosphate-mannose (Dol-P-Man) also do not express on their surface GPI-anchored proteins such as Thy-1 and Ly-6A. These mutants cannot form mannose-containing GPI precursors. Transfection with the yeast Dol-P-Man synthase gene rescues the synthesis of both Dol-P-Man and mannose-containing GPI precursors, as well as the surface expression of Thy-1 and Ly-6A, suggesting that Dol-P-Man is the donor of at least one mannose residue in the GPI core.

A NUMBER OF EUKARYOTIC PROteins, such as Thy-1, Ly-6A, and the variant surface glycoprotein (VSG) of the protozoan *Trypanosoma brucei*, are attached to the cell membrane by a GPI anchor (*1, 2*). The GPI anchors of *T. brucei* VSG and rat brain Thy-1 have a remarkably conserved core structure (*3, 4*), which suggests that a common biosynthetic pathway may have been conserved throughout eukaryotic evolution. For VSG, the first step in GPI anchor biosynthesis is the transfer of *N*-acetylglucosamine (GlcNAc) from uridine 5′-diphosphate (UDP)–*N*-acetylglucosamine to phosphatidylinositol, which is followed by N-deacetylation to form glucosaminylphosphatidylinositol (*5, 6*). Subsequently, three mannose residues are added from either guanosine 5′-diphosphate (GDP)–mannose or Dol-P-Man, after which phosphoethanolamine is added. The completed GPI core is then transferred en

bloc to the COOH-termini of proteins with appropriate cleavage and attachment signals (*2, 7–9*).

A panel of T cell hybridoma mutants that synthesize Thy-1 and Ly-6A but cannot express them on the cell surface, presumably due to defects in the synthesis or attachment of the GPI anchor, has been described (*10*). To define the precise defect, we incubated wild-type (YH16.33) and mutant (M38/20) hybridoma cells with [^{3}H]mannose in the presence of tunicamycin—to block the formation of dolichol-linked oligosaccharides. [^{3}H]Mannose-labeled glycolipids were then extracted and examined by thin layer chromatography (TLC) (Fig. 1). The wild-type hybridoma synthesized at least four distinct mannose-containing glycolipids (referred to as glycolipids 1 to 4) (Fig. 1A), whereas the mutant hybridoma did not (Fig. 1B). Glycolipid 1 could also be labeled with ^{3}H-labeled inositol, glucosamine, or ethanolamine (*11*). Glycolipid 1 was sensitive to treatment with nitrous acid (HNO_2), but resistant to treatment with dilute hydrochloric acid and α-mannosidase (Table 1); it therefore satisfied the proposed criteria for the completed GPI core (*5, 12*). Glycolipid 1, however, was resistant to phosphatidylinositol-specific phospholipase C (PI-PLC) treatment, suggesting that it may be acylated with a fatty acid in the inositol residue (*13*) (Table 1). The absence of glycolipid 1 in the mutant indicated that the latter's failure to express Thy-1 and Ly-6A was attributable to a defect in GPI anchor synthesis.

Glycolipids 2 and 3 were sensitive to treatment with HNO_2 and α-mannosidase, indicating that they were mannose-containing GPI compounds (Table 1). The oligosaccharides liberated from HNO_2 treatment of glycolipids 2 and 3 were reduced with sodium borohydride and analyzed by high-pressure liquid chromatography (HPLC) (*14*). Reference oligosaccharides were isolated from the urine of a patient with α-mannosidosis; these compounds, with composition $Man_xGlcNAc$ (where x = 2 to 9), were used because of their close structural similarity to the products of HNO_2 treatment and their similar chromatographic properties (*15*). The major compounds derived from glycolipids 2 and 3 had the same retention times as the trisaccharide $Man_2GlcNAc$ and the chemically synthesized disaccharide ManGlcNAc (*16*), respectively. A similar analysis of material eluting between glycolipids 1 and 2 suggested the presence of a compound containing three mannose residues. Glycolipid 4 was not a GPI precursor because it was resistant to treatment with HNO_2 and α-mannosidase. This glycolipid was identified as Dol-P-Man because it comigrated with an authentic Dol-P-Man standard and released mannose quantitatively after mild acid hydrolysis (*17*). The absence of glycolipids 1, 2, and 3 in a mutant that cannot synthesize Dol-P-Man suggested that Dol-P-Man was required for the synthesis of mannose-containing GPI precursors. This was further supported by the analysis of [^{3}H]mannose-labeled glycolipids in two independently derived hybridoma mutants defective in Dol-P-Man synthesis. In both cases, no mannose-containing GPI precursors were detected (*11*).

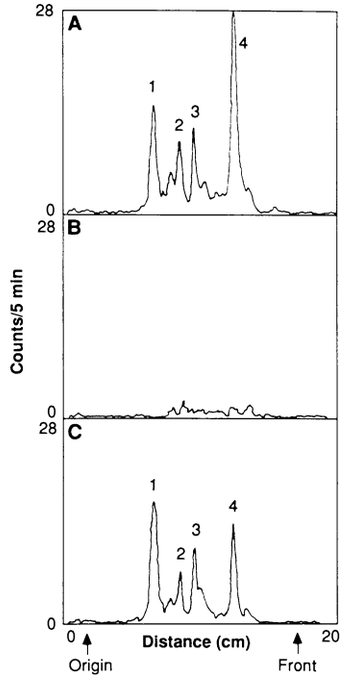

Fig. 1. Mannose-containing glycolipids in wild-type (YH16.33) (**A**), mutant (M38/20) (**B**), and transfected (BL20) (**C**) cells. Hybridoma cells (20×10^6) were labeled for 45 min with [^{3}H]mannose (20 μCi/ml) in low glucose RPMI containing tunicamycin (1 μg/ml). Lipids were extracted with $CHCl_3$:CH_3OH:H_2O (10:10:3), and the lipid extract was dried, dissolved in water-saturated butanol, and partitioned with H_2O. In a typical experiment, 15,000 cpm were extracted from the wild type and 10,000 cpm from the transfectant. Portions of the butanol phases corresponding to the same number of cells were analyzed by TLC on silica gel 60 plates. The plates were developed in $CHCl_3$:CH_3OH:H_2O (10:10:3) and analyzed with a linear radioisotope scanner (Berthold, Hanover, New Hampshire). Each lane was scanned for 5 min, and the tracing was smoothed electronically.

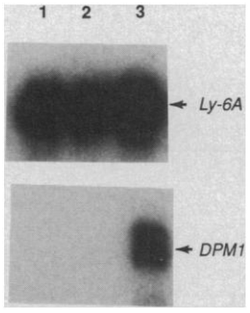

Fig. 2. Northern blot analysis of mRNA isolated from wild-type (YH16.33) (lane 1), mutant (M38/20) (lane 2), and transfected (BL20) (lane 3) cells. Cell pellets were lysed in 4 M guanidinium thiocyanate and extracted with phenol as described (*30*). RNA was size-fractionated on 1% agarose–formaldehyde gels and transferred to nitrocellulose filters in 0.1× SSC (standard saline citrate). The DNA probes specific for *DPM1* and *Ly-6A* were excised by digestion with appropriate restriction enzymes and purified on agarose gels. Purified inserts were labeled with ^{32}P by random primer extension (*31, 32*). Baked nitrocellulose filters were prehybridized and then hybridized with ^{32}P-labeled probes for 20 hours at 42°C in 50% formamide, 5× SSC, 1× Denhardt's solution, 20 mM sodium sulfate, and 10% dextran sulfate. Hybridized blots were then washed at 65°C in 0.1% SDS and decreasing concentrations of SSC (2× to 0.1×), and finally autoradiographed at −70°C.

Dol-P-Man is synthesized from dolichyl phosphate and GDP-mannose in a reaction catalyzed by Dol-P-Man synthase (*18*). Our mutants were not deficient in either substrate because they accumulated truncated N-linked glycan precursors (*19*). To ascertain whether the defect involved Dol-P-Man synthase, we tested the ability of the yeast Dol-P-Man synthase gene, *DPM1*, to complement the biosynthetic defect in the mutant (*20*). For this purpose, we cloned *DPM1* into a cytomegalovirus (CMV) vector (*21*). This construct was introduced by electroporation into three independently derived mutants defective in Dol-P-Man synthesis. Transfectants resistant to antibiotic G418 were then subcloned, and 31 stable transfectants were obtained. Analysis was performed on subclones that were grown in regular culture medium for several weeks. Results from BL20, a representative transfectant derived from M38/20, are described.

Northern blot analysis of mRNA isolated from the wild-type (YH16.33), mutant (M38/20), and transfected (BL20) cells showed that they all expressed similar amounts of Ly-6A mRNA (Fig. 2). How-

R. DeGasperi, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Massachusetts General Hospital, Boston, MA 02114.
L. J. Thomas, E. Sugiyama, C. D. Warren, E. T. H. Yeh, Arthritis Unit, Department of Medicine, Harvard Medical School, Massachusetts General Hospital, Boston, MA 02114.
H. M. Chang, Pain Unit, Department of Anesthesia, Harvard Medical School, Massachusetts General Hospital, Boston MA 02114.
P. J. Beck and J. F. Sambrook, Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX 75235.
P. Orlean and C. Albright, Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139.
G. Waneck, Department of Surgery, Harvard Medical School, Massachusetts General Hospital, Boston, MA 02114.
*To whom correspondence should be addressed.

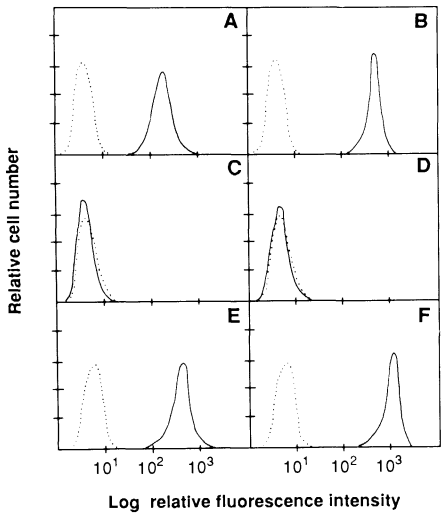

Fig. 3. Immunofluorescence analysis of Ly-6A (**A**, **C**, and **E**) and Thy-1 (**B**, **D**, and **F**) expression in wild-type (YH16.33) (A and B), mutant (M38/20) (C and D), and transfected (BL20) (E and F) cells. Staining and analysis were performed as described (*10*). Solid traces, with specific antibody; dotted traces, negative controls. Scale for the vertical axis is from 0 to 250.

ever, the *DPM1* transcript could only be detected in the transfectant. The *DPM1* product was functional in the transfectant because Dol-P-Man synthesis was restored (glycolipid 4, Fig. 1C). The ability of the yeast enzyme to function in the mammalian system suggested conservation in the synthesis of Dol-P-Man, a crucial substrate in the N-linked glycosylation pathway (*21*). Despite the lower amount of Dol-P-Man in the transfectant, formation of the mannose-containing GPI precursors—glycolipids 1, 2, and 3—was completely restored (Fig. 1C). This result provided direct evidence that Dol-P-Man was required for GPI anchor synthesis in vivo. The restored GPI anchor allowed the expression of Thy-1 and Ly-6A on the cell surface (Fig. 3). Because the phenotype of the transfectant was stable in regular culture medium for over 2 months, the expression of Thy-1 was not a result of metabolic correction by G418 (*22*).

To show that Thy-1 and Ly-6A were indeed GPI-anchored, we treated both the wild-type and transfected cells with PI-PLC and analyzed them by immunofluorescence. Although the expression of the CD3 ϵ subunit, a non–GPI-anchored protein, was not

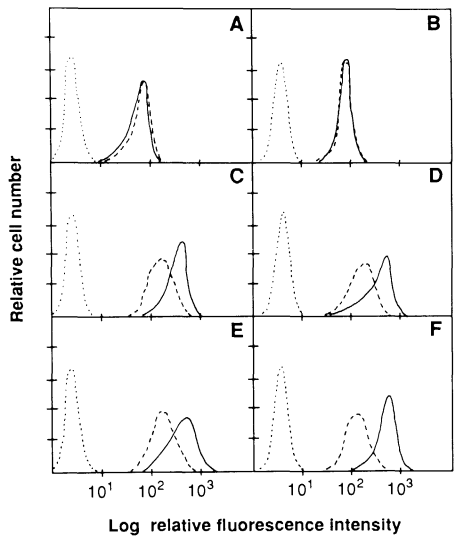

Fig. 4. PI-PLC sensitivity of membrane proteins CD3 ϵ subunit (**A** and **B**), Ly-6A (**C** and **D**), and Thy-1 (**E** and **F**), on wild-type (YH16.33) (A, C, and E) and transfected (BL20) (B, D, and F) cells. Solid traces, no treatment; dashed traces, PI-PLC treatment; dotted traces, negative controls. Cells (5×10^6/ml) were incubated for 60 min at 37°C in RPMI 1640 with bovine serum albumin (2 mg/ml) and 0.1% NaN_3. PI-PLC (0.4 unit/ml) was used for the treatment group. Staining and analysis were performed as described (*10*). Scale for the vertical axis is from 0 to 250.

altered by PI-PLC treatment, both Thy-1 and Ly-6A in the wild-type and transfected cells were removed by PI-PLC to the same degree (Fig. 4). Cross-linking of Thy-1 and Ly-6A on the wild-type hybridoma results in interleukin-2 production (*23*). Thus, we also tested the functional capacity of Thy-1 and Ly-6A on the transfectant. Indeed, both antibodies to Thy-1 and to Ly-6A stimulated interleukin-2 production in the transfectant, but not in the mutant (*24*). Taken together, our results show that yeast *DPM1* was capable of restoring the surface expression of functional, GPI-anchored proteins.

The participation of Dol-P-Man in GPI anchor synthesis was previously suggested on the basis of experiments with the class E mutant derived from BW5147 cells (*25, 26*). The class E mutant does not synthesize Dol-P-Man and cannot express Thy-1 on the cell surface (*27, 28*). Similar results were obtained with an inhibitor of Dol-P-Man synthesis in the *T. brucei* VSG system in vitro (*29*). Complementation of our mutants by the structural gene for the yeast Dol-P-Man synthase suggests that Dol-P-Man donates at least one mannose residue to the GPI core. The complete absence of any mannose-containing GPI precursors in our mutants suggests that Dol-P-Man is probably the donor of the first mannose added to the GPI core. Mutants such as these will provide an important tool for the genetic analysis of GPI anchor synthesis and attachment.

Table 1. Characteristics of glycolipids to 1 to 4. [^{3}H]Mannose-labeled glycolipids prepared from 1×10^8 wild-type hybridoma cells were scraped from a preparative TLC plate, extracted with $CHCl_3$:CH_3OH:H_2O (10:10:3), dried, dissolved in water-saturated butanol, partitioned with H_2O, and dried again. Purified glycolipids were redissolved in 400 μl of 25 mM sodium acetate (pH 3.5) and 0.1% SDS, and incubated with 10 μl of 2.5 M $NaNO_2$ for 3 hours at 37°C or in 150 μl of 0.1 M sodium acetate (pH 4.2) and 2 mM $ZnSO_4$ containing 0.5 unit of α-mannosidase (Jack bean) for 3 hours at 37°C. The samples were then extracted twice with water-saturated butanol, and the butanol phase was analyzed by TLC as described (Fig. 1). Sensitivity to treatment is defined as a greater than 50% reduction in the total counts. Glycans released after HNO_2 treatment were analyzed by HPLC on a Varian 5020 instrument (Varian Associates, Walnut Creek, California) and a 5-μm Amino Spherisorb column (Regis Chemical, Morton Grove, Illinois). Elution was performed with 74% acetonitrile-water at a flow rate of 2 ml/min. Fractions were collected every 30 s and analyzed by scintillation counting. As internal standards, a mixture of oligosaccharides [$Man_xGlcNAc$ (x = 2 to 9)] or the synthetic disaccharide ManGlcNAc were co-injected and detected by absorbance at 195 nm. E, ethanolamine; P, phosphate; GlcN, glucosamine.

Glycolipid	Labels with:		Sensitive to:		Suggested structure
	Ethanolamine	Mannose	HNO_2	α-Mannosidase	
1	+	+	+	−	E-P-Man_3GlcN-PI
2	−	+	+	+	Man_2GlcN-PI
3	−	+	+	+	ManGlcN-PI
4	−	+	−	−	Dol-P-Man

REFERENCES AND NOTES

1. M. G. Low, *FASEB J.* **3**, 1600 (1989).
2. M. A. J. Ferguson and A. F. Williams, *Annu. Rev. Biochem.* **57**, 285 (1988).
3. S. W. Homans *et al.*, *Nature* **333**, 269 (1988).
4. M. J. Ferguson, S. W. Homans, R. A. Dwek, T. W. Rademacher, *Science* **239**, 753 (1988).
5. W. J. Masterson, T. L. Doering, G. W. Hart, P. T. Englund, *Cell* **56**, 793 (1989).
6. T. L. Doering, W. J. Masterson, G. W. Hart, P. T. Englund, *J. Biol. Chem.* **265**, 611 (1990).
7. M. A. J. Ferguson, M. Duszenko, G. S. Lamont, P. Overath, G. A. M. Cross, *ibid.* **261**, 356 (1986).
8. I. W. Caras, G. N. Weddell, M. A. Davitz, V. Nussenzweig, D. W. J. Martin, *Science* **238**, 1280 (1987).
9. G. L. Waneck, M. E. Stein, R. A. Flavell, *ibid.* **241**, 697 (1988).
10. E. T. H. Yeh, H. Reiser, A. Bamezai, K. Rock, *Cell* **52**, 665 (1988).
11. R. DeGasperi and E. T. H. Yeh, unpublished results.
12. A. K. Menon, S. Mayor, M. A. J. Ferguson, M. Duszenko, G. A. M. Cross, *J. Biol. Chem.* **263**, 1970 (1988).
13. W. L. Roberts, J. J. Myher, A. Kuksis, M. J. Low, T. L. Rosenberry, *ibid.*, p. 18766.
14. C. D. Warren *et al.*, in *Swainsonine and Related Glycosidase Inhibitors*, L. F. James, A. D. Elbein, R. J. Molyneux, C. D. Warren, Eds. (Univ. of Iowa Press, Amls, 1989), pp. 304–315.
15. C. D. Warren, A. S. Schmit, R. W. Jeanloz, *Carbohydrate Res.* **116**, 171 (1983).
16. C. D. Warren *et al.*, *ibid.* **82**, 71 (1981).
17. C. D. Warren, I. Y. Liu, A. Herscovics, R. W. Jeanloz, *J. Biol. Chem.* **250**, 8069 (1975).
18. S. C. Hubbard and R. J. Ivatt, *Annu. Rev. Biochem.* **50**, 555 (1981).

19. L. Thomas and E. T. H. Yeh, in preparation.
20. P. Orlean, C. Albright, P. W. Robbins, *J. Biol. Chem.* **263**, 17499 (1988).
21. P. J. Beck *et al.*, *Mol. Cell. Biol.* **10**, 4612 (1990).
22. D. Gupta, A. Tartakoff, E. Tisdale, *Science* **242**, 1446 (1988).
23. E. T. H. Yeh, H. Reiser, J. Daley, K. L. Rock, *J. Immunol.* **138**, 91 (1987).
24. H. M. Chang and E. T. H. Yeh, unpublished results.
25. S. H. Fatemi and A. M. Tartakoff, *Cell* **46**, 653 (1986).
26. A. Conzelmann, A. Spiazzi, R. Hyman, C. Bron, *EMBO J.* **5**, 3291 (1986).
27. A. Chapman, K. Fujimoto, S. Kornfeld, *J. Biol. Chem.* **255**, 4441 (1980).
28. I. S. Trowbridge and R. Hyman, *Cell* **17**, 503 (1979).
29. R. T. Schwartz, S. Mayor, A. K. Menon, G. A. M. Cross, *J. Cell. Biochem.* Suppl. 13A, 143 (1989).
30. J. M. Chirgwin, A. E. Przbyla, R. J. MacDonald, W. J. Rutter, *Biochemistry* **18**, 5294 (1979).
31. A. P. Feinberg and B. Vogelstein, *Anal. Biochem.* **132**, 6 (1983).
32. ———, *ibid.* **137**, 266 (1984).
33. We thank J. Bonventre for the use of the linear radioisotope scanner; P. Englund for GPI-specific PLC; R. Kashi for PI-PLC; A. Bothwell for Ly-6A cDNA; P. Robbins for critical reading of the manuscript; and S. Krane, D. Robinson, R. Kitz, and R. Jeanloz for encouragement. Supported by the Welch Foundation (J.F.S.) and by NIH grants AR-03564 (E.T.H.Y.); HD-21087, HD-16942, and S07 RR0548-26 (C.D.W.); and GM-31318 (P. Robbins). P.J.B. is a postdoctoral fellow of the American Cancer Society. E.T.H.Y. is an Upjohn Scholar and Investigator of the Arthritis Foundation.

18 April 1990; accepted 23 July 1990

Tissue, Developmental, and Tumor-Specific Expression of Divergent Transcripts in Wilms Tumor

ANNIE HUANG, CHRISTINE E. CAMPBELL, LAURA BONETTA, MONICA S. MCANDREWS-HILL, SUSAN CHILTON-MACNEILL, MAX J. COPPES, DAVID J. LAW, ANDREW P. FEINBERG, HERMAN YEGER, BRYAN R. G. WILLIAMS*

The Wilms tumor locus on chromosome 11p13 has been mapped to a region defined by overlapping, tumor-specific deletions. Complementary DNA clones representing transcripts of 2.5 (WIT-1) and 3.5 kb (WIT-2) mapping to this region were isolated from a kidney complementary DNA library. Expression of WIT-1 and WIT-2 was restricted to kidney and spleen. RNase protection revealed divergent transcription of WIT-1 and WIT-2, originating from a DNA region of <600 bp. Both transcripts were present at high concentrations in fetal kidney and at much reduced amounts in 5-year-old and adult kidneys. Eleven of 12 Wilms tumors classified as histopathologically heterogeneous exhibited absent or reduced expression of WIT-2, whereas only 4 of 14 histopathologically homogeneous tumors showed reduced expression. These data demonstrate a molecular basis for the pathogenetic heterogeneity in Wilms tumorigenesis.

WILMS TUMOR (WT) IS A HERITABLE embryonal renal malignancy with complex genetic (*1*) and pathological (*2*) features. Although earlier statistical and molecular genetic analyses implicated a single recessive oncogene locus in the etiology of WT (*3, 4*) recent studies suggest mutations in at least three loci (two mapping to 11p) can cause or predispose to the development of WT (*5, 6*). Heterogeneity also characterizes WT histopathology, such that these tumors can be categorized into: (i) intralobar WTs, which feature heterogeneous tumor histology and association with the WAGR (Wilms tumor, aniridia, genitourinary dysplasia, mental retardation) syndrome region on 11p13; or (ii) perilobar WTs, which are characterized by homogeneous histology and association with the Beckwith-Wiedemann syndrome mapped to 11p15 (*7*).

In Bonetta *et al.* (*8*) we describe the identification and mapping of three transcripts expressed in kidney tissues to a homozygous 175-kb WT deletion, using chromosome walking and yeast artificial chromosome (YAC) cloning. On the basis of data by Gessler *et al.* (*9*), it appears that one of these transcripts (WIT-3) maps outside the putative WT locus and thus seems less likely to be involved in WT. Here we report the biological characterization of cDNA clones corresponding to the two remaining transcripts, WIT-1 and WIT-2. These genes are transcribed in divergent directions from a single CpG island, and their expression shows a striking correlation with tumor histopathology.

Two phylogenetically conserved DNA fragments, AvH1 and E9, which detected distinct transcripts of 2.5 (WIT-1) and 3.5 kb (WIT-2), respectively, in human fetal kidney RNA (*8*), were used to isolate two corresponding cDNA clones, GB16 (WIT-1) and 31E1 (WIT-2). Southern blot hybridization and DNA sequencing confirmed that these cDNAs mapped to the homozygously deleted region in the WiT-13 cell line (*10, 11*). The WIT-1 transcript corresponding to GB16 is novel, whereas 31E1 identifies the same gene as two other previously described cDNAs (*12*). This gene encodes a potential zinc finger protein and is deleted in WiT-13 and in two other cases of sporadic Wilms tumor (*9, 12, 13*).

Using GB16 and 31E1 as probes for Southern blot hybridization of cloned genomic DNA, we found that the transcription units for these two genes were tightly linked. RNase protection analyses (*14*) determined that the WIT-1 mRNA was transcribed in a telomeric-to-centromeric direction, while transcription of the WIT-2 mRNA was in the opposite direction (Fig. 1) DNA sequencing mapped the 5′ termini of GB16 and 31E1 to a single genomic fragment of <3 kb. As both GB16 (2 kb) and 31E1 (2.7 kb) did not represent full-length cDNA clones, we used RNase protection analysis of the intervening genomic region to identify and map transcribed sequences corresponding to the uncloned 5′ regions of each gene. RNA probes complementary to both strands of genomic DNA were used to show that the most 5′ exons detected for each transcript were nonoverlapping and divergently mapped within approximately 600 bp. Sequence analysis revealed the presence of two putative TATA boxes and a CCAAT box within this intervening region (Fig. 1A) that could potentially function as a bidirectional promoter.

When we examined the sequence of GB16 (Fig. 1B), we found the WIT-1 transcript spanned CpG island 6, which we had identified previously by pulsed-field gel electrophoresis (*8, 15*). Although RNase protection experiments indicated GB16 contained only exon sequences, the largest open reading frame (ORF) discernible within the 2025-bp insert of GB16 was 276 bp (Fig. 1B). Subsequent isolation (from independent libraries) of three shorter cDNA clones

A. Huang, C. E. Campbell, B. R. G. Williams, Department of Genetics, Research Institute, Hospital for Sick Children, Toronto, Ontario, M5G 1X8, and Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario, M5S 1A8, Canada.
M. S. McAndrews-Hill and S. Chilton-MacNeill, Department of Genetics, Research Institute, Hospital for Sick Children, Toronto, Ontario, M5G 1X8, Canada.
M. J. Coppes, Division of Hematology-Oncology, Hospital for Sick Children, Toronto, Ontario, Canada.
H. Yeger, Department of Pathology, Hospital for Sick Children, Toronto, Ontario, M5G 1X8, Canada.
L. Bonetta, Department of Genetics, Research Institute, Hospital for Sick Children, Toronto, Ontario, M5G 1X8, and Department of Microbiology, University of Toronto, Toronto, Ontario, Canada.
D. J. Law and A. P. Feinberg, Howard Hughes Medical Institute and Departments of Internal Medicine and Human Genetics, University of Michigan, Ann Arbor, MI 48109.

*To whom reprint requests should be addressed.