Blood group P1/P2 is a glycolipid antigen system for which the genetic mechanism has not yet been clarified. We analyzed the potential of the cloned Gb3/CD77 synthase to synthesize P1 antigen, because Gb3/CD77 and P1 share a common structure, GalNAC1,4Glc1,4Gal1,4Glc (NAC)–. L cell transfectants with Gb3/CD77 synthase cDNA expressed marginal levels of P1 on the cell surface but contained high levels of P1 in the cytoplasm. P2-type erythrocytes, which were serotyped as P2, also contained definite P1 antigen inside cells, although the amounts were lower than those of P1 cells. Only p erythrocytes lacked P1 antigen corresponding with functioning mutations in the Gb3/CD77 synthase gene. Synthesis of P1 antigen from paragloboside in vitro was demonstrated using membrane fraction of the transfec-
tants and a fusion enzyme with protein A. These results strongly suggested that P1 synthase is identical to Gb3/ CD77 synthase and appear to propose a clue for the solution of the long-pending P1/P2/p puzzle. The P1/P2 difference might result from the difference in P1 quantity based on either different enzyme activity or the presence/absence of other enzyme modulators. Because P2 erythrocytes showed lower levels of Gb3/CD77 synthase mRNA than P1, 5′-upstream promoter regions were analyzed, resulting in the identification of two P2-specific homozygous mutations. Differences in the transcrip-
tional regulation in erythrocytes might be a major factor determining P1/P2.

The molecular basis of many histo-blood group antigen sys-
tems such as A/B/O, Lewis a/Lewis b, XY, or Se/se has recently been clarified (1–7). However, the genetic basis of the blood group P antigen system including P, P1/P2, P6, and p has not yet been clearly disclosed. The genetic basis of P, P6 and p was recently elucidated with molecular cloning of the P6 synthase gene (α1,4-galactosyltransferase, α1,4Gal-T6; Gb3/CD77 synthase) (8–10) and P synthase gene (β1,3-N-acetylgalactosami-
nyltransferase; Gb4 synthase) (11). Namely, P6 is globotria-
syleramidase (Gb3) synthesized from lactosylceramide with the action of Gb3/CD77 synthase. Lack of Gb3 synthase results in p phenotype expressing neither Gb3 nor P (Gb4), because P is generated from Gb3 with Gb4 synthase (12). In fact, multiple mutations in the Gb3/CD77 synthase gene leading to functional loss of the enzyme activity were identified in the individuals with p phenotype (9, 13). On the other hand, P1/P2 (P1 negative) is the last glycolipid antigen system for which the genetic mechanisms has not yet been clarified, because the P1 synthase gene has not been isolated to date.

P1 is a member of the neolecto-series glycosphingolipids with α1,4-linked galactose at the non-reducing end (14) (Table I), sharing a very similar structure with P6, Gb3/CD77 antigen. This similarity of the antigen structures between P1 and Gb3/ CD77 suggests that a single α1,4-galactosyltransferase gene is responsible for the synthesis of both antigens. The P1/P2 polymorphism is linked to 22q11.3-ter (15), and the Gb3/CD77 synthase gene is assigned at 22q13.2, supporting the idea that these two syntheses are identical or closely linked. However, the approximate frequencies of P1 and P2 are 80 and 20%, respectively, in Caucasians (16, 17), and the ratio in Asians is almost the reverse. On the other hand, the occurrence of the p phenotype is very rare in all nationalities (18, 19), suggesting that the genetic basis of p and P2 phenotypes is not directly linked. Actually, no relevant polymorphic sequences in the coding region of the Gb3/CD77 synthase gene could be detected between P1 and P2 individuals, and no P1 synthase activity could be found in cell lysates transfected with a Gb3/CD77 synthase expression vector (8, 9). Consequently, it appears difficult to accept that P1 synthase and Gb3/CD77 synthase are identical. One puzzling finding is that p individuals could never synthesize or express P1 antigen, suggesting the presence of a certain linkage between P1 and Gb3/CD77.

In the present study, we analyzed the biosynthesis of the P1 antigen in transfected cells of Gb3/CD77 synthase cDNA. We
demonstrated here that Gb3/CD77 synthase could generate P1 antigen, and P2 individuals except for p were also able to synthesize P1, although the levels of the product might be different. Furthermore, we analyzed 5’-upstream regulatory regions of Gb3/CD77 synthase gene and identified two P2-specific mutations that may determine the differential expression levels of the gene in P1/P2 individuals. Thus, the genetic basis of P1 synthesis has been clarified, whereas fine mechanisms for the transcriptional regulation need to be investigated.

EXPERIMENTAL PROCEDURES

Materials—Anti-P1 monoclonal antibody (mAb) HIRO-59 (3D4) and anti-Gb4 mAb HIRO-34 (9H6) were established. Anti-paragloboside mAb H11 (20) was provided by Dr. T. Taki (Otsuka Pharmaceutical Company, Tokushima, Japan). Biotinylated anti-human IgM was from Vector Laboratories, Inc. (Burlingame, CA). Anti-Gb3/CD77 mAb 38–13 was as described previously (21). α-Galactosidase (from coffee beans) and papain were from Sigma. β-Galactosidase (from Jack beans) and Pronase were purchased from Seikagaku Corp. (Tokyo, Japan) and Calbiochem, respectively. The P1- and P2-type bloods were from healthy donors with their consent. The p (little p)-type bloods were obtained from the Department of Transfusion Medicine, Umeå University Hospital. All glycolipid structures and specificities of antibodies reactive with them used in this study are summarized in Table I.

Cell Lines—A mouse fibroblast line L cell was provided by Dr. A. P. Albino (Sloan-Kettering Cancer Center, New York) and was maintained in Dulbecco’s modified Eagle’s minimal essential medium containing 7.5% fetal bovine serum. A stable transfectant of L cells (L-VTR) was established as described previously (9) and maintained in Dulbecco’s modified Eagle’s minimal essential medium containing 7.5% fetal bovine serum and G418 (Invitrogen) (300 μg/ml).

Flow Cytometry—Cell surface expression of Gb3/CD77 and P1 was analyzed by flow cytometry (BD Biosciences) as described previously (22). mAbs 38.13 or HIRO-59 were used with fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgM or anti-human IgM (ICN Pharmaceuticals, Aurora, OH), respectively.

Glycolipid Extraction—Glycolipids were extracted as described previously (23). For TLC immunostaining, a neutral glycolipid fraction derived from 0.25 ml of each blood sample was spotted on each lane.

TABLE I

| Glycolipids                        | Structures                           | MAbs          |
|------------------------------------|--------------------------------------|---------------|
| Lactosylceramide (arrested here in p) | Galβ1,4Glc-Cer                        | HIRO-59       |
| Globotriacylsceramide (Gb3, Pk)    | Galα1,4Galβ1,4Glc-Cer                 | HIRO-34       |
| Globotetraacylsceramide (Gb4, P)   | GalNAcβ1,3Galα1,4Galβ1,4Glc-Cer       | 38–13         |
| Paragloboside (nLe4)               | Galβ1,4GlcNAcβ1,3Galβ1,4Glc-Cer       | H11           |
| P1 (lacked in P2 ?)                | Galα1,4Galβ1,4GlcNAcβ1,3Galβ1,4Glc-Cer | +             |

FIG. 1. L cell transfectants of the Gb3/CD77 synthase gene express P1 antigen. A, L cells were transfected with pMIKneo/VTR, and a transfectant was analyzed with flow cytometry using anti-P1 mAbHIRO-59 and anti-Gb3/CD77 mAb38.13 as described under “Experimental Procedures.” B, neutral glycolipid fractions from L cells and the transfectant cells (L-VTR) were separated on TLC and immunostained with mAbHIRO-59. C, the transfectants (b) and L cells (a) were stained with mAbHIRO-59 without fixation using FITC-anti-human IgM as described under “Experimental Procedures.” In d and c, they were fixed with 90% acetone and were stained with mAbHIRO-59 and FITC-conjugated goat anti-human IgM. The staining pattern was observed using a μ Radiance® confocal imaging system. Original magnification is ×200 except for d, inset (×400).

* M. Uchikawa, Y. Suzuki, T. Toyoda, and M. Satake, manuscript under preparation.
TLC and TLC Immunostaining—TLC was performed on high-performance TLC plates (Merck) using the solvent system of chloroform/ methanol/water (60:35:8) and sprayed by orcinol. The identity of P1 was confirmed by TLC immunostaining using a universal-backed silica plate (Merck) as described previously (24). After TLC, the plate was heat-blotted onto polyvinylidene difluoride membrane. After blocking in 5% skim milk in PBS, the plate was incubated with mAb and then antibody binding was detected with an ABC kit (Vector Laboratories, Burlingame, CA) combined with an enhanced chemiluminescence system (PerkinElmer Life Sciences).

Glycosidase Treatment—A neutral glycolipid fraction from erythrocytes was treated with α- or β-galactosidases according to the methods described by Bally et al. (25) with modification. Hydrolysis of neutral glycolipids was carried out with 0.05 units of α-galactosidase in 20 mM citrate-phosphate buffer, pH 5.5, containing 1 mg/ml of α-galactosidase with or without 1% BSA. Hydrolysis was carried out with 0.1 unit of β-galactosidase in 50 mM citrate-phosphate buffer, pH 3.5, with or without 1% BSA, 2 mM EDTA. After incubation for 20 h at 37 °C with shaking, the products were isolated using a C18 Sep-Pak cartridge (Waters, Milford, MA) and analyzed by TLC immunostaining using anti-Gb4 mAb HIRO-34. The intensity of the P1 bands was corrected with the intensity of the Gb4 bands using cricket graph software.

Immunofluorescence Assay and Immunocytochemistry—For immunofluorescence assay, cells were plated in 60-well plates (Greiner) in Dulbecco's modified Eagle's minimal essential medium containing 7.5% fetal bovine serum. On the following day, cells were stained with mAbs and corresponding secondary antibodies as described in flow cytometry and observed under fluorescence microscopy (BX60; Olympus, Tokyo). For immunocytochemistry, cells were plated on cover glasses. On the following day, they were fixed with 90% acetone containing 10% PBS and then were stained with anti-P1 mAb HIRO-30 and FITC-conjugated goat anti-human IgM. The staining pattern was observed using a μ Radiance® confocal imaging system (Bio-Rad).

Enzyme Treatment of Erythrocytes—Erythrocytes from P2- and p-type donors were washed with PBS and then treated with trypsin (0.25%) for 7 min at 37 °C, with papain (80 mg/ml) in 67 mM phosphate buffer, pH 5.4, for 7 min, or with Pronase (10 μg/ml) in PBS containing CaCl2 and MgCl2 at 37 °C for 30 min. After treatment, they were washed twice and then used for flow cytometry as described above.

Enzyme Assay—Gb3/CD77 synthesis activity was measured as described previously (8). The enzyme activity of α1,4Gal-T to generate P1 structure was measured as described previously (26). Briefly, membrane fractions were prepared as described (8). The reaction mixture for the assay contained the following in a volume of 50 μl: 0.2 mM UDP-Gal (Sigma), UDP-[14C]Gal (2.5 × 104 dpm)/PerkinElmer Life Sciences), 2.5 μg of 20 μM UDP-Gal, and 1 μg of phosphatidylglycerol (Sigma), and after evaporation, 20 mM sodium cacodylate-HCl, pH 6.8, 10 mM MnCl2, 20 mM galactonolactone (Sigma), 0.3% Triton X-100 (Sigma), 250 μg of α-lactalbumin, and membrane fraction containing 100 μg of protein. The protein concentration was determined by the methods of Lowry et al. (35). The products were isolated by a C18 Sep-Pak cartridge (Waters, Milford, MA) and analyzed by TLC and autoradiography using a Bio-Imaging Analyzer BAS2000 (Fuji Film, Tokyo). Reaction products were detected by TLC immunostaining using anti-P1 mAb.

Construction and Generation of α1,4Gal-T Proteins Fused with Protein A—The putative catalytic domain of α1,4Gal-T was expressed as a secreted recombinant protein encoded by a C-terminal portion of α1,4Gal-T was amplified by PCR. The PCR was performed using 5′- and 3′-primer sequences flanked with EcoRI and XhoI sequences, respectively, forming a DNA fragment that codes 306 amino acids of α1,4Gal-T at the C-terminal region. The primer sequences were as follows: forward primer, 5′-GGGAATTCCTCCAGGAGAAAGGGCAGGGTGGTGACG-3′; reverse primer, 5′-GGCTCGAGGGGAATTCCCCAAGGTTTTGGTACG-3′ corresponding to nucleotides +550 to +569 and +825 to +844 were used, and PCR was carried out as follows: 94 °C for 1 min, 24–39 cycles of 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 1 min with a final extension of 72 °C for 5 min using Taq DNA polymerase (Promega, Madison, WI). For amplification of GAPDH cDNA, the sense primer 5′-CACCCACATGGCAAAATCCGAC-3′ and the antisense primer 5′-TCTTAGAGGCGGCTAGGC-3′ were used. The reactions were performed using the following conditions: 94 °C for 1 min and then 24–36 cycles of 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 1 min, with a final extension of 72 °C for 5 min. The PCR products were electrophoresed in agarose gel and visualized using ethidium bromide. The intensities of bands of products in RT-PCR were quantified by scanning the bands in pictures of gels using the public domain NIH Image program. Relative intensities of α1,4Gal-T gene amplified from P1 and p-type erythrocytes were determined by comparing with the most intense band of P1-type individual and were plotted. For GAPDH, relative intensities of bands compared with the maximum in individual samples were determined and plotted. The cycle numbers of PCR with which band intensities reach 50% of the plateau were obtained from the graphs.

5′-Rapid Amplification of cDNA Ends (RACE) Analysis—A modified RACE analysis was performed to clone gene-specific 5′-ends using the SMART® RACE cDNA amplification kit (Clontech) according to the manufacturer’s instructions. Human prostate sample was obtained from the patients consent. First strand cDNAs were synthesized by avian myeloblastosis virus reverse transcriptase from 0.7 μg of total RNA oligo(dT) primer with two degenerate nucleotide positions at the 3′-end was used. Then, 5′-RACE was performed with primers consisting of a gene-specific primer and long or short 5′-primers as provided in the kit.
Fig. 3. **P1 structure exists in P2 erythrocytes at lower levels than in P1 cells.** A, TLC/corcinol spray pattern of neutral glycolipids extracted from pP2/P1 erythrocytes *lanes* 1–10. St contained standard Gb3 and Gb4. TLC was performed using a solvent system of chloroform/methanol/water (60:35:5). B, TLC immunostaining of the neutral glycolipids from p, P2, and P1 erythrocytes as shown in A. The results revealed the presence of P1 antigen in all P2 erythrocytes examined (as indicated by 3–7). Neutral glycolipids derived from 0.25 ml blood samples were applied in the individual *lanes*. C, intensities of P1 bands in B were quantified with NIH Image and presented as relative amounts. Note that P1 antigen in P2 erythrocytes is less than 50% of that in P1 cells.

The gene-specific antisense reverse transcription primer corresponding to nucleotides +71 to +95 (5'-ACGGTGAACTGGCCGATGATGA-3'); position +1 = A of ATG of P1,4Gal-T cDNA (8) was used. The reactions were performed by the following conditions: five cycles of 94°C for 5 s and 72°C for 3 min, five cycles of 93°C for 5 s, 70°C for 10 s, and 72°C for 3 min and then 25 cycles of 94°C for 5 s, 68°C for 10 s, and 72°C for 3 min. The amplified product was TA-cloned into pCR2.1-TOPO vector (Invitrogen) and sequenced.

**Sequence Analysis of the P1,4Gal-T Gene**—Peripheral blood samples were obtained from healthy volunteers who had given informed consent. After isolation of mononuclear cells by density gradient centrifugation, genomic DNA was extracted with 100 µl EDTA, 0.5% SDS, and 500 µg of proteinase K in 50 mM Tris-HCl, pH 8.0, overnight at 55°C. After treatment with RNase and phenol-chloroform extraction, DNA was precipitated with ethanol and dissolved in water. About 2 kb upstream genomic region from the transcriptional start site of P1,4Gal-T gene was amplified by PCR using LA Taq (Takara, Otsu, Japan). Two sets of primers used are as follows: 1) 5′-primer, 5′-TCTCGATCTCCT-GCCCTTGT-3′ (nt –1991 to –1972) and 3′-primer, 5′-CCCAAGGGCAATGAAAAATG-3′ (nt –804 to –785); 2) 5′-primer, 5′-AGCCTGTGATGGGAATTCCAGT-3′ (nt –1075 to –1056) and 3′-primer, 5′-ACAAATGTCGCCTCCAGAAC-3′ (nt +180 to +199); position +1 = G of the first nucleotide of exon 1, which was determined as the transcription initiation site by 5′-RACE. Subsequently they were subcloned into pCR2.1-TOPO vector (Invitrogen), and inserts were sequenced by the dyeoxo terminator method using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA).

**Direct Sequence**—The promoter region of P1,4Gal-T gene was amplified by PCR using genomic DNAs extracted from peripheral mononuclear cells and PfuTurbo™ DNA polymerase (Stratagene). Two sets of primers used are as follows: 1) 5′-primer, 5′-GAGTTCATTTTCTCAGTG-3′ (nt –811 to –792) and 3′-primer, 5′-GCGGAATTCCAGT-TATTTGC-3′ (nt –481 to –462); 2) 5′-primer, 5′-ATGGGGAAAACGGGATGTTTA-3′ (nt –306 to –287) and 3′-primer, 5′-ACAAATGTCGCCCTCCAGAAC-3′ (nt +180 to +199). PCR was carried out as follows: 94°C for 1 min, 35 cycles of 94°C for 30 s, annealing at 53°C for 30 s, extension at 72°C for 1 min, with final extension of 72°C for 5 min. The amplified products were sequenced directly by the dyeoxo termination method. A strong antisense product was detected in Fig. 3 lane 1, which is the expected size of the amplified 4Gal-T gene.

**Fig. 4. Digestion of the P1-like band with α- or β-galactosidases.** The identity of the bands detected in Fig. 3B was confirmed by digestion with two (α- or β-) galactosidases as described under “Experimental Procedures.” The digestion was performed with BSA (lane 1) or without enzyme (lane 2). Lane 3 is of no treatment. The products were immunostained with anti-P1 and anti-Gb4 mAbs. Only the sample treated with α-galactosidase in the presence of BSA showed reduction in the intensity of the P1 band.

**Fig. 5. P1 antigen synthesis in vitro with a cell membrane fraction of the transf ectant cells.** Enzyme assay to find the activity of P1 antigen synthesis was performed using membrane fractions for L cells (L) and a transfectant (L-VTR). Paragloboside was used as an acceptor, and UDP-Gal with/without radiolabeling was used as a sugar donor as indicated. A, an autoradiogram of the enzyme products. A strong band detected just above P1 should be Gaβ1,3-paragloboside as explained in text. B, TLC immunostaining of P1 antigen was performed as described under “Experimental Procedures.” Note that products with the membrane fraction from L-VTR showed clear bands.

**Note:** The identity of the bands detected in Fig. 3B was confirmed by digestion with two (α- or β-) galactosidases as described under “Experimental Procedures.” The digestion was performed with BSA (lane 1) or without enzyme (lane 2). Lane 3 is of no treatment. The products were immunostained with anti-P1 and anti-Gb4 mAbs. Only the sample treated with α-galactosidase in the presence of BSA showed reduction in the intensity of the P1 band.
primer, 5'-CCAAGCTTTAGCTCCAGCGG-CGGCGGGC-3' (nt 1 to 20); 2) 5'-primer, 5'-CCCTCGAGGAGGTT-CCATTTTCTCAGTG-3' (nt 811 to 792) and 3) 5'-primer, 5'-CCAAGCTT-ACAAATGTCGC-CTCCAGAAC-3' (nt 180 to 199); 3) 5'-primer, 5'-CCCTCGAGAGC-CTGTGATGGGAATGACC-3' (nt 1075 to 1056) and 3) 5'-primer, 5'-CCAAGCTTACAAATGTCGCCTCCAGAAC-3' (nt 180 to 199); position +1 = G of first nucleotide of exon 1, which was determined by 5'-RACE. PCR was carried out as follows: 94 °C for 1 min, 35 cycles of 94 °C for 30 s, annealing at 53 °C for 30 s, and then extension at 72 °C for 1 min, and 72 °C for 5 min. Subsequently PCR products were TA-cloned into pCR2.1-TOPO vector, and inserted were sequenced for confirmation. Inserts were digested with XhoI and HindIII restriction enzymes and cloned into the XhoI and HindIII sites of the pGL3-Basic vector (Promega).

Luciferase Assay for Promoter Activity—The Dual Luciferase™ reporter assay (Promega, Madison, WI) was used to evaluate the promoter activity. Luciferase expression vectors described above were co-transfected with pRL-TK vector for normalization of transfection efficiency into NCC-IT cells using LipofectAMINETM (Invitrogen). After 48 h of transfection, the medium was removed, the cells were washed twice with PBS, and lysates were prepared to independently measure luciferase activity. The luciferase assay was performed using Pica

**Fig. 6. P1 antigen synthesis with Prot/α1,4Gal-T soluble enzyme.** A soluble form of Gb3/CD77 synthase, Prot/α1,4Gal-T (PA/GalT), was generated as described under “Experimental Procedures” and used for in vitro enzyme assay. A, an autoradiogram of the enzyme products performed under the conditions either for Gb3 synthase or P1 synthase as indicated. Concentrated culture medium from L cells transfected with a control vector (mock) or PA/GalT was used, as well as an extract from L-VTR. B, long exposure of the plate was performed, and a faint band was found in the sample with PA/GalT. C, TLC immunostaining of the same plate in A using an anti-P1 mAb was performed as described under “Experimental Procedures.”

**Fig. 7. Quantitative comparison of mRNA levels of Gb3/CD77 synthase gene.** RT-PCR was performed to compare the mRNA levels of Gb3/CD77 synthase gene between P1 and P2 erythrocytes as described under “Experimental Procedures.” Three P1 and three P2 samples were compared with 24, 27, 30, 33, 36, and 39 cycles of PCR for α1,4Gal-T gene (upper) and the similar cycles for GAPDH (lower) as a control. The gel was stained with ethidium bromide, and the picture was taken under UV. A, examples of one each of P1 and P2 cases. B, plots of the relative intensities of bands measured as described under “Experimental Procedures.” Calculated cycles showing 50% of relative intensities for Gb3/CD77 synthase gene and GAPDH gene were compared in Table II.
Gene™ (Toyo Ink Corp., Tokyo) according to the manufacturer’s instructions. The ratio of firefly luciferase activity to Renilla luciferase activity was calculated.

Statistical Analysis—Significance of the obtained luciferase activities was examined with Student’s t test, taking the case of $p < 0.05$ as statistically significant.

RESULTS

Expression of P1 Antigen on Gb3/CD77 Synthase Gene Transfectant Cells—L-VTR1 cells (transfectant cells with pMII/neo-VTR-1) expressed a high level of Gb3/CD77 antigen as expected (Fig. 1A, left). In these cells, P1 was also expressed, although the positive population was low (Fig. 1A, right).

P1 Antigen Was Significantly Synthesized in Gb3/CD77 Gene Transfectant Cells—To analyze the presence of P1 antigen in the transfected cells of Gb3/CD77 synthase cDNA, glycolipids were extracted from the parent L cells and from L-VTR and served for TLC immunostaining. L-VTR cells definitely showed a clear band stained with anti-P1 mAb as did the extracts from erythrocytes of P1-type individuals (Fig. 1B).

P1 Antigen Was Present Mainly in Cytoplasm—To examine the localization of P1 antigen in the transfectant cells, immunocyto-staining was performed for unfixed and fixed cells using anti-P1 mAb. In immunofluorescence assay, a few cells in the transfec-ants were stained, whereas L cells were completely negative (Fig. 1C, a and b). On the other hand, the majority of fixed L-VTR cells were clearly stained, and P1 antigen appeared to be localized more abundantly in cytoplasm rather than at the surface membrane (Fig. 1C, d).

Expression of P1 Antigen in pP1/p2 Group Erythrocytes—Serological analysis of P1 antigen expression on various erythrocytes was performed with flow cytometry. P1 erythrocytes showed fairly high levels of P1 expression, whereas P2 cells showed completely negative or marginal levels of P1 antigen (Fig. 2A). Erythrocytes from p individuals were also negative as expected.

P1 Antigen in P2 Erythrocytes Is Cryptic—To analyze the crypticity of P1 antigen in erythrocytes, p and P2 erythrocytes were treated by three kind of proteases and then P1 antigen expression was analyzed by flow cytometry compared with untreated cells. Only P2 erythrocytes showed low levels of P1 antigen on the cell surface after treatment with papain or Pronase (Fig. 2B), suggesting that P2 erythrocytes contained cryptic P1 antigen, which might be masked with cell surface proteins.

P2 Erythrocytes Contain P1 Antigen at Lower Levels Than P1 Erythrocytes—To examine the presence/absence of P1 antigen in p and P2 individuals, glycolipids of erythrocytes from two p donors, in whom function-losing mutations were found in the Gb3/CD77 synthase gene (13), and five P2 samples, as well as three P1 samples, were analyzed. In TLC, Gb3 and Gb4 were not found in the two p samples, whereas bands of lactosylceramide were increased in intensity as detected with orcinol (Fig. 3A). In TLC immunostaining, all five P2 individuals showed definite bands migrating at the same levels of the P1 bands detected in P1 individual erythrocytes with anti-P1 mAb (Fig. 3B). However, the intensity of the bands in P2 erythrocytes was generally weaker than that in P1 erythrocytes, suggesting the difference in the P1 quantity between P1 and P2 erythrocytes. Neutral glycolipids from p individuals showed no P1 band (Fig. 3B), confirming that p individuals really lack P1 antigen. The relative levels of P1 antigen contained in various erythrocytes as determined in TLC immunostaining are summarized in Fig. 3C.

| Genes          | P1(1) | P1(2) | P1(3) | P2(1) | P2(2) | P2(3) |
|----------------|-------|-------|-------|-------|-------|-------|
| a,4GalT       | 30.8% | 31.7% | 28.0% | 36.0% | 42.0% | 39.0% |
| (B) GAPDH      | 27.2% | 25.5% | 24.8% | 26.6% | 27.7% | 26.7% |
| (A)+(B)        | 3.6%  | 6.2%  | 3.2%  | 9.4%  | 14.3% | 12.3% |

* Cycles at which band intensities reach 50% of the maximum. An example is presented in Fig. 7.
digest the band stained with anti-P1 mAb (Fig. 4), confirming that the component contained α-linked galactose at the non-reducing end.

**Enzyme Assay with Membrane Fraction**—Enzyme assay to detect P1 synthase activity was performed with extracts from L cells or an L cell transfectant (L-VTR) with/without UDP-[14C]Gal (in the presence of cold UDP-Gal). An autofluorogram revealed a common strong band at the migration site close to that of P1. This band seemed to be digested by anti-P1 mAb (Fig. 4), confirming that the component contained α-linked galactose at the non-reducing end.

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**Enzyme Assay with Membrane Fraction**—Enzyme assay to detect P1 synthase activity was performed with extracts from L cells or an L cell transfectant (L-VTR) with/without UDP-[14C]Gal (in the presence of cold UDP-Gal). An autofluorogram revealed a common strong band at the migration site close to that of P1. This band seemed to be digested by anti-P1 mAb (Fig. 4), confirming that the component contained α-linked galactose at the non-reducing end.
Direct Sequencing Revealed Two Mutations That Clearly Distinguish P1 and P2—To avoid the PCR errors and artificial deviations to either clones, we directly sequenced the PCR products with two sets of primers that covered either upper four candidate sites or lower two candidate sites. Finally, two mutations distinctly found in P2 samples were identified, i.e., an insertion (551C–550) and a mutation (A160G) (Table IV). Both of them were homozygous mutation in P2. Actual sequencing results were shown in Fig. 9. The expression levels of Gb3/CD77 synthase gene should be differentially regulated with the sequence differences in one or both of these mutational regions.

Promoter Activity in the Upstream Region of Gb3/CD77 Synthase Gene—By constructing three kinds of luciferase vectors inserting the regulatory regions from P1 and P2, all of which contained the mutation sites, luciferase assay was performed. Among many cell lines, we selected NCC-IT (germ cell tumor) as a recipient cell of the transfection, because it expressed Gb3/CD77 synthase gene at a very high level and showed high transfection efficiency (data not shown). To our surprise, no essential differences in the promoter activities were detected in any of three constructs between P1 and P2 genes (Fig. 10).

DISCUSSION

Although P1 and P2 blood groups have been considered to be absolutely distinct from each other based on the hemagglutination typing, substantial differences in the presence/absence of P1 antigen could not be recognized in the results obtained in the present study. The most puzzling and interesting point has been the relation between P2 and p (27). The results in the present study showed that P2 erythrocytes can synthesize P1 antigen and can express marginal levels of P1. Therefore, it appears quite reasonable to consider that P1 synthase and Gb3/CD77 synthase are encoded by a single gene located at chromosome 22q13.2. Results of L-VTR strongly supported this point. It also turned out that only p individuals are unable to synthesize either Gb3/CD77 or P1 antigen, and P1 and P2 erythrocytes have other quantitative or qualitative differences in the enzyme levels or relevant factors than in the coding region of the a1,4Gal-T gene.

Steffensen et al. (9) suggested three possibilities about the puzzling issue of P1/P2/p: 1) a1,4Gal-T gene can function as a P1 synthase in the presence of another polymorphic gene product; 2) a second polymorphic a1,4Gal-T gene, which is non-homologous to the a1,4Gal-T gene, exists; or 3) an alternatively spliced version of the a1,4Gal-T gene can function as a P1 synthase. The results obtained in the present study suggest that the first explanation is most likely, provided the difference between P1 and P2 is based on an unknown factor regulating the surface expression of P1 antigen. However, the capability of the surface expression of P1 might be determined simply by the
amount of P1 generated in erythrocytes as shown in Fig. 3C. The findings of Fletcher et al. (28) that P1 erythrocytes contain more Gb3 and less lactosylceramide than P2 strongly suggest that enzyme activity of Gb3/CD77 synthase is higher in erythrocytes of P1 individuals than those of P2, suggesting that the same enzyme synthesizes P1 antigen with higher efficiency in P1 erythrocytes. When the amount of P1 antigen in erythrocytes becomes greater than some threshold, the antigen may be exposed at an adequate level to be recognized by anti-P1 antibodies. The finding observed by Bailly et al. (26) that kidney extracts from P2 could not synthesize P1 antigen might be because of the relatively low activity of Gb3/CD77 synthase in P2 individuals.

Consequently, the final issue is whether the P1/P2 difference is only because of the quantity of the P1 antigen and what the mechanisms are that cause the different levels of Gb3/CD77 enzyme activity. Because the coding sequence of the Gb3/CD77 synthase gene showed no mutations corresponding to the polymorphism of P1/P2 (9, 13), either the regulatory region of the gene such as promoter/enhancer or the presence/absence of another factor including transcription factors or enzyme modifiers should be responsible for the P1/P2 polymorphism.

The results of quantitative RT-PCR indicated that the expression levels of Gb3/CD77 gene were generally lower in P2 erythrocytes than in P1 and might cause the distinction between P1 and P2 phenotypes. The analysis of 5′-upstream regions of Gb3/CD77 synthase gene revealed that there is a distinct allele that contains two mutations, i.e., one insertion and one mutation. These two mutations exist on one allele and appear as homozygous in P2 individuals and heterozygous in P1 individuals. These results are in good accordance with the fact that P1 phenotype is dominant, and P1/P2 phenotypes follow Mendelian inheritance. Because these mutation sites are at very distant distances from the transcription initiation sites as the regulatory regions for the gene expression, we expected that the luciferase activity of P1-derived sequence would be much higher than that of P2-derived one. However, comparison of the promoter activity in these regions showed no significant differences in repeated experiments. The most likely explanation for this discrepancy between the presence/absence of mutations and similar promoter activities is the difference of cell lineage between erythrocytes and NCC-IT used in the transfection. Namely, the mutations in P2 might affect the transcriptional efficiency only in erythrocytes. If this is the case, promoter activities of the regulatory regions should be investigated using P1/P2 erythrocytes at an immature stage, although it is technically hard. If the P1/P2 blood group is based mainly on the differences in the gene regulatory region, it should be the first example among blood group carbohydrate antigens in terms that the defects in the responsible glycosyltransferase are not in the structure of the coding region.

Results of flow cytometry analysis, immunocytostaining, and protease treatment of P2 erythrocytes suggest that the major portion is expressed on the membrane with a cryptic nature. However, it is interesting that even P1 antigen belonging to the neurato-series of glycolipids often be have as a cryptic antigen similar to many other erythrocyte antigens (32–34). The general regulatory mechanisms for the determination of the intracellular localization and membrane crypticity of glycolipids remain to be investigated. The biological implications of P/Pk/P1 antigens are not well understood at present; however, many of these antigens including P1 are considered to be involved in urinary tract infection (29). The findings of the present study could greatly promote the analysis of the molecular mechanisms for the initial phase of bacterial infection and also the clinical application of glycosyltransferase genes and their products in the manipulation of disastrous infection.

The enzyme activity in vitro has been difficult to detect. In the past experiments of our group and others (8, 9), the trial to find P1 synthase activity resulted in failure. The fact that in vitro synthesis of P1 antigen is not well achieved has disturbed fine analysis of the substrate specificity of Gb3/CD77 synthase. However, the assay system of Bailly et al. (26) enabled us to detect the P1 synthesis activity with either membrane fraction of the transfected cells or the fusion proteins with protein A. P1 antigen generated in the in vitro assay was clearly detected only with a specific mAb, suggesting that the assay condition was not optimal, and the best conditions for the synthesis of Gb3/CD77 and P1 appear fairly different as shown in Fig. 4. Various factors determining the substrate specificity of Gb3/CD77 synthase, such as pH, cationic ion, detergent, and phospholipids are now under investigation. The fact that no cell lines except for P1-type erythrocytes definitely express P1 antigen suggests that there might be an important factor(s) that is exclusively present in erythrocytes and enables Gb3/CD77 synthase to generate P1 structure in erythrocytes. Furthermore, the results of the promoter assay strongly suggested the presence of erythrocyte-specific transcriptional regulation for Gb3/CD77 synthase gene. Thus, tissue-specific regulatory mechanisms for the function of Gb3/CD77 synthase at the transcriptional level and with enzymologic environments are quite important and remain to be investigated.

REFERENCES

1. Yamamoto, F., Clausen, H., White, T., Marken, J., and Nakomori, S. (1990) Nature 345, 229–233
2. Rajan, V. P., Larsen, B. D., Ajmera, S., Ernst, L. K., and Lowe, J. B. (1989) J. Biol. Chem. 264, 11158–11167
3. Kukowska-Latallo, J. F., Larsen, R. D., Nair, R. P., and Lowe, J. B. (1990) Gene 94, 1288–1303
4. Larsen, R. D., Ernst, L. K., Nair, R. P., and Lowe, J. B. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6674–6678
5. Nishihara, S., Narimatsu, I., Iwasaki, H., Yazawa, S., Akamatsu, S., Ando, T., Seno, T., and Narimatsu, I. (1994) J. Biol. Chem. 269, 29271–29276
6. Kudo, T., Iwasaki, H., Nishihara, S., Shinya, N., Ando, T., Narimatsu, I., and Narimatsu, H. (1996) J. Biol. Chem. 271, 9830–9837
7. Kano, M., Nishihara, S., Shinya, N., Kudo, T., Iwasaki, H., Seno, T., Okubo, Y., and Narimatsu, H. (1997) Blood 90, 839–849
8. Kojima, Y., Fujimoto, S., Furukawa, K., Okajima, T., Wels, J., Yosakaya, K., Suzuki, Y., Urao, T., Otta, M., and Furukawa, K. (2000) J. Biol. Chem. 275, 15152–15156
9. Steffensen, R. C., Carlier, K., Wels, J., Levery, S. B., Stroud, M., Cedergren, B., Nilsson, S. B., Bennett, E. P., Jersild, C., and Clausen, H. (2000) J. Biol. Chem. 275, 16723–16729
10. Leuch, J. J., Manzella, S. M., Nyame, K. A., Cummings, R. D., and Baenziger, J. U. (2000) J. Biol. Chem. 275, 25315–25321
11. Okajima, T., Nakamura, T., Uchikawa, M., Haslam, D. B., Numata, S. I., and Furukawa, K. (2000) J. Biol. Chem. 275, 40498–40503
12. Naiki, M., and Marcus, D. M. (1975) Biochemistry 14, 4387–4481
13. Furukawa, K., Iwamura, K., Uchikawa, M., Sjöka, B., N., Wels, J., Okajima, T., Urao, T., and Furukawa, K. (2000) J. Biol. Chem. 275, 37752–37756
14. Nishihara, S., Narimatsu, H., and Iwasaki, H. (1994) J. Biol. Chem. 269, 6289–632
Zelinski, T. (1999) Vox Sang. 77, 52–57
18. Watkins, W. M. (1980) Adv. Hum. Genet. 10, 1–136
19. Marcus, D. M. (1989) Immunol. Ser. 43, 701–712
20. Myoga, A., Taki, T., Arai, K., Sekiguchi, K., Ikeda, I., Kurata, K., and Matsumoto, M. (1988) Cancer Res. 48, 1512–1516
21. Wiels, J., Fellous, M., and Tursz, T. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 6483–6488
22. Yamashiro, S., Haraguchi, M., Furukawa, K., Takamiya, K., Yamamoto, A., Nagata, Y., Lloyd, K. O., Shiku, H., and Furukawa, K. (1995) J. Biol. Chem. 270, 6149–6155
23. Furukawa, K., Clausen, H., Hakomori, S., Sakamoto, J., Look, K., Lundblad, A., Mattes, M. J., and Lloyd, K. O. (1995) Biochemistry 24, 7820–7826
24. Ishikawa, D., and Taki, T. (2000) Methods Enzymol. 312, 157–159
25. Bailly, P., Chevalley, J., Sandag, D., Francois-Gerard, C., Piquet, Y., Vezon, G., and Carton, J.-P. (1997) Mol. Immunol. 24, 171–176
26. Bailly, P., Pillier, F., Gillard, B., Veyrieres, A., Marcus, D., and Cartron, J. P. (1992) Carbohydr. Res. 228, 277–287
27. Watkins, W. M., and Morgan, W. T. (1976) J. Immunogenet. 3, 15–27
28. Fletcher, K. S., Bremer, E. G., and Schwarting, G. A. (1979) J. Biol. Chem. 254, 11196–11198
29. Lomberg, H., Jodal, U., Eden, C. S., Leffler, H., and Samuelsson, B. (1981) Lancet i, 551–552
30. Wiels, J., Holmes, E. H., Cochran, N., Tursz, T., and Hakomori, S. (1984) J. Biol. Chem. 259, 14783–14787
31. Furukawa, K., Yokoyama, K., Sato, T., Wiels, J., Hirayama, Y., Ohta, M., and Furukawa, K. (2002) J. Biol. Chem. 277, 11247–11254
32. Dahr, W., Uhlenbruck, G., and Bird, G. W. (1974) Vox Sang. 27, 29–42
33. Rearden, A., and Maseuredis, S. P. (1981) Vox Sang. 41, 160–164
34. Feizi, T., Childs, R. A., Hakomori, S. I., and Powell, M. E. (1978) Biochem. J. 173, 245–254
35. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275