Cloning, Expression, and Characterization of a Novel UDP-galactose:β-N-Acetylglucosamine β1,3-Galactosyltransferase (β3Gal-T5) Responsible for Synthesis of Type 1 Chain in Colorectal and Pancreatic Epithelia and Tumor Cells Derived Therefrom*

(Received for publication, November 30, 1998, and in revised form, February 1, 1999)

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The sialyl Lewis α antigen is a well known tumor marker, CA19-9, which is frequently elevated in the serum in gastrointestinal and pancreatic cancers. UDP-galactose:N-acetylglucosamine β1,3-galactosyltransferase(s) (β3Gal-Ts) are required for the synthesis of the sialyl Lewis α epitope. In the present study, a novel β3Gal-T, named β3Gal-T5, was isolated from a Colo205 cDNA library using a degenerate primer strategy based on the amino acid sequences of the four human β3Gal-T genes cloned to date. Transfection experiments demonstrated that HCT-15 cells transfected with the β3Gal-T5 gene expressed all the type 1 Lewis antigens. In gastrointestinal and pancreatic cancer cell lines, the amounts of β3Gal-T5 transcripts were quite well correlated with the amounts of the sialyl Lewis α antigens. The β3Gal-T activity toward agalacto-lacto-N-neotetraose was also well correlated with the amounts of β3Gal-T5 transcripts in a series of cultured cancer cells, and in Namalwa and HCT-15 cells transfected with the β3Gal-T5 gene. Thus, the β3Gal-T5 gene is the most probable candidate responsible for the synthesis of the type 1 Lewis α antigens in gastrointestinal and pancreatic epithelia and tumor cells derived therefrom. In addition, β3Gal-T5 is a key enzyme that determines the amounts of the type 1 Lewis α antigens including the sialyl Lewis α antigen.

CA19-9 in serum is a well known tumor marker, which is frequently used for the clinical diagnosis of cancer, in particular, colorectal, pancreatic, and gastric cancers (1, 2). The 1116NS19-9 (19-9)1 monoclonal antibody detects a CA19-9 antigen, of which the antigenic epitope has been defined as the carbohydrate structure of sialyl Lewis α (αLeα) (2–4). Besides its usefulness as a tumor marker, αLeα antigen is known to be a ligand for selectins (5, 6). Clinical statistical analysis demonstrated that cancer patients who express abundant αLeα antigens have a worse prognosis as to liver metastasis than patients who do not express αLeα antigens (7, 8). Thus, it is of interest that αLeα antigens may confer some metastatic capacity on cancer cells.

At least three glycosyltransferases are required for the synthesis of the αLeα epitope. First, N-acetylglucosamine:β1,3-galactosyltransferase (β3Gal-T) transfers a galactose (Gal) to an N-acetylglucosamine (GlcNAc) with a β1,3-linkage, resulting in the synthesis of a type 1 chain, Galβ1GlcNAc, and then galactose:α2,3-sialyltransferase (ST3Gal) transfers a sialic acid (SA) to the Gal residue of the type 1 chain with an α2,3-linkage, resulting in sialyl-type 1 (sialyl Lewis a; sLeα) chain, SAα2Glcβ1GlcNAc. Finally, α1,3-fucosyltransferase (Fuc-TII, FUT3, Lewis enzyme) transfers a fucose (Fuc) to the GlcNAc residue of the sialyl-type 1 chain with an α1,4-linkage to complete the synthesis of the structure, SAα2Glcβ1Fucα1GlcNAc. Previous studies, demonstrated that Fuc-TII (FUT3) is the only enzyme determining the expression of αLeα antigens in colorectal cancer (3, 4), and that ST3GalIV, one of the ST3Gals, mainly participates in the αLeα synthesis in colorectal cancer (9).

Regarding β3Gal-Ts, we have reported for the first time the cloning of a β3Gal-T gene from human WM266–4 melanoma cells using an expression cloning method (10). The recent rapid growth of data bases of expressed sequence tags (ESTs) and the Human Genome Project enabled us to find novel genes homologous to the original one. Thus, three human β3Gal-T genes homologous to the original one were cloned very recently (11, 12). The four β3Gal-Ts, including the original one, are named β3Gal-T1 to -T4 (12). Expression studies on the four human β3Gal-Ts demonstrated that two of them, β3Gal-T1 and T2, apparently transfer Gal to GlcNAc with a β1,3-linkage resulting in type 1 chain synthesis, but β3Gal-T4 transfers Gal to an N-acetylglucosamine:β1,3-galactosyltransferase; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose.

* This work was supported in part by Grant-in-aid for Scientific Research on Priority Areas 10178104, from the Ministry of Education, Science, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: 19-9, 1116NS19-9; αLeα, sialyl Lewis α;
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N-acetylgalactosamine (GalNAc) residue, resulting in the synthesis of the type 3 chain, Galβ3,3GalNAc (12). The human β3Gal-T4 did not transfer Gal to a GlcNAc residue for the type 1 chain synthesis (12). The human β3Gal-T4 is likely to be the human homologue of the rat GM3/GD1 synthase (13), since the amino acid sequence of human β3Gal-T4 shows very high homology, 79.4%, to that of the rat GM3/GD1 synthase, and the human β3Gal-T4 apparently transfers Gal to the GalNAc residue of asialo-GM2 and GM2, resulting in the asialo-GM1 and GM1 synthase, respectively (12). The activity of human β3Gal-T3 has not been detected toward any of the acceptor substrates used in their study (12). Three mouse β3Gal-T genes have been cloned and named mβ3GalT-I, mβ3GalT-II, and mβ3GalT-III, corresponding to human β3Gal-T1, β3Gal-T2, and β3Gal-T3, respectively (14). mβ3Gal-TII and mβ3Gal-III were found to exhibit the β3Gal-T activity toward both GlcNAc and GalNAc residues; however, they showed quite low activities for the type 1 chain synthesis, i.e., about 3% of the activity of mβ3Gal-TI (14).

It has not been elucidated which β3Gal-T determines the expression of the sLeα epitopes in gastrointestinal and pancreatic cancers. The tissue distributions of the four β3Gal-Ts were determined by Northern analysis (11, 12), it being found that neither β3Gal-T1 nor -T2 is expressed in the pancreas, which indicated that there may be unknown β3Gal-T(s) synthesizing the type 1 chain in the pancreas. They did not examine the expression of those β3Gal-Ts in the gastrointestinal tissues, such as colon and stomach, which frequently produce the sLeα antigens when they become cancerous.

In this study, we first noticed that none of the four human β3Gal-Ts cloned to date, β3Gal-T1 to -T4, is responsible for the sLeα expression in gastrointestinal and pancreatic cancers, and successfully cloned a novel β3Gal-T gene, named β3Gal-T5, from Colo205 cells. β3Gal-T5 is the most probable candidate participating in the synthesis of the sLeα epitopes, i.e. CA19-9 antigens, in gastrointestinal and pancreatic cancer cells.

**Experimental Procedures**

**Tumor Cell Lines and Monoclonal Antibodies**—Various tumor cell lines were cultured in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum. The monoclonal antibodies used in this study were as follows: 1116NS19-9 (19-9), which was used for the detection of tumor marker CA19-9, anti-sLeα (lgG) (2); DU-PAN-2, anti-Leα (lgG) (4); 7LE, anti-Leα (lgG) (16); and TT42, anti-Leα (lgM), of which the specificity against Leα was recently defined by us, and will be published elsewhere. 19-9 and TT42 were kind gifts from Fujirebio Inc. (Tokyo, Japan), and Diagnostic Division, Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan), respectively. DU-PAN-2 and 7LE were purchased from Kyowa Medex Co., Ltd. (Tokyo, Japan), and Seikagaku-Kogyo Co., Ltd. (Tokyo, Japan), respectively.

**Cloning of the Four Cloned β3Gal-T Genes from Various Human cDNA Libraries and Construction of Expression Plasmids**—The cDNA encoding β3Gal-T1 was cloned by the expression cloning method used in our previous study (10). We found three sequences homologous to that of β3Gal-T1 in the EST data bases. The full-length cDNAs encoding the other three homologous sequences were cloned from various human cDNA libraries using probes encoding the fragment sequence in the EST data base. They were identical to β3Gal-T2, -T3, and -T4, which were reported by Amado et al. (12), and Kolbinger et al. (11).

Thus, the four human cDNAs encoding the respective full-length open reading frames (ORFs) of β3Gal-T1, -T2, -T3, and -T4 were subcloned into the pMa vector for expression in cultured cells (17, 18).

**Construction of a cDNA Library from Colo205 Cells**—Total cellular RNA was isolated from Colo205 cells using acid guanidium thiocyanate-phenol-chloroform method (19). Poly(A)^+^-rich RNA was isolated with OligotexTM-dT30 (Super) (Roche, Tokyo, Japan). Complementary DNAs were synthesized with oligo(dT) primers from poly(A)^+^-rich RNA using a Superscript Choice System for cDNA Synthesis (Life Technologies, Inc.). A cDNA library was constructed by inserting size-fractionated cDNAs (more than 1.5 kilobase pairs) into an expression vector, pMaO, using SfiI adaptors (17, 18). We obtained about 1 × 10^6 independent clones as a cDNA library and extracted plasmid DNAs from the library.

**Cloning of a Fragment Encoding a Novel β3Gal-T—**On alignment of the amino acid sequences of the four cloned β3Gal-Ts, we found conserved amino acid sequences at three positions, and named them Motif 1, 2, and 3 (Table I). The conserved amino acid sequences of Motifs 1, 2, and 3 were employed for the design of degenerate primer sequences, i.e., primers 1, 2, and 3 (Table I). The amplified PCR products were inserted into a pBluescript KS (-) (pBS) vector (Stratagene, La Jolla, CA), and the DNA fragments obtained were sequenced by the dideoxynucleotide chain termination method using an ALF DNA sequencer. We obtained a genome sequence from the Genome Project and found a genome sequence of the cloned β3Gal-T gene. On an additional PCR involving Colo205 cDNAs as templates using primers encompassing the two fragment sequences, both fragments were found to be encoded by one species of cDNA.

**Cloning of Full-length cDNAs Encoding a Novel β3Gal-T**—The two DNA fragments obtained through the two PCRs, i.e., those with the Motif 1 and 2 primers, and the Motif 2 and 3 primers, respectively, were mixed and used as the probe for hybridization to isolate full-length cDNA clones. We screened the Colo205 cDNA library and isolated several distinct clones having inserts of different sizes. All inserts encoded the same sequence of one species of cDNA, this sequence being found to be homologous to those of the known four β3Gal-Ts. Thus, we named this novel gene the human β3Gal-T5 gene. After the cDNA sequences had been completed, we searched the data base of the Human Genome Project to determine whether the same sequence or homologous ones were registered or not. We found a genome sequence completely identical to the cDNA sequences in the data base, which was very recently registered (June 2nd, 1998). Its registration number is AF064860. By comparison between the cDNA sequences and the genome one, we determined the genomic organization of the β3Gal-T5 gene.

**Identification of Alternatively Spliced Isoforms of β3Gal-T5 Transcripts and the Transcription Initiation Site**—By the 5’-rapid amplification of cDNA ends (5’-RACE) method using two primers, i.e. si-1 (5’-GAAAAGTATGAGCTGATGCCG-3’), which was positioned close to the ATG codon in the ORF, and si-2 (5’-GGTGAAATCTTCTGTCTGTCG-3’), we obtained five different types of amplified fragments, and subcloned them into a pBS (-) vector for sequencing. Thus, five isoforms, isoforms 1, 2, 3, 4, and 5, of β3Gal-T5 transcript were identified. Further RT-PCR experiments were performed to determine the abundance of each isoform of the β3Gal-T5 transcript expressed in Colo205 cells, for which the following primers were employed, primers si-1, si-2, si-3 (5’-GAAAAGTATGAGCTGATGCCG-3’), and si-4 (5’-GAAAAGTATGAGCTGATGCCG-3’).

**Quantitative Analysis of the five β3Gal-T5 Transcripts in Human Tumor Cell Lines and Human Tissues by Competitive RT-PCR**—The principle of the competitive RT-PCR method was described in detail in our previous papers (9, 18). Competitor DNA plasmids each carrying a small deletion within the respective full-length ORF cDNA were constructed by appropriate restriction enzyme digestion, as shown in Table II. For instance, a competitor DNA plasmid of the β3Gal-T1 gene was prepared by deleting the 212-bp BsmI-EcoRV fragment from the standard plasmid DNA containing the full-length cDNA of β3Gal-T1.

Total cellular RNA was isolated from various tumor cell lines and human tissues. Complementary DNAs were synthesized with an oligo(dT) primer from 6 μg of RNA treated total RNA in a 20-μl reaction. The amplified products were subcloned into a pMa vector.
TABLE II

| Target gene | Primer sets | Sizes of PCR products | Restriction enzymes for competitor DNA | Annealing temperature (°C) |
|-------------|-------------|-----------------------|---------------------------------------|--------------------------|
| β3Gal-T1    | F: 5’-TTAGGGACCTAATTGATCGCTAAAG-3’ | 495 | BanII-EcoRV | 60 |
| β3Gal-T2    | R: 5’-AATCCCTCTTTGACCTGCGGAGGAG-3’ | 283 |                          |  |
| β3Gal-T3    | F: 5’-ACCTGGCCGAGTGTGAACACAAAC-3’ | 461 | AfIII-BstII | 60 |
| β3Gal-T4    | R: 5’-ACCTGCGGAGTGTGAACACAAAC-3’ | 358 | StyI | 60 |
| β3Gal-T5    | F: 5’-ACCACCGGAGCTGCGCGGAGAAGGGA-3’ | 291 | AccIII-Styl | 68 |
| β-Actin     | R: 5’-ACGAGCAGCGAGCGGAGGAGGGA-3’ | 337 | EcoRII-XcmI | 65 |

a F, forward primer; R, reverse primer.

Discrepancy between the Expression Levels of three β3Gal-T Transcripts, i.e. the β3Gal-T1, -T2, and -T3 Transcripts, and the Amounts of Type 1 Lewis Antigens Expressed in Various Tumor Cells—Various tumor cell lines derived from different human tissues were examined as to the transcript levels of the four β3Gal-T genes that were cloned previously, and their expression levels were compared with the amounts of type 1 Lewis antigens, i.e. the sLeα (19-9), Leα (7LE), and Leβ (TT42) antigens, expressed in these cancer cells (Fig. 1). Flow cytometry analysis revealed that Colo205, Colo201, and SW1116 (colon cancer) cells, and Capan-2 (pancreatic cancer) cells expressed large amounts of type 1 Lewis antigens, whereas Capan-1 cells expressed slightly lower levels. The amounts of type 1 Lewis antigens in these cancer cells were measured by a flow cytometry assay, and the expression levels were compared with the amounts of type 1 Lewis antigens in the four β3Gal-T genes that were cloned previously. The results showed that the expression levels of the four β3Gal-T genes were comparable with the amounts of type 1 Lewis antigens in these cancer cells. The expression levels of the four β3Gal-T genes were also compared with the amounts of type 1 Lewis antigens in the four β3Gal-T genes that were cloned previously. The results showed that the expression levels of the four β3Gal-T genes were comparable with the amounts of type 1 Lewis antigens in these cancer cells. The expression levels of the four β3Gal-T genes were also compared with the amounts of type 1 Lewis antigens in the four β3Gal-T genes that were cloned previously. The results showed that the expression levels of the four β3Gal-T genes were comparable with the amounts of type 1 Lewis antigens in these cancer cells.
blastoma) cells at intermediate levels, and faintly expressed in some cell lines from gastrointestinal cancers. β3Gal-T3 was abundantly expressed in some gastric cancer cells, i.e., KATO III and MKN45 cells, and PC-3 cells, and intermediately expressed in Capan-1 and Capan-2 (pancreatic cancer) cells, HCT-15 cells, and SK-N-MC and SK-N-SH (neuroblastoma) cells; however, it was not detected at all in Colo201 or Colo205 cells.

We observed a significant discrepancy between the expression of the three β3Gal-Ts, i.e., β3Gal-T1, -T2, and -T3, and the expression of the type 1 Lewis antigens on these cells. β3Gal-T3 was abundantly expressed in some gastric cancer cells, i.e., KATO III and MKN45 cells, and PC-3 cells, and intermediately expressed in Capan-1 and Capan-2 (pancreatic cancer) cells, HCT-15 cells, and SK-N-MC and SK-N-SH (neuroblastoma) cells; however, it was not detected at all in Colo201 or Colo205 cells.

We observed a significant discrepancy between the expression of the three β3Gal-Ts, i.e., β3Gal-T1, -T2, and -T3, and the expression of the type 1 Lewis antigens on these cells. In contrast, the expression of β3Gal-T4 appeared to be correlated with the type 1 Lewis antigen expression, i.e., the cells expressing type 1 Lewis antigens, i.e., Colo201, Colo205, SW1116, HT-29, and Capan-2 cells, also expressed substantial amounts of the β3Gal-T4 transcript.

β3Gal-T1, -T2, and -T3 could synthesize the type 1 chain (11, 12, 14); however, they were not correlated with the expression of type 1 Lewis antigens in the present study. The expression of β3Gal-T4 seemed to be correlated with the expression of type 1 Lewis antigens in some tumor cells; however, it could not synthesize the type 1 chain (12). From these results, we concluded that none of the four β3Gal-Ts is responsible for type 1 chain synthesis, resulting in sLeα (CA19-9) antigen expression, in gastrointestinal and pancreatic cancer cells.

**Cloning and Sequence of a Novel cDNA Homologous to the Cloned β3Gal-Ts**

As described under “Experimental Procedures,” we obtained two DNA fragments encoding novel sequences, which, however, are homologous to the corresponding regions of the four cloned β3Gal-T genes. The nucleotide sequences of the two DNA fragments were found to be encoded by a single cDNA species. We named this gene β3Gal-T5. By use of the DNA fragments as probes, full-length cDNA clones were obtained from the Colo205 cDNA library. Complementary DNA sequenc-
ing analysis revealed that the β3Gal-T5 cDNA contains an ORF encoding a protein of 310 amino acids (Fig. 2). The position of the AUG start codon was assigned according to the Kozak consensus sequence (21). A hydropathy profile based on the Kyte and Doolittle method (22) indicated that the ORF encodes a type II membrane protein, which is a typical feature of glycosyltransferases (data not shown). The three motifs of amino acid sequences, Motifs 1, 2, and 3, which we employed for the design of degenerate primers in this study, were conserved in the sequence of β3Gal-T5. Four cysteine residues were conserved in the five β3Gal-Ts, which indicates that some of these cysteines are essential for maintenance of the tertiary structures of β3Gal-Ts. Fifty-four of the 310 amino acid residues of β3Gal-T5 were conserved in comparison with the sequences of the other four β3Gal-Ts.

### Table III

| Exon no. | Nucleotide no. in AF064860 | Length | Splice acceptor site sequence | Splice donor site sequence |
|----------|----------------------------|--------|-------------------------------|----------------------------|
| 1        | 85153–85425                | 273    | CTGTCAAGGGTTTTCCC            | CCAAGGGAGCCTTTCTGG          |
| 1'       | 85153–85292                | 140    | CTCTCTTGGAGGCTTTCTGG         | GTTTGGAGGCTTGGGCT           |
| 2        | 85611–85719                | 109    | TTTCTTTGTACACCTT             | AGCAAAAGATGTGA             |
| 3        | 87579–87738                | 160    |                          |                             |
| 4        | 88336–90714                | 2329   |                          |                             |

![Genomic structure of the β3Gal-T5 gene. A, the four exons are shown as boxes, and introns as lines. The restriction sites in exon 2, XbaI, and in exon 3, BsmI, are indicated. ORF is indicated by a hatched box. B, structures of five isoforms of β3Gal-T5 transcripts. *, ratio means the abundance as a percentage of abundance of each isoform in Colo205 cells. C, RT-PCR analysis to determine abundance of the five isoforms of β3Gal-T5 transcripts. Molecular weight markers (100-bp ladders) are on the leftmost lane.](image-url)
indicated that isoforms 3, 4, and 5 are minor transcripts in Colo205 cells. RT-PCR with primers si-4 and si-1 gave a single band for isoform 3, which was digested by both BsmI and XbaI. A band for isoform 4 was not detected on this RT-PCR, because the amount of isoform 4 transcripts may be very small. RT-PCR with primers si-2 and si-3 gave a single band for isoform 5. Isoform 1 and 2 were abundant among the five isoforms, and both isoforms amounted to approximately 50% of the total transcripts of the β3Gal-T5 gene, respectively. The other three isoforms, i.e. isoforms 3, 4, and 5, were only present in trace amounts.

Correlation of the Expression Levels of the β3Gal-T5 Transcripts with the Amounts of Type 1 Lewis Antigens in Various Tumor Cells—As can be seen in Fig. 4, the expression levels of β3Gal-T5 transcripts and the amounts of CA19-9 antigens in various cancer cells were determined by the competitive RT-PCR method and Western blot analysis, respectively. The results of flow cytometry analysis in Fig. 1 are well consistent with those of Western blot analysis in this section, i.e. the four types of cells, i.e. Colo205, Colo201, SW1116, and Capan-2 cells, that were stained strongly with 19-9 on flow cytometry also gave strong positive bands, which were smear ones with high molecular weights indicating they are mucins, with 19-9 on Western blotting (Fig. 4). These four cell lines also expressed abundant β3Gal-T5 transcripts (Fig. 4). The other cell lines, HT-29, WiDr, and Capan-1 cells, intermediatedly expressed the β3Gal-T5 gene.

The Ability of Type 1 Chain Synthesis of β3Gal-T5 in Transfected Cells—Namalwa cells do not possess the type 1 chain and lack α1,3/4-fucosyltransferase (Fuc-TIII), which is the only enzyme capable of the synthesis of type 1 Lewis antigens, such as the Lea, Leb, and sLea epitopes. Therefore, Namalwa cells transfected stably with the β3Gal-T5 gene were stained with DU-PAN-2 (anti-sLea), which recognizes the precursor structure, SAα2,3Galβ1,3GlcNAc, of the sLea epitope (3, 4). As can be seen in Fig. 5, Namalwa cells stably transfected with the β3Gal-T5 gene gave positive peaks with DU-PAN-2. HCT-15 cells were chosen as the host cells for the transfection experiment with the β3Gal-T5 gene for the following reasons. First, they are cancer cells derived from colon tissue. Second, they are known to express substantial amounts of Fuc-TIII and ST3GalIV (data not shown), but not to express β3Gal-T5 at all (Fig. 4). A single transformant clone of HCT-15 cells, which had been transfected stably with the β3Gal-T5 gene, was obtained by the limiting dilution method and named HCT-3GT5H. Flow cytometry analysis of HCT-3GT5H cells apparently showed positive peaks with the antibodies against all type 1 Lewis antigens (Fig. 5). These results confirmed that β3Gal-T5 can synthesize the type 1 chain in transformant cells, i.e. not only in Namalwa cells but also in colon cancer (HCT-15) cells.

β3Gal-T Activity toward Agalacto-LNnT in Various Tumor Cells and HCT-3GT5 Cells—Agalacto-LNnT-PA was used as an acceptor substrate to measure β3Gal-T activity, resulting in the synthesis of lacto-N-tetraose-PA (LNT-PA). The β3Gal-T activity, i.e. the LNT-PA synthesizing activity, in Namalwa cells transfected with the β3Gal-T5 gene, Namalwa-3GT5, was strongest among all samples of transfected cells and cultured cancer cells examined. Thus, the activity of Namalwa-3GT5 is expressed as 100% activity, and the β3Gal-T activities of the other samples relative to that of Namalwa-3GT5 cells are presented in Table IV. The amounts of transcripts for individual β3Gal-T genes were measured by competitive RT-PCR, and the relative amounts of individual transcripts normalized as to the amounts of β-actin transcripts are shown in Table IV. The level of β3Gal-T activity synthesizing LNT-PA was quite parallel to the amounts of β3Gal-T5 transcripts, i.e. the cell homogenate of Colo205 showed the strongest activity among the cultured cancer cells, followed by those of SW1116 and Capan-2. This indicated that the LNT-PA synthesizing activity is mainly directed
by β3Gal-T5. Mock-transfected HCT-15 (HCT-mock) cells did not exhibit any activity, but HCT-3GT5H cells exhibited strong activity almost equal to that of Colo205 cells. HCT-3GT5L cells, which expressed almost one-third of the amount of β3Gal-T5 transcripts in the HCT-3GT5H cells, showed one-third of the β3Gal-T activity of HCT-3GT5H cells. The cells expressing substantial amounts of transcripts for the other four β3Gal-T genes, i.e. the β3Gal-T1, T2, T3, and T4, genes, did not exhibit LNT-PA synthesizing activity at all. Namalwa cells stably expressing β3Gal-T1, T2, T3, and T4, which were named Namalwa-3GT1, 3GT2, 3GT3, and 3GT4 cells, respectively, also did not exhibit any LNT-PA synthesizing activity.

The above results confirmed that the β3Gal-T activity synthesizing LNT-PA is mainly directed by β3Gal-T5 in these cells, not by the other four β3Gal-Ts.

**Tissue Distribution and Quantitative Measurement of the β3Gal-T5 Transcripts**—As can be seen in Fig. 6, β3Gal-T5 transcripts were substantially detected in the stomach, jejunum, colon, and pancreas, which are known to express sLeα antigens frequently when they become cancerous. On the other hand, they were hardly detected in the lungs, liver, spleen, adrenal glands, and peripheral blood leukocytes, which rarely produce sLeα antigens when they become malignant. This strongly suggested that β3Gal-T5 is responsible for the type 1 chain synthesis, resulting in the sLeα antigen synthesis in gastrointestinal and other tissues.

**Multiple Sequence Alignment (ClustalW) of the Five Members of the Human β3Gal-T Family**—Multiple amino acid sequence alignment of the five β3Gal-Ts was constructed by ClustalW method (Fig. 7). Three conserved amino acid motifs, which were employed for design of the degenerate primers, and four conserved cysteine residues were indicated in Fig. 7. One possible N-glycosylation site was also conserved in all five hβ3Gal-Ts.

A phylogenetic tree of the five β3Gal-Ts was constructed by means of the neighbor-joining method based on the amino acid sequences (data not shown) (23). The position of β3Gal-T5 in the tree is closer to those of β3Gal-T1 and -T2, which can synthesize the type 1 chain as reported by us and others (10–12), than to those of β3Gal-T3 and -T4. These three enzymes, β3Gal-T1, T2, and T5, form a subfamily on the phylogenetic tree.

**DISCUSSION**

Expression of the sLeα and sLeβ epitopes, which are mainly carried on the carbohydrate chains of mucins, is frequently elevated in gastrointestinal and pancreatic cancers. The sLeα antigens are known to be some of the factors determining the prognoses of colorectal and gastric cancer patients (7, 8, 24). In this sense, it is very important to identify the β3GalT(s) responsible for type 1 chain synthesis, which results in the synthesis of the sLeα and sLeβ epitopes in gastrointestinal and pancreatic cancers. The novel β3Gal-T, i.e. β3Gal-T5, isolated in the present study was demonstrated to be responsible for the type 1 chain (including the sLeα and sLeβ antigen) synthesis in gastrointestinal and pancreatic cancers by the following evidence. 1) β3Gal-T5 could synthesize type 1 chains, leading to expression of Leα, Leβ, sLeα and sLeβ in transfected cells, i.e. in Namalwa-3GT5 and HCT-3GT5H cells. 2) β3Gal-T5 was expressed in cultured colon and pancreatic cancer cells expressing substantial amounts of type 1 chains, whereas the other three β3Gal-Ts, i.e. β3Gal-T1, T2, and T3, were not expressed in these cells. 3) The expression levels of β3Gal-T5 transcripts were well correlated with those of type 1 Lewis antigens in cultured cancer cells. 4) In the transfection experiment involving Namalwa cells, only β3Gal-T5 exhibited the LNT-PA synthesizing activity, whereas the other four β3Gal-Ts did not. 5) The expression level of β3Gal-T5 transcript in cultured cancer cells was well correlated with the LNT-PA synthesizing activity.

Kolbinger et al. demonstrated that β3Gal-T2 was able to synthesize type 1 chains in CHO cells through a transfection experiment (11). Amado et al. detected the β3Gal-T activities of β3Gal-T1 and -T2 (12). Hennet et al. demonstrated the β3Gal-T
activities of three mouse β3Gal-Ts, i.e. mβ3GalIT-1, -II and -III (14). In contrast, we could not detect LNT-PA synthesis activity in the Namalwa cells transfected stably with each of the β3Gal-T1, -T2, or -T3 genes. This was probably due to the amounts or structures of the enzymes used or the sensitivity of the assay system. In a previous study, we detected the activity of β3Gal-T1 using purified β3Gal-T1 that was expressed as a secreted form fused with the IgG binding domain of Staphylococcus aureus protein A (10). The other three groups, i.e. Hennet et al. (14), Kolbing et al. (11), and Amado et al. (12), assayed β3Gal-T activity by measuring radioisotope incorporation using recombinant enzymes produced in soluble forms with a baculo-expression system or the S. aureus protein A fusion system, whereas we used cell homogenates as enzyme sources and a pyridylaminated acceptor substrate in the present study.

We apparently detected LNT-PA synthesizing activity in Namalwa-3GT5 cells and cultured cancer cells expressing β3Gal-T5, whereas Namalwa cells transfected with the other four β3Gal-T genes and cultured cells endogenously expressing β3Gal-T1 (PC-1), β3Gal-T2 (Namalwa), or β3Gal-T3 (MKN45) did not exhibit LNT-PA synthesizing activity at all. This indicated that β3Gal-T5 possesses the strongest activity as to type 1 chain synthesis among the 12 was correlated with the amounts of sLe α antigens and the other type 1 Lewis antigens in the cultured cancer cells. This means that β3Gal-T5 is responsible for expressing sLe α antigens when those tissues become cancerous.

We previously determined the expression levels of 12 glycosyltransferases, i.e. those of five α1,3-fucosyltransferases (Fuc-TIII, -TIV, -TV, -TVI) and three α1,4-galactosyltransferases (GalNAcT-I, -II, and -IV), one α1,3-galactosyltransferase, one α1,3-fucosyltransferase, one α1,4-galactosyltransferase, one α1,2-fucosyltransferase, and one α2,3-sialyltransferase in colorectal cancer tissues in order to correlate them with the amounts of the sLe α and sLe β antigens (9). Although Fuc-TIII and ST3Gal IV are essentially required for the sLe α synthesis in colorectal cancers, no single enzyme among the 12 was correlated with the amounts of sLe α antigens. Therefore, we conjectured in the previous study that the combinatorial up-regulated expression of multiple enzymes determines the amounts of the antigens. However, it is a noteworthy finding in the present study that the expression levels of β3Gal-T5 were well correlated with the amounts of sLe α antigens and the other type 1 Lewis antigens in the cultured cancer cells. This means that β3Gal-T5 is a key enzyme determining the expression levels of type 1 Lewis antigens including sLe α antigens in those cells. The above, together with the results of the present study, indicated that β3Gal-T5 and ST3Gal IV are responsible for sLe α synthesis, and Fuc-TIII is further required for sLe α synthesis in colorectal cancers in addition to β3Gal-T5 and ST3Gal IV.

On the other hand, the type 2 chain, Galβ1,4GlcNAc, was found to be expressed in all cell lines examined in the present study, since β4Gal-T1 is a ubiquitous enzyme, and was substantially expressed in all cell lines (data not shown). It is of interest to determine whether or not the up-regulation of the β3Gal-T5 gene expression determines the levels of sLe α antigens in native cancer tissues. If this is the case, transcriptional regulation of the β3Gal-T5 gene will be an
attractive subject in the future. In this study, we determined the transcription initiation site of the β3Gal-T5 gene in Colo205 cells. The nucleotide sequence in the upstream region was examined for the binding sites of transcription factors using the TFSEARCH (transcription factor search) program, based on the data bases deposited by Heinemeyer et al. (27). We searched the 1-kilobase pair upstream region from the transcription initiation site of the β3Gal-T5 gene, but found no TATA box. Within 150 bp upstream of the transcription initiation site, two CdxA sites, an AP-1 site, and a myeloid zinc finger 1 protein, MZF1, site were found. AP-1 and MZF1 have been reported to be potential targets of neoplastic transformation (28, 29). CdxA, known as a chicken homeobox-containing gene related to caudal in Drosophila, was previously shown to be expressed in the endoderm-derived gut epithelium during early embryogenesis (30). In the future, we will examine whether or not these transcription factors function in regulation of the β3Gal-T5 gene.

Finally, the results of the present study strongly indicate that β3Gal-T5 is the most probable candidate responsible for the sLea antigen synthesis in gastrointestinal and pancreatic cancer cells. In the future, it will be interesting to determine whether or not expression of the β3Gal-T5 gene changes some characteristics of cancer cells, especially those related to malignancy.

Acknowledgments—We thank Dr. Nobuyuki Imai, Dr. Kyoko Takeuchi, Dr. Satoshi Nakagawa, Sachiko Kodama, Hiromi Inagaki, Reiko Ito, Dr. Satoshi Ito, of Fujirebio Inc., and Dr. Tetsuya Chi, Dr. Satoshi Nakagawa, Sachiko Kodama, Hiromi Inagaki, Reiko Ito, Dr. Satoshi Ito, of Fujirebio Inc., and Dr. Tetsuya Tachikawa, of Diagnostic Division, Otsuka Pharmaceutical Co. Ltd. for the kind gifts of the 19-9 and TT42 antibodies, respectively.

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3. The TFSEARCH program was developed by Y. Akiyama and is available via the World Wide Web (http://www.rwcp.or.jp/lab/pdappl/papia.html).

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