The O-linked oligosaccharides of the cloned, murine cytotoxic T cell line B6.1.SF.1 were compared with the corresponding oligosaccharides from a Vicia villosa lectin-resistant mutant of B6.1.SF.1 called VV6 (Conzelmann, A., Pink, R., Acuto, O., Mach, J.-P., Dolivo, S., and Nabholz, M. (1980) Eur. J. Immunol. 10, 860–868). The VV6 mutant cells are deficient in binding sites for this GalNAc-specific lectin. Cells were grown in the presence of [3H]glucosamine and [3H]galactose to label the glycoproteins, and the desialylated, alkaline borohydride-released oligosaccharides were isolated and characterized. The VV6 cells contained a series of O-linked oligosaccharides ranging in size from a disaccharide to a pentasaccharide. These were composed of galactose, N-acetylgalactosamine, and N-acetylhexosaminidol, the latter sugar being derived from the reducing terminus. The predominant oligosaccharide had the partial structure GalβGlcNAcβ-(Galβ)N-acetylhexosaminidol. In contrast, the analogous oligosaccharides of the parental cells contained additional β-linked GalNAc residues located at nonreducing termini. The smallest of these had the structure GalNAcβ1,4Galβ-N-acetylhexosaminidol. Neither cell line contained significant amounts of terminal GalNAc linked to Ser/Thr which is the main binding site for the parental line. The VV6 cells are missing the N-acetylgalactosaminyltransferase that is responsible for the synthesis of these unusual oligosaccharides.

In vitro generated murine CTL express a surface glycoprotein termed T145 which is absent on other types of lymphocytes and lymphomas (1, 2). It has been found that T145 interacts with high affinity with a GalNAc-specific lectin from Vicia villosa and this property has been used to separate CTLs from other lymphocytes (2). Previously, one of us (A. C.) reported that cloned murine CTL lines which are independent of feeder cells but dependent on Interleukin 2 have high levels of binding sites for V. villosa lectin (3). These cell lines express T145 and several other surface glycoproteins which bind to V. villosa lectin-Sepharose and are specifically eluted with the haptenic sugar GalNAc. V. villosa lectin was shown to be highly cytotoxic for these cloned cell lines and this property formed the basis for selecting V. villosa-resistant mutants from a mutagenized culture of a parent CTL-line called B6.1.SF.1 (3). Based on the degree of resistance to the lectin, the mutants could be subdivided into two subclasses, one with intermediate resistance (30–100-fold) and the other with high resistance (1000-fold). The clones with intermediate resistance had a partial loss of V. villosa lectin-binding sites whereas a clone with high resistance had a 100-fold decrease in lectin-binding sites compared to the parental line. Interestingly, both classes of mutants retained susceptibility to the cytotoxic effects of Helix pomatia lectin, another GalNAc-specific lectin.

These findings suggested that the parental clones of CTLs expressed an unusual type of oligosaccharide that contained GalNAc residues and that the V. villosa-resistant clones had specific defects in the assembly of this oligosaccharide. In this paper we analyze the GalNAc-containing oligosaccharides made by the parental CTL-line B6.1.SF.1 and one of the highly resistant mutants (VV6).

**EXPERIMENTAL PROCEDURES**

**Materials**—[2-3H]Mannose (15 Ci/mmol), [6-3H]galactose (15 Ci/mmol), and [6-3H]glucosamine hydrochloride (20 Ci/mmol) were from New England Nuclear. Ambersite MB-3 ion-exchange resin was from Mallinckrodt. Sodium borohydride, sodium metaperiodate, sodium metaborate, Sephadex G-25-80 and bovine serum albumin were from Sigma Chemical Co. Bio-Gel P-6 (200–400 mesh) was from Bio-Rad Laboratories. ConA-Sepharose and ConA were from Pharmacia Fine Chemicals. Silica Gel G-coated (250 μm) thin layer chromatography plates were from Analtech. Scintiverse I scintillation mixture and acetonitrile (HPLC grade) were from Fisher Chemical Company. V. villosa B6 lectin was provided by Dr. S. E. Tollefsen of this institution (4). Methylated galactose standards were a gift of Dr. P. Stoffyn (Eunice Kennedy Shriver Center for Mental Retardation, Inc., Wallingford, Conn.)

1 The abbreviations used are: CTL, cytotoxic T lymphocytes; B6 lectin, isolectin B from V. villosa; ConA, concanavalin A; HPLC, high-performance liquid chromatography; VV, V. villosa lectin; GalNAcol, N-acetylgalactosaminidol.

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Enzymes—β-Galactosidase and β-N-acetylgalactosaminidase from jack bean meal were prepared as described (5). α-N-Acetylgalactosaminidase from Charonia lampas was from Miles. It contained 0.8% β-N-acetylgalactosaminidase activity relative to its activity toward the α-N-acetylgalactosaminidase from Aspergillus niger was prepared as described (6). β-Galactosidase from Escherichia coli was from Sigma. Pronase was from Calbiochem-Behring Corp.

Preparation of O-Linked Oligosaccharides—The Pronase-digested material was desalted by gel filtration on Sephadex G-25 and the glycopeptides were then heated at 80 °C for 1 h in 10 mM HCl to remove sialic acid residues. The reaction mixtures were lyophilized, dissolved in 0.9% NaCl, 10 mM KPO₄, pH 7.4, 1 mM MgCl₂, 1 mM CaCl₂ and applied to a ConA-Sepharose column. The material which passed through the ConA-Sepharose was subjected to gel filtration on a Bio-Gel P-6 column followed by treatment with E. coli β-galactosidase. The material consisted of N-linked glycopeptides, O-linked glycopeptides, and proteoglycans. The material was then passed through a column of ConA-Sepharose which retained some of the O-linked glycopeptides and the glycopeptides were desalted and subjected to mild acid treatment in order to remove sialic acid residues. This was done to simplify the subsequent workup. At this stage the labeled material consisted of N-linked glycopeptides, O-linked glycopeptides, and proteoglycans. The material was then passed over a column of Amberlite X-80 which retained some of the N-linked glycopeptides. The run-through material (85% of the starting radioactivity), which contained tri- and tetra-antennary N-linked oligosaccharides as well as the O-linked glycopeptides, proteoglycans, and free sialic acid, was further resolved by gel filtration on Bio-Gel P-6 (Fig. 1, A and B). Most of the radioactivity eluted in the void volume of the column (pool A) or was slightly included (pool B). The A and B fractions were subjected to alkaline borohydride treatment to release O-linked oligosaccharides and rerun on the Bio-Gel P-6 columns (Fig. 1, B–E). Approximately 13% of the radioactivity was released from both parent and mutant material as small oligosaccharides and these fractions were pooled as noted on the figure. The oligosaccharides released from the pool A material were used for most of the subsequent experiments.

Amino Sugar Content of the A Fractions—Aliquots of fractions A1–A4 were hydrolyzed in strong acid, and the resulting monosaccharides were separated by paper chromatography in solvent C. The results are summarized in Table I. The N-acetylgalactosamine residues which are O-linked to serine or threonine are reduced during the alkaline borohydride treatment and therefore are recovered in the N-acetylgalactosaminol area. Each fraction contained N-acetylgalactosaminol, al-
**FIG. 1.** Gel filtration of glycopeptides from B6.1.SF.1 and VV6 before and after alkaline borohydride treatment. [3H]Glucosamine-labeled glycopeptides which passed through ConA-Sepharose were loaded onto Bio-Gel P-6 columns (A and D). Fractions A and B were pooled as noted, subjected to alkaline borohydride treatment, and reapplied to the same columns (B, C, E, and F). Two different columns of identical size (1.4 × 100 cm) were used for the B6.1.SF.1 and VV6 glycopeptides. The fraction size was 1.5 ml. The arrows indicate the elution position of mannose as detected by the phenol-sulfuric acid assay (18). A, B, and C, B6.1.SF.1; D, E, and F, VV6.

**TABLE I**

Amino sugar content of the A fractions from Bio-Gel P-6

Aliquots of the indicated fractions were hydrolyzed, reacetylated, and separated by paper chromatography. Numbers in the first column indicate the percentage of radioactivity recovered in each fraction as compared to the total amount of radioactivity in the original Pronase digest of the starting material; numbers in the other columns indicate the percentage of radioactivity recovered in each fraction as compared to total recovery in the paper chromatogram.

| Source of fraction | % total radioactivity | N-Acetylhexosaminol | GalNAc | GlcNAc | % cpm in monosaccharide |
|--------------------|-----------------------|---------------------|--------|--------|-------------------------|
| B6.1.SF.1          |                       |                     |        |        |                         |
| A1                 | 29.5                  | 12                  | 47     | 41     |                         |
| A2                 | 3.7                   | 34                  | 43     | 23     |                         |
| A3                 | 3.7                   | 39                  | 37     | 25     |                         |
| A4                 | 1.6                   | 26                  | 42     | 32     |                         |
| VV6                |                       |                     |        |        |                         |
| A1                 | 20.1                  | 13                  | 44     | 43     |                         |
| A2                 | 2.4                   | 21                  | 22     | 57     |                         |
| A3                 | 2.1                   | 61                  | 10     | 28     |                         |
| A4                 | 0.4                   | 84                  | 6      | 7      |                         |

though the percentage was low in the A1 fractions. Since GalNAc is the predominant N-acetylatedosamine linked to serine or threonine in established structures, it is likely that GalNAcol is being recovered in the N-acetylatedosaminol region. However, we cannot exclude the presence of GlcNAcol. The fractions also contained GalNAc and GlcNAc but the ratio of GalNAc to N-acetylatedosaminol and to GlcNAc was decreased in fractions A2–A4 in the mutant cells relative to the parental cells.

Since these cells synthesize considerable amounts of chondroitin and chondroitin sulfate, it seemed likely that most of the GalNAc in fraction A1 was derived from these proteoglycans. Approximately 80% of the oligosaccharide material in parental fractions A2 and A3 and mutant fraction A2 bound to Amberlite MB-3 even after reacetylation. This charged material was considered to be most likely derived from the proteoglycans and was not analyzed further. The material in fraction A4 from parental cells and fractions A3 and A4 from the mutant cells did not bind to Amberlite MB-3. The oligo-

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\(^2\)Andreas Conzelmann, unpublished observation.
a di- to a pentasaccharide with the tetrasaccharide being the major peak (Fig. 2, B and C). No material was detected in the position expected of a hexasaccharide.

The amino sugar content of these various fractions is shown in Fig. 3. Each fraction contained N-acetylhexosaminol as would be expected if these species were derived from O-linked oligosaccharides. However, no GalNAc was detected in the tetra- and pentasaccharides from the mutant cells whereas this sugar was a prominent component of the equivalent fractions from the parental cells. GalNAc was also present in the tri- and hexasaccharides derived from the parental cells.

Isolation of [3H]Galactose-labeled Oligosaccharides—The protocol described for the preparation of [3H]glucosamine-labeled oligosaccharides was also used to prepare the analogous fractions from [3H]galactose-labeled cells. The behavior of alkaline borohydride-released oligosaccharides on HPLC is shown in Fig. 4. As in the case of [3H]glucosamine-labeled material, the major oligosaccharide from the mutant cells migrated as a tetrasaccharide and no hexasaccharide was detected. In contrast, the predominant oligosaccharide in parental cell material was a pentasaccharide and a significant hexasaccharide peak was also present.

Structure of A4b from Parental Cells—Parental fraction A4b from the HPLC column migrated as a trisaccharide and yet it contained N-acetylhexosaminol, GalNAc, GlcNAc, and galactose. This indicated that most likely consisted of a mixture of trisaccharides. The [3H]glucosamine- and the [3H]galactose-labeled fractions were therefore subjected to paper chromatography in solvent C which separated the mixture into three components, termed α, β, and γ (Fig. 5). The γ peak contained most of the [3H]galactose. In addition, hydrolysis of the [3H]glucosamine-labeled γ peak revealed the presence of N-acetylhexosaminol and GalNAc in a ratio of 1:0.85 and only a trace of GlcNAc (Table II). Taken together, these data indicate that the γ fraction consists of a trisaccharide containing N-acetylhexosaminol, galactose, and GalNAc. The α and β fractions did not appear to be pure, so they were not analyzed further (Table II).

Methylation of [3H]galactose-labeled A4b by followed by acid hydrolysis and separation of the methylated monosaccharides using thin layer chromatography gave rise to a single species which migrated with the 2,3,6-trimethylgalactose standard (Fig. 6). This indicates that the trisaccharide contains a single galactose residue substituted at C-4 by GalNAc. Since 2,3,6-trimethylgalactose does not separate well from 2,4,6-trimethylgalactose in this TLC system, an independent approach was used to confirm the C-4 substitution. An aliquot of [3H]galactose-labeled material was subjected to periodate oxidation which would destroy the galactose if the substitution is C-4 but not destroy it if the substitution is C-3. As shown in Fig. 7, periodate oxidation completely destroyed the [3H]galactose.

Aliquots of the [3H]galactose-labeled trisaccharide were also digested with a series of exoglycosidases as shown in Fig. 8. The trisaccharide was resistant to E. coli β-galactosidase (Fig. 8B), but treatment with jack bean β-N-acetylhexosaminidase converted the trisaccharide to a disaccharide which migrated with the mobility of Galβ1,3GalNAc (Fig. 8, C and F). This disaccharide can be acted upon by E. coli β-galactosidase which released [3H]galactose (Fig. 8C). These
than one GalNAc residue. The oligosaccharides also contain the trisaccharide and indicate that it is linked to the demonstrating that the GalNAc is not released by the a-N- results confirm that the GalNAc is at the nonreducing end of the trisaccharide and indicate that it is linked to the galactose. Further evidence for the b-linkage was obtained by demonstrating that the GalNAc is not released by the a-N-acetylgalactosaminidases from A. niger and C. lampas (Fig. 8, D and E, respectively).

These findings indicate that the structure of the trisaccharide is GalNAcβ1,4Galβ-N-acetylgalactosaminitol.

Partial Structure of A4e from Parental Cells—A4e eluted from the HPLC column in the position expected of a hexasaccharide. The ratio of N-acetylgalactosaminitol:GalNAc:GlcNAc was determined to be 1:1.5:0.6 (see Fig. 3). This composition indicated that the fraction consisted of a mixture of oligosaccharides with at least one of these containing more than one GalNAc residue. The oligosaccharides also contain galactose since the A4e material was isolated from [3H]galactose-labeled cells (Fig. 4). The material gave rise to a single, symmetrical peak on paper chromatography in solvent systems A and B, so it was not possible to separate the various components that were presumed to be present in the mixture. Digestion of [3H]glucosamine-labeled A4e material with β-N-acetylgalactosaminidase released 47% of the radioactivity as free GalNAc (Fig. 9). The ratio of N-acetylgalactosaminitol:GalNAc:GlcNAc in the residual oligosaccharides was 1.0:1.0:0.7 for α and 1:0.1:0.2 for β. This indicates that virtually all of the GalNAc present in the A4e oligosaccharides is in a terminal position.

Partial Structure of O-Linked Oligosaccharides from Mutant VV6 Cells—Several of the HPLC fractions derived from the mutant VV6 cells were analyzed further. Each of these fractions was obtained from [3H]glucosamine- and [3H]galactose-labeled cells, so they contain both galactose and one or more hexosamines. Fraction A4a contained a single component which co-migrated with authentic Galβ1,3GalNAc when analyzed by paper chromatography in solvents A and B (data not shown). The disaccharide was resistant to jack bean β-galactosidase which is known to be unable to cleave Galβ1,3GalNAc linkages. These data indicate that the disaccharide in A4a is probably Galβ1,3GalNAc.

Fraction A4c gave rise to a single symmetrical peak when analyzed by paper chromatography in solvents C and A (Fig. 10A). Treatment of [3H]galactose-labeled material with jack bean β-galactosidase released 56% of the radioactivity as free galactose and the residual oligosaccharide migrated in the position expected of a trisaccharide (Fig. 10B). Treatment with both jack bean β-galactosidase and β-N-acetylgalactosaminidase released the same amount of galactose (54%), but now the residual oligosaccharide co-migrated with the Galβ1,3GalNAc standard (Fig. 10C). Methylation of intact, [3H]galactose-labeled A3c gave rise to tetramethylgalactose as the sole methylated species, indicating that all of the galactose residues are unsubstituted (Fig. 6). Analysis of fraction A3c from [3H]glucosamine-labeled cells revealed the presence of N-acetylgalactosaminitol and GlcNAc (Fig. 3B). Taken together, the most likely structure of A3c is Galβ1,4Glc-
N-acetylgalactosamine has previously been found as a constituent of the very rare blood group determinant called Cad (28). Glycophorin from erythrocytes of Cad-positive individuals was shown to contain O-linked oligosaccharides with the structure GalNAcβ1,4(NeuAcα2,3)-Galβ1,3(NeuAcα2,6)GalNAc-Ser/Thr which differs from the O-linked structures on glycophorin from Cad-negative individuals only by having the additional β-linked GalNAc residue. Erythrocytes from Cad-positive individuals react strongly with antibodies against another blood group determinant, Sda, which is an antigen of varying strength present on erythrocytes of more than 90% of Caucasians. This finding led to the suggestion that Sda and Cad might be related (29).

In fact, a difference in GalNAc content of the Tamm-Horsfall urinary glycoprotein between Sda-positive and negative individuals has been reported (30) and treatment of Sda-positive Tamm-Horsfall glycoprotein with Escherichia freundii endo-β-galactosidase released a pentasaccharide that strongly inhibited the agglutination of Sda-positive erythrocytes by human anti-Sda antiserum (31). The structure of this pentasaccharide was found to be GalNAcβ1,4(NeuAcα2,3)Galβ1,4GlcNAcβ1,3Gal (31). In addition to erythrocytes and the Tamm-Horsfall glycoprotein, Sda activity has been found in urinary mucin (32) and various tissues from other species (33). β-linked GalNAc has also been detected in fish egg glycoproteins (34–36). For instance, the oligosaccharide GalNAcβ1,4Galβ1,4(NeuNGc2,3)GalNAcβ1,3Galβ1,3GalNAcol is present in trout egg glycoproteins. In this sequence the galactose substituted β1,4 by the GalNAc is not substituted by sialic acid as it is in the Cad- and Sda-positive oligosaccharides.

\[ \text{Galβ1,4} \text{-N-acetylgalactosamine residues} \]

**Discussion**

The major finding in this study is that a cloned, murine cytotoxic T cell line with numerous binding sites for the V. villosa lectin contains O-linked oligosaccharides with GalNAc residues at their nonreducing termini. The smallest of these is a trisaccharide with the sequence GalNAcβ1,4Galβ1,4-N-acetylgalactosaminyl. Since these oligosaccharides were desialyzed in order to facilitate the workup, it is probable that the native oligosaccharides also contain sialic acid residues. In sharp contrast, a V. villosa lectin-resistant clone that fails to bind this lectin contains O-linked oligosaccharides that are devoid of the GalNAc residues at the nonreducing termini. This finding suggests that these oligosaccharides on the parental cells may serve as the V. villosa binding sites.

N-acetylgalactosamine has previously been found as a constituent of cell surface glycoproteins in a variety of structural configurations. GalNAc or sialyl-GalNAc linked O-glycosidically to serine or threonine (a structure classically found on mucins) has been described on rat AH66 ascites hepatoma cells (19), on epiglycanin, which is the major surface glycoprotein of the TA3-Ha murine mammary carcinoma cell line (20), and on human Tn+ erythrocytes (21). Other sugars may be added so that a family of oligosaccharides results, as in AH66 cells, epiglycanin, glycophorin (22), the W3/13 glycoprotein of rat thymocytes (23) and the low density lipoprotein receptor (24). α-linked GalNAc also occurs as a constituent of the blood group A determinant, GalNAcα1,3[Fucose1,2]Galβ1, which is located at the nonreducing termini of oligosaccharides linked either N- or O-glycosidically to peptides or to glycolipids (25). This determinant, apart from being the antigen of blood group A-positive human red blood cells, occurs in other species such as hog (26) and rat (27). By contrast, oligosaccharides containing β-linked GalNAc residues are quite unusual. Recently, such residues have been found to be a constituent of the very rare blood group determinant called Cad (28). Glycophorin from erythrocytes of Cad-positive individuals was shown to contain O-linked oligosaccharides with the structure GalNAcβ1,4(NeuAcα2,3)-Galβ1,3(NeuAcα2,6)GalNAc-Ser/Thr which differs from the O-linked structures on glycophorin from Cad-negative individuals only by having the additional β-linked GalNAc residue. Erythrocytes from Cad-positive individuals react strongly with antibodies against another blood group determinant, Sda, which is an antigen of varying strength present on erythrocytes of more than 90% of Caucasians. This finding led to the suggestion that Sda and Cad might be related (29).

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**Fig. 6 (left). Methylation of [3H]galactose-labeled oligosaccharides.** Aliquots of A4b7 from B6.1.SF.1 (○) and A3c from VV6 (□) were methylated, hydrolyzed, and analyzed by thin layer chromatography as described under "Experimental Procedures." The standards are: 1, 2,3-di-O-methylgalactose; 2, 2,4,6-tri-O-methylgalactose; 3, 2,3,6-tri-O-methylgalactose; 4, 2,3,4,6-tetra-O-methylgalactose. The arrow indicates the expected position of 2,4,6-tri-O-methylgalactose.

**Fig. 7 (right). Periodate degradation of the [3H]galactose-labeled trisaccharide from B6.1.SF.1.** An aliquot of [3H]galactose-labeled fraction A4b (600 cpm) from B6.1.SF.1 was incubated with 0.08 M Na metaperiodate in 0.05 M sodium acetate, pH 4.6, for 20 h at 4 °C. The sample was then hydrolyzed in 2 N H2SO4 for 4 h at 100 °C, lyophilized, passed over a column of Amberlite MB-3 (0.6 x 10 cm), and chromatographed on paper in solvent B (4). The sample shown in B was treated similarly except that the periodate was not included in the original incubation. The arrow indicates the position of the galactose standard.

\[ \text{P. Stoffen, personal communication.} \]
The fact that the trisaccharide isolated from the parental cytotoxic T cells also contains the GalNAc\(\beta\)1,3Gal\(\alpha\) sequence is of particular interest. Since this material was desialylated prior to its isolation, it is possible that it contained sialic acid in its native state. This similarity suggested to us that the \(\beta\)N-acetylgalactosaminyltransferase responsible for the synthesis of the Cad and Sda determinants may also be involved in the synthesis of the \(\alpha\)-linked GalNAc-containing \(\beta\)-linked oligosaccharides on the cytotoxic T cells and that the mutant VV6 cells may be deficient in this enzyme activity. This proved to be the case as documented in the following paper (37).

In addition to the relationship of the GalNAc-containing oligosaccharides to the Cad and Sda determinants, our data suggest that these oligosaccharides may serve as the main binding sites for the V. villosa lectin. In a binding assay with purified V. villosa B4 lectin, we obtained a linear Scatchard plot and calculated that the parental cell line contained 9 \(\times\) 10^4 binding sites/cell and bound the B4 lectin with an association constant of 1.5 \(\times\) 10^7 M^-1 (data not shown). Moreover, although the lectin preparation used to select mutants contained both A and B subunits,\(^4\) pure B4 lectin was as cytotoxic for the parental cell line as the less pure preparation. While \(\alpha\)GalNAc1-\(\alpha\)Ser/Thr sequences can act as binding sites for the B4 lectin (16), the parental cytotoxic T cells contained very little of this structure relative to the larger \(\beta\)-linked oligosaccharides with the \(\beta\)-linked GalNAc. The absence of \(\beta\)-linked GalNAc-containing \(\alpha\)-linked oligosaccharides in the lectin-resistant VV6 cells provides the strongest evidence that these residues serve as B4 lectin-binding sites in this cell type. Recently, Tollefsen and Kornfeld have shown that V. villosa B4 lectin binds to Cad-positive erythrocytes whereas it is unable to bind to Cad-negative cells.\(^5\) This

\(^4\) S. Tollefsen, personal communication.

\(^5\) S. Tollefsen and R. Kornfeld, manuscript in preparation.
finding provides direct evidence that the Cad determinant serves as a V. villosa lectin-binding site. These findings demonstrate that the B, lectin is capable of binding to both α- and β-linked GalNAc residues. This is compatible with the observation of Tollefsen and Kornfeld that p-nitrophenyl α- and β-GalNAc are equally potent as inhibitors of the binding of the B, lectin to TN erythrocytes (16).

A previous analysis of desialylated O-linked oligosaccharides on the surface of murine lymphocytes revealed Galβ1,3GalNAc-Ser/Thr as the predominant structure (38). The larger O-linked oligosaccharides detected in the present study were not found.

In the following paper, we characterize the glycosyltransferase responsible for the synthesis of the β-linked GalNAc residues present on the O-linked oligosaccharides and demonstrate the absence of this enzyme in the mutant VV6 cells.

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