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Distinct Substrate Specificities of Five Human α-1,3-Fucosyltransferases for *in Vivo* Synthesis of the Sialyl Lewis x and Lewis x Epitopes

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Five different human α-1,3-fucosyltransferase genes, *the Fuc-TIII, Fuc-TIV, Fuc-TV, Fuc-TVI* and *Fuc-TVII* genes, have been cloned to date. We transfected HeLa cells and Namalwa cells with each of the five different genes, and established a series of stable cloned transformant cells. Thin-layer chromatography immunostaining analysis revealed that all five enzymes were able to synthesize sialyl Lewis x (sLe^x) epitopes on glycolipids in HeLa cells, but each enzyme showed a different preference as to the carbohydrate chain length on glycolipids as acceptor substrates. Fuc-TIII and Fuc-TV showed very similar patterns of sLe^x positive bands, which indicated that the enzymes have similar acceptor substrate specificities. Fuc-TVI exhibited a little different pattern from those of the former two enzymes. Fuc-TIV and Fuc-TVII showed similarity in the positive bands, however, their patterns were quite different from those of the former three enzymes. Four enzymes except for Fuc-TVII were able to synthesize the Lewis x (Le^x) epitope on glycolipids in HeLa cells. Fuc-TV alone showed a little different pattern of Le^x positive bands from those of the other three enzymes. Flow cytometric analysis of HeLa cells and Namalwa cells again demonstrated the similar specificities of Fuc-TIII and Fuc-TV. They exhibited similar stronger staining with FH6 (anti-sLe^x) antibodies than that with the other enzymes. A phylogenetic tree of the five enzymes constructed using the neighbor-joining method showed good agreement with the similarities in the enzyme substrate specificity.

The sLe^x epitope, consisting of four sugar residues, NeuAcα2-3Galβ1-4 (Fucα1-3) GlcNAc-R, is well known to play an important role during early steps of the inflammatory response as a ligand of E- and P-selectins resulting in the attachment and rolling of leukocytes to blood endothelial cells (1, 2). It was previously shown that colon carcinoma tissues from patients at advanced stages contained higher levels of sLe^x antigens than tissues from patients with early diseases (3, 4). Such malignant phenotype might be attributed to the altered metastatic potentials (5-7). The Le^x epitope, consisting of three sugar residues, Galβ1-4(Fucα1-3)GlcNAc-R, has also been attracting much attention because of its biological function as an intercellular interaction molecule, although its function remains ambiguous (8, 9).

The synthesis of sLe^x and Le^x is finally completed by the addition of fucose from guanosine diphosphate-fucose (GDP-Fuc) to the *N*-acetylglucosamine residue in the precursor structures. This final step is catalyzed by an enzyme exhibiting α-1,3-fucosyltransferase (α1,3Fuc-T) activity. Five homologous human genes encoding α1,3Fuc-Ts have been cloned to date. The enzymes encoded by the five genes are named Fuc-TIII (10), Fuc-TIV (11-13), Fuc-TV(14), Fuc-TVI (15-17), and Fuc-TVII (18, 19). The three genes encoding Fuc-TIII, Fuc-TV and Fuc-TVI among them are known to be localized at the close physical positions on chromosome 19p13.3 (17, 20). Therefore, it was predicted that the three enzymes would have more similar substrate specificities than the other two enzymes, Fuc-TIV and Fuc-TVII. Minimal knowledge of the enzyme capacity for sLe^x and Le^x synthesis has accumulated at the flow cytometric analysis level by detecting the cell surface expression of sLe^x and Le^x epitopes using gene-transfected cells (1, 10-15, 18, 19, 21). These studies demonstrated that all five members are able to synthesize the sLe^x epitope although one of them, Fuc-TIV, exhibits

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very little sLe^x synthesis activity. Four of them, Fuc-TIII, IV, V and VI, the exception being Fuc-TVII, are known to be able to synthesize the Le^x epitope. Fuc-TVII is known to be unable to synthesize the Le^x epitope.

There has been one paper describing the enzyme kinetics of three α1,3Fuc-Ts, i.e. Fuc-TIII, IV and V (22). They assayed the *in vitro* enzyme specificities, using the recombinant enzymes, towards a panel of oligosaccharide, glycolipid and glycoprotein acceptor substrates. Notwithstanding several studies have concerned the substrate specificities of the five enzymes, the glycan structures bearing sLe^x and Le^x epitopes which are actually synthesized in the cells by the five α1,3Fuc-Ts have not been analyzed at all.

In this study, we examined the distinct substrate specificities of the five enzymes in an *in vivo* situation, e.g., in stable transformant cells. Each α1,3Fuc-T exhibits a distinct specificity as to the synthesis of glycolipids bearing the sLe^x and Le^x epitopes.

MATERIALS AND METHODS

Monoclonal antibodies. Monoclonal antibodies (mAbs), CSLEX-1 (anti-sLe^x) (23), FH6 (anti-sLe^x) (24) and PM 81 (anti-Le^x) (25), were used to detect antigens in the present study. All mAbs used were IgM.

Establishment of stable transformant clones transfected with each of the five α1,3Fuc-T cDNAs. The stable transformant Namalwa cells (Burkitt lymphoma) with each of the five α1,3Fuc-T genes were previously described in detail (26, 27). The same cDNA fragment encoding the full-length sequence of each α1,3Fuc-T gene was inserted into another expression vector, pCXN2 (28), which was kindly provided by Dr. J. Miyazaki. HeLa cells (human cervical cancer cell line) were transfected with each cDNA in the pCXN2 vector using the Lipofectin (GIBCO-BRL, Grand Island, NY) system, as described in the supplier's manual. The transfected cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). After 24-hr incubation, geneticin (G418; GIBCO-BRL) was added to the culture at a final concentration of 1.2 mg/ml to obtain stable transformants. After 10 days exposure to geneticin, the cells were subjected to limited dilution in 96 well microtiter plates to obtain single clones. Thus, a series of clones were established, and the expression levels of transcripts in the cells were determined by the competitive RT-PCR method (18).

Determination of the expression levels of transcripts by the competitive RT-PCR method. The competitive RT-PCR method for measurement of the five α1,3Fuc-T mRNAs was originally established by Sasaki et al., and the method was previously described in detail in their paper (18). In this study, we employed almost the same materials and methods as they used, and determined the expression level of each α1,3Fuc-T transcript in the stable transformant clones. The amount of β-actin transcript in each transformant clone was also determined by the competitive RT-PCR method (18). The number presented at the bottom column of transcript level in FIGs. 1 and 2 indicates the relative ratio of the amount of each Fuc-T transcript to the amount of β-actin transcript expressed in each transformant clone. It is also easy to compare the relative amount between each Fuc-T transcript. In addition, to certify how accurately the amount of transcripts is measured by the competitive RT-PCR method employed in this study, we performed Western blotting analysis using two monoclonal antibodies, one of which specifically recognizes Fuc-TIII and another of which recognizes three of Fuc-Ts, Fuc-TIII, -V and -VI (data not shown). We observed the clear correlation between the amount of transcripts measured by the competitive RT-PCR method and the amount of the proteins detected by Western blotting analysis.

Purification of glycolipids. Stable transformant clones were cultured in DMEM supplemented with 10% FCS. Crude lipids were extracted from the harvested cells with isopropanol-hexane-water, 55:25:20, and then dried under reduced pressure. Each dried sample was dissolved in chloroform-methanol-water, 30:60:8, and then applied to a QMA Sep-Pak cartridge (Waters, MA). Neutral lipids were recovered in the fraction passing through the QMA Sep-Pak cartridge. Acidic fractions containing gangliosides were eluted with chloroform-methanol-4M sodium acetate, 30:60:8, from the cartridge, and were desalted with a C18 Sep-Pak cartridge (Waters, MA). The neutral lipids were incubated in a mixture of 3 volumes of pyridine and 2 volumes of acetic anhydride, and then applied to a Florisil Sep-Pak cartridge (Waters, MA). Acetylated neutralglycolipids were eluted from the cartridge with 1,2-dichloroethane-acetone, 1:1, deacetylated with sodium methoxide, and then desalted with a C18 Sep-Pak cartridge.

Immunoblotting analysis of glycolipids by thin-layer chromatography (TLC). Gangliosides and neutral glycolipids were separated by TLC (HPTLC Kieselgel 60, 5641; MERK, Germany) with mixtures of chloroform-methanol-0.2% calcium chloride, 55:45:10, and chloroform-methanol-water, 62.5:30:6, respectively. The TLC plates were coated with 0.1% poly-iso-butylmethacrylate (Polyscience, Warrington, PA) in hexane, and then blocked with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (1% BSA-PBS). Each mAb, diluted adequately, was overlaid on the TLC plates in a humidified chamber, followed by incubation overnight at 4 C. The TLC plate was washed with 1% BSA-PBS and then incubated with biotinylated anti-mouse IgM (Vectastain Elite ABC kit; Vector, CA) for 2 hr at room temperature. After washing with 1% BSA-PBS, the TLC plate was incubated in a mixture of avidin and biotinylated peroxidase (Vectastain Elite ABC kit; Vector, CA) for 30 min at room temperature. Finally, the TLC plate was washed with PBS and then treated with an ECL detection kit (Amersham, UK).

Flow cytometric analysis. The sLe^x and Le^x expression on the cell surface was examined by flow cytometric analysis using an Epics Elite (Coulter, FL). One million cells were incubated with the first antibody (10 μg/ml) for 1 hr on ice, and then washed twice with 1% BSA-PBS, followed by incubation with FITC-conjugated anti-mouse IgM. Then, the cells were washed again with 1% BSA-PBS and finally subjected to flow cytometric analysis.

Phylogenetic tree of the five human α-1,3-fucosyltransferases. For the five human *α1,3Fuc-T* genes, the amino acid sequence data of Kukowska-Latallo et al. (10), Lowe et al. (13), Weston et al. (14), Weston et al. (15) and Sasaki et al. (18) were used. A phylogenetic tree was constructed using the neighbor-joining method (29).

RESULTS AND DISCUSSION

TLC-immunostaining analysis of glycolipids bearing the sLe^x and Le^x epitopes in stable transformant HeLa cells. The expression level of each α1,3Fuc-T transcript in a series of stable transformant cells was first determined by the competitive RT-PCR method (18). Various levels of transcript expression was observed with the stable transformant cells. We named the clones obtained by the limitting dilution method, with suffixes L (low) to HH (high and high), according to the transcript expression level. For example, we established four different HeLa clones transfected with the *Fuc-TV* gene, which were named HeLa-FT5L, -FT5M,

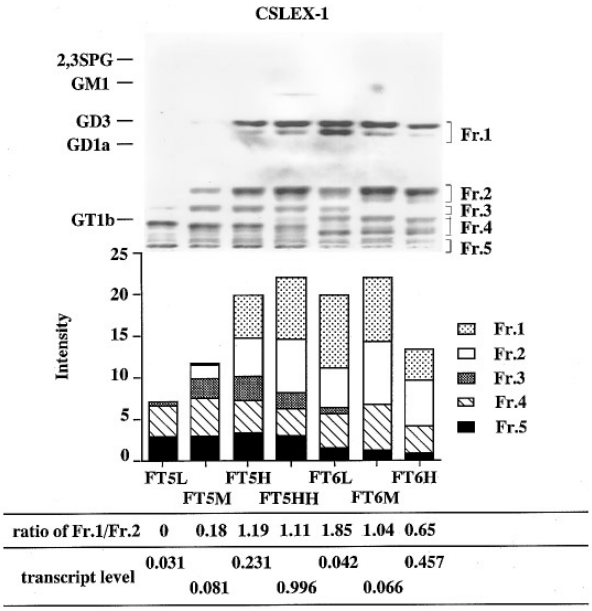

FIG. 1. TLC-immunostaining with CSLEX-1 (anti-sLe^x) of the acidic fraction glycolipids of HeLa-FT5 and HeLa-FT6 clones expressing various amounts of the transcripts. The positive bands were divided into five fractions depending on the Rf value. The ratio of the band intensity for fraction No. 1 to that for fraction No. 2 was calculated and is presented at the bottom. The transcript level determined by the competitive RT-PCR method is also presented at the bottom.

-FT5H and -FT5HH, in order of the Fuc-TV transcript expression level. These four HeLa-FT5 clones and three HeLa clones transfected with the *Fuc-TVI* gene (HeLa-FT6 clones) were analyzed for the expression of the sLe^x antigens carried on glycolipids by TLC-immunostaining analysis (FIG. 1). The transcript expression level in each clone determined by the competitive RT-PCR method is presented at the bottom of the FIGs. 1 and 2. Mock-transfected HeLa cells did not give any positive bands when probed with an anti-sLe^x antibody, CSLEX-1 (data not shown). As shown in FIG. 1, the sLe^x antigens carried on glycolipids of the transfected clones appeared as multiple bands, which we divided into five fractions depending on the Rf values. The intensity of each band was measured with a densitometer and is presented as bars at the bottom. The dual bands with the highest Rf values for fraction No. 1 were considered to represent the minimal length of sugar chain carrying the sLe^x epitope, and the difference in the fatty acid backbone, one probably being hydroxylated and the other not, may give rise to the dual bands. Interestingly, Fuc-TV-transfected clones exhibited a different pattern of multiple bands from Fuc-TVI-transfected cells, i.e., the HeLa-FT5L cells with a very low level of Fuc-TV transcripts were able to synthesize the sLe^x epitope present in the lower Rf value fractions, Nos. 3 to 5, which probably contain rather longer carbohydrate chains than the higher Rf value fractions, whereas no visible bands were observed for the high Rf value fractions, Nos. 1 and 2, for the HeLa-FT5L cells. According to the increase in Fuc-TV transcript expression, a positive band clearly appeared for No. 2, as seen in FT5M cells, and the positive bands for fraction No. 1 then appeared in FT5M and FT5H cells. The intensity ratio of No. 1 to No. 2 was calculated and is presented at the bottom of FIG. 1. It gradually increased in good accordance with the increase in Fuc-TV. In contrast, a series of stable tranformant cells transfected with the *Fuc-TVI* gene apparently exhibited a different pattern of positive bands from that of Fuc-TV transfected cells. The Hela-FT6L cells expressing Fuc-TVI transcripts at a level equal to that of Fuc-TV transcripts in Hela-FT5L cells showed strong positive bands for fraction No. 1. According to the increase in the Fuc-TVI transcript level, the ratio of No. 1 to No. 2 gradually decreased, as shown at the bottom of FIG. 1. These results demonstrated the following points. First, Fuc-TV preferably utilizes glycolipids having rather longer carbohydrate chains as acceptor substrates than shorter chains. Second, Fuc-TVI seems to prefer shorter chains than longer ones or may utilize acceptor substrates indiscriminately irrespective of the carbohydrate chain length. Third, they seem to have different substrate specificities, as shown by fraction Nos. 3 and 4. The band for No. 3 apparently disappeared in FT6M and FT6H cells. The bands for No. 4 showed different patterns in HeLa-FT5 and HeLa-FT6 cells.

The transcript levels in the above HeLa cells under the strong promotor control of the pCXN2 vector were considered to be extremely high compared with those in native human tissues. We measured the α1,3Fuc-T transcript levels in some native human tissues, such as colon, stomach, lung and uterus, by the competitive RT-PCR method (data not shown). In comparison with the expression level in a native tissue, the physiological level of α1,3Fuc-T expression is around the level in FT6L or less than it. Therefore, the HeLa-FT5L and HeLa-FT6L cells would have a more physiological carbohydrate structure than the others. To summarize the results in FIG. 1, Fuc-TV physiologically synthesizes the sLe^x epitopes on considerably longer chains, as in fraction Nos. 4 and 5, whereas Fuc-TVI mainly synthesizes sLe^x epitopes on the shortest chains of glycolipids, as in fraction No. 1, in addition to the sLe^x synthesis on longer chains.

We established a series of HeLa cells transfected stably with other *α1,3Fuc-T* genes, such as Fuc-TIII, Fuc-TIV and Fuc-TVII. The transcript expression levels were measured by the competitive RT-PCR method, and we selected one clone from each group which expressed transcripts at almost an equal levels to those by HeLa-FT5M and HeLa-FT6M cells. The expression level of each transcript is presented at the bottom of

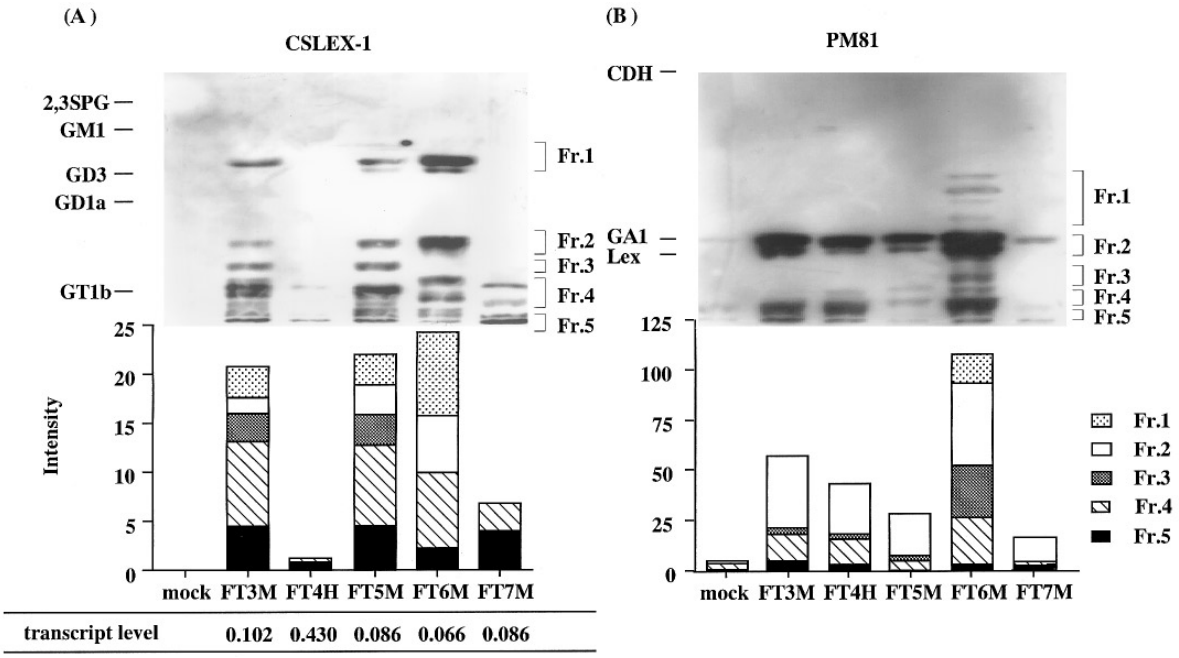

FIG. 2. (A) TLC-immunostaining with CSLEX-1 (anti-sLe^x) of the acidic fraction glycolipids of representative HeLa clones transfected with each *α1,3Fuc-T* gene. The transcript level determined by the competitive RT-PCR method is presented at the bottom. (B) TLC-immunostaining with PM81 (anti-Le^x) of the neutral glycolipids of the same HeLa clones as used in the experiment (A).

FIG. 2 (A). FIG. 2 (A) shows the sLe^x positive bands probed with the CSLEX-1 antibody. We again confirmed the lack of expression of the sLe^x epitope in mock-transfected HeLa cells, as seen in the far left lane in FIG. 2 (A). HeLa-FT5M and Hela-FT6M cells were again examined in this experiment. They showed reproducible patterns of positive bands, as can be seen in FIG. 1. HeLa-FT3M cells expressing Fuc-TIII revealed a very similar pattern of positive bands to that of the FT5M cells, indicating that the two enzymes, Fuc-TIII and Fuc-TV, should have similar substrate specificities. As can be seen in the far right lane in FIG. 2 (A), HeLa-FT7M cells expressing Fuc-TVII exhibited a quite different pattern from those of the former three types of cells. Fuc-TVII preferentially synthesized the sLe^x epitopes on rather longer carbohydrate chains of glycolipids in fraction Nos. 4 and 5 and was not able to synthesize them on the shorter chains in fraction Nos. 1, 2 and 3. Fuc-T IV expressed in HeLa-FT4M cells exhibited very weak but apparently positive sLe^x synthesis activity. This was coincident with the results reported by others (21), i.e. that mouse Fuc-TIV is able to synthesize the sLe^x antigens with very weak but apparently positive activity. It was of interest that Fuc-TIV also exhibited preferential activity towards the longer carbohydrate chains, with almost the same pattern as that of Fuc-TVII. This indicated that Fuc-TIV is more closely related to Fuc-TVII phylogenetically than the other three.

Regarding Le^x epitope synthesis, the TLC-immunostaining analysis was performed by probing with the PM-81 antibody. Mock-transfected HeLa cells showed very faint but apparently positive bands for fraction Nos. 2 and 4. The same Rf value bands as observed for the mock-transfected cells were observed for the Hela-FT7M cells, and the intensity of band for fraction No. 2 for the FT7M cells was slightly augmented as compared with that for the mock-transfected cells. The inability of Le^x synthesis by Fuc-TVII has generally been accepted. However, Fuc-TVII may have a little Le^x synthesis activity. It was of interest that Fuc-TIII and Fuc-TIV had very similar patterns of Le^x positive bands, and Fuc-TV exhibited a little different pattern from those of Fuc-TIII and Fuc-TIV. Fuc-TV did not give strong signals of the positive bands in fraction Nos. 4 and 5. Fuc-TVI exhibited the strongest intensity of the Le^x positive bands, and some additional bands in fraction Nos. 1 and 3. This suggested that Fuc-TVI may have the highest specific activity for the synthesis of Le^x-bearing glycolipids compared with the others. As judged from the Rf values of standard markers, the bands for fraction No. 2 are considered to represent Le^x epitopes on the minimal length of glycolipid carbohydrate chains. The multiple bands obtained for fraction No. 1 are therefore thought to represent different structures from Le^x, and were probably probed by crossreaction of PM81. However, these bands in fraction No. 1 would be fucosylated by Fuc-TVI.

Flow cytometric anlaysis of sLe^x and Le^x expression on stable transformant HeLa and Namalwa cells. FIG. 3

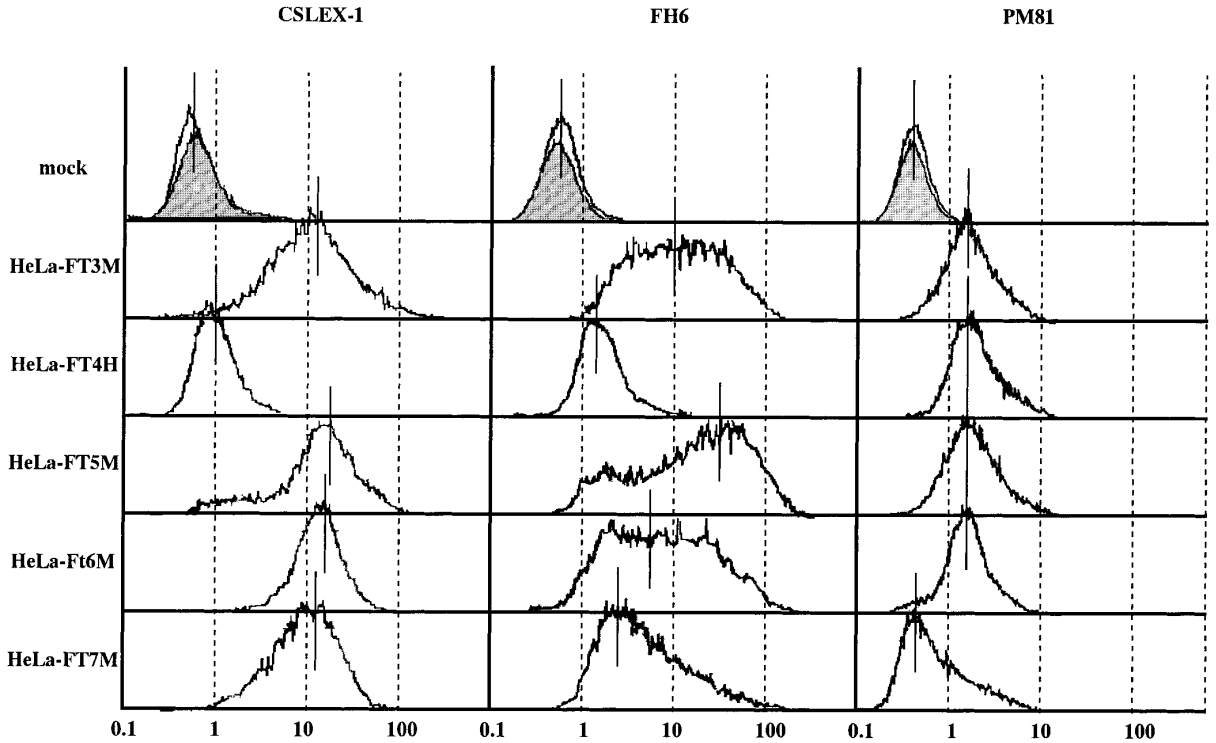

FIG. 3. Flow cytometric analysis of the same HeLa clones as used in the FIGs. 2 (A) and (B) experiments with three mAbs, CSLEX-1 (anti-sLe^x), FH6 (anti-sLe^x), and PM81 (anti-Le^x). The tops of the peaks are indicated by vertical bars. The shadowed areas represent the peaks stained without the first antibody.

shows the profiles of sLe^x and Le^x expression on the cell surface of the stable HeLa transformants on flow cytometric analysis. On Western blotting analysis, all HeLa transformant clones in FIG. 3, except for the mock-transfected clone, were found to possess the sLe^x epitopes carried by glycopoteins (data not shown). Therefore, the flow cytometry profiles reflect the positive staining on both of glycoproteins and glycolipids. Fuc-TIV was again confirmed to have little ability to express the sLe^x epitopes when probed with CSLEX-1 and FH6. It was a noteworthy finding that FH6 (anti-sLe^x) exhibited the order of positive staining intensity of Fuc-TV, Fuc-TIII, Fuc-TVI, Fuc-TVII and Fuc-TIV, while CSLEX-1 (anti-sLe^x) staining did not reveal any distinguishable positive staining. The defined epitope structures recognized by CSLEX-1 and FH6, although both are anti-sLe^x antibodies, were previously determined (23, 24). CSLEX-1 binds to the minimal sLe^x structure consisting of four sugars, e.g., NeuAcα2-3Galβ1-4 (Fucα1-3)GlcNAc-R, whereas FH6 preferably binds to a sLe^x structure having repeating units of lactosamine, NeuAcα2-3Galβ1-4 (Fucα1-3)GlcNAc-Galβ1-4GlcNAc-R. This flow cytometric analysis again demonstrated that Fuc-TIII and Fuc-TV have similar specificities, i.e. they synthesize the FH6 epitope more effectively than the other enzymes. The PM81 profiles did not reveal any differences between the four enzymes, Fuc-TIII, IV, V and VI, however, they again confirmed that the activity of Fuc-TVII for Le^x synthesis was very low.

To determine whether the FH6 epitopes are on glycolipids or glycoproteins, stable transformant Namalwa cells transfected with each *α1,3Fuc-T* gene were analyzed by flow cytometry. In preliminary experiments involving TLC immunostaining, none of the stable transformant Namalwa cells with each *α1,3Fuc-T* gene exhibited any positive bands of sLe^x and Le^x, which indicated that Namalwa cells do not possess the precursor structures of sLe^x and Le^x glycolipids (data not shown). Therefore, the flow cytometry profiles of the Namalwa transformants in FIG. 4 definitely reflect the sLe^x and Le^x epitopes on glycoproteins. We obtained similar results to those for HeLa transformants, i.e. Fuc-TIII and Fuc-TV again exhibited stronger staining with FH6 than the other enzymes, whereas no differences were observed for CSLEX-1. It was again confirmed that Fuc-TIV and Fuc-TVII have a little activity of Le^x and sLe^x epitope synthesis on glycoproteins, respectively.

In conclusion, all five enzymes are able to synthesize sLe^x and Le^x epitopes on both glycolipids and glycoproteins with different specific activities. In particular, it was a noteworthy finding that the FH6 epitope is more effectively synthesized by the two enzymes, Fuc-TIII

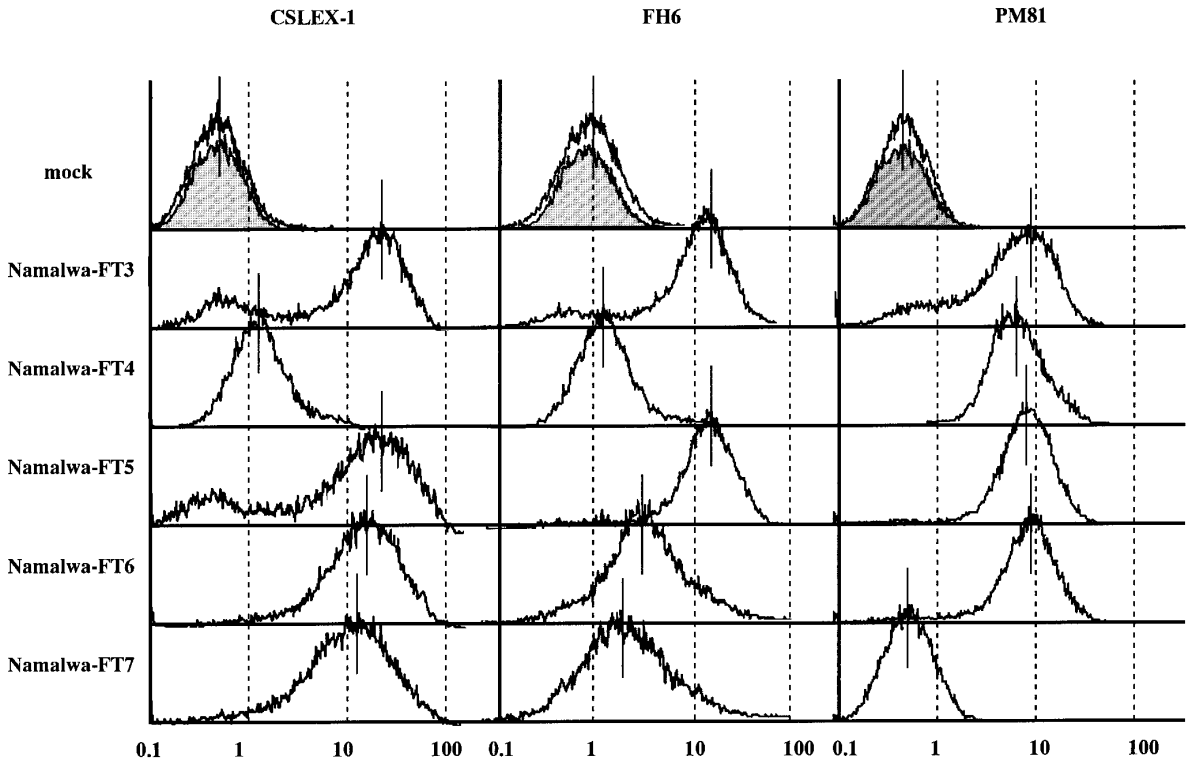

FIG. 4. Flow cytometric analysis of the stable transformant Namalwa cells with each *α1,3Fuc-T* gene with three mAbs, CSLEX-1 (anti-sLe^x), FH6 (anti-sLe^x), and PM81 (anti-Le^x). The tops of the peaks are indicated by vertical bars. The shadowed areas represent the peaks stained without the first antibody.

and Fuc-TV. Although there is little knowledge of the tissue distribution of the five enzymes, one paper reported the human tissue distributions of three enzymes, Fuc-TIII, Fuc-TV and Fuc-TVI, determined by detection using the RT-PCR method (30). According to their results, Fuc-TV is scarcely expressed in any tissue, whereas Fuc-TIII is relatively abundant in many tissues. On immunohistochemical analysis throughout the whole human body using a mAb against Fuc-TIII (26), we found that Fuc-TIII is ubiquitously expressed in the epithelial cells of digestive organs (a manuscript is in preparation). Taking the tissue distributions of the enzymes into consideration, Fuc-TIII seems to be more responsible and more important for FH6 epitope synthesis in native tissue.

It will be quite interesting in the future to determine the actual carbohydrate structures on glycolipids and glycoproteins in the transformants which are synthesized by each of the five enzymes. Each enzyme probably produces distinct structures with fucosylated modification.

A phylogenetic tree of the five α1,3Fuc-Ts. A phylogenetic tree of the five α1,3Fuc-Ts, constructed by the neighbor-joining method, showed good accordance with their similarities in substrate specificity. As can be seen in the FIG. 5, Fuc-TIII and Fuc-TV are most

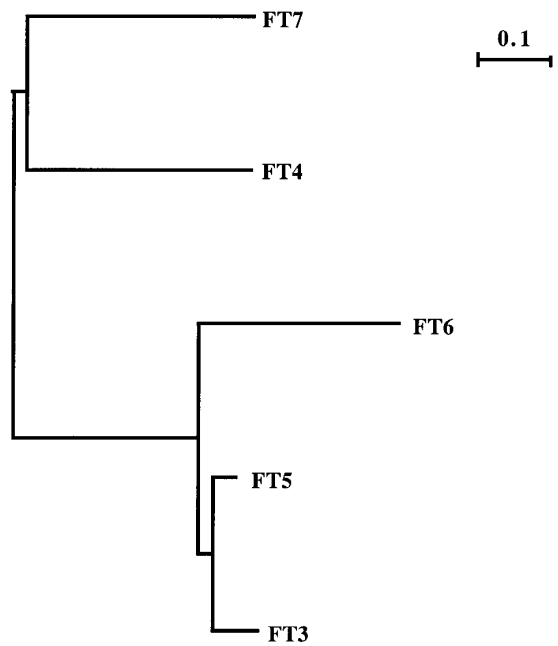

FIG. 5. A phylogenetic tree of the five α1,3Fuc-Ts constructed by the neighbor-joining method. Branch lengths indicate evolutionary distance between different sequences.

closely related to each other in the tree, and Fuc-TVI is relatively distant from the former two enzymes. Fuc-TIV and Fuc-TVII form another gene cluster.

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