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G. 知的所有権の取得状況

特許出願

「B型肝炎ウイルスのpseudotypeウイルスを作成し、その感染性を定量的に検出する方法」
(特願 2005-263575)

表1　お茶の粗抽出物の抗HIV-1活性

試料名		50%抑制濃度（IC50：μ		
		感染抑制	GFP発現阻害	細胞毒性濃度
タイのお茶粗成分	A	16	>100	>30
	B	＞100	>100	>30
	C	22	>100	>100
	D	31	>100	>30
カテキン	EGCG	9.8	>100	>10
Tat阻害剤	DMP	57	>100	>10
アジドチミジン	AZT	0.017	>100	>100

表２　緑茶成分の抗HIV-1活性

薬剤名	50%抑制濃度（IC50：μg/ml）		
	感染抑制	細胞融合抑制	細胞毒性濃度
Geraniin	3	18	>100
GTE	20	53	>100
EGCG	12	23	>75
Epigalocatechin	15	20	>30
Epicatechin	76	35	>300
Epicatechin galate	20	53	>100
デキストラン硫酸Na	—	15	>1000
AZT	0.02	—	>1000

図1 緑茶粗抽出物の抗HIV活性

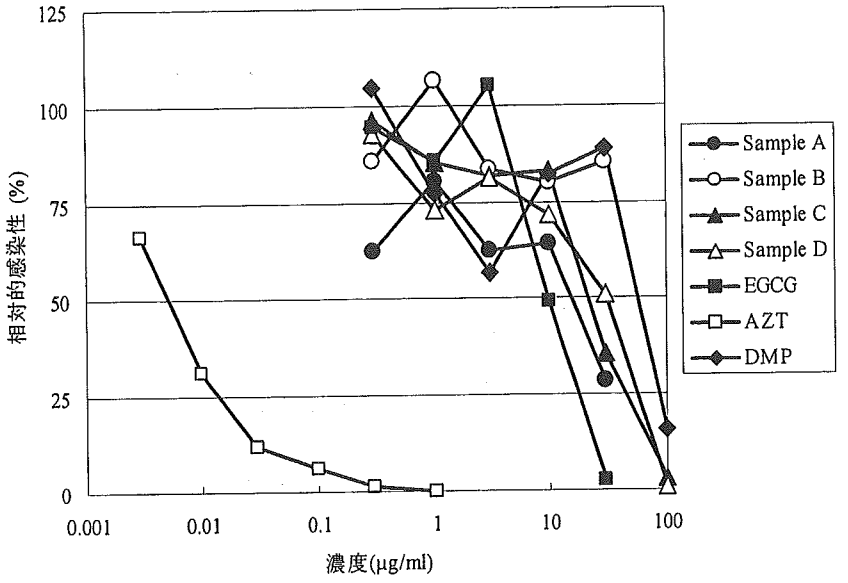


図2 緑茶粗抽出物のGFP発現への影響

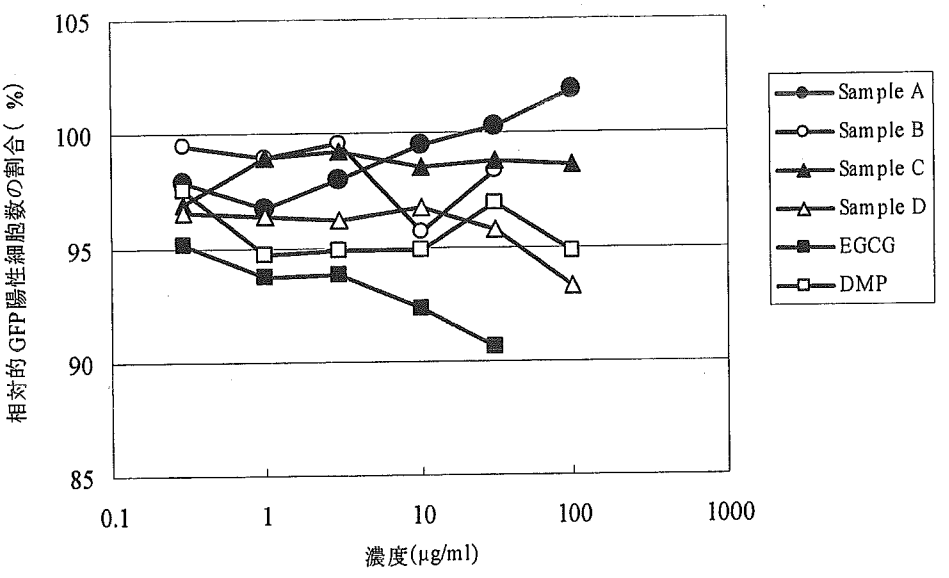


図3 緑茶成分によるHIV-1感染抑制効果

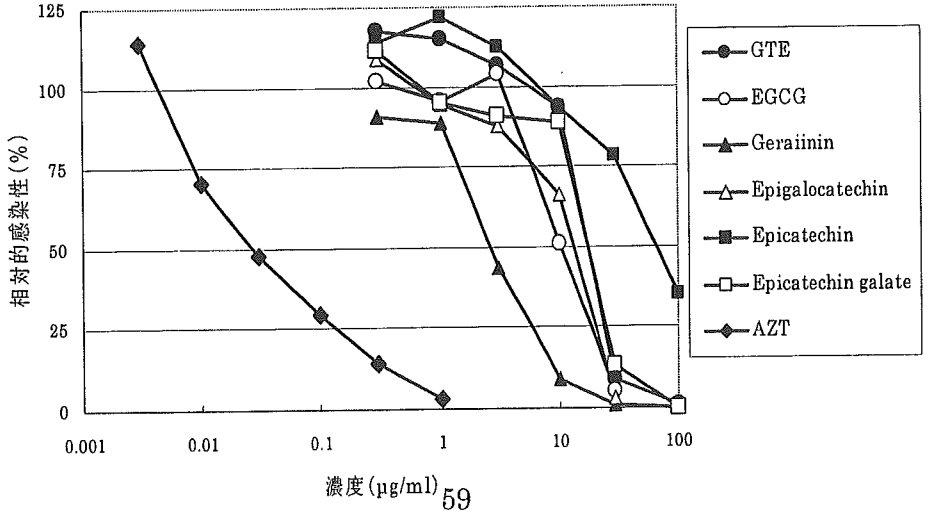


図4 緑茶成分の合胞体形成抑制効果

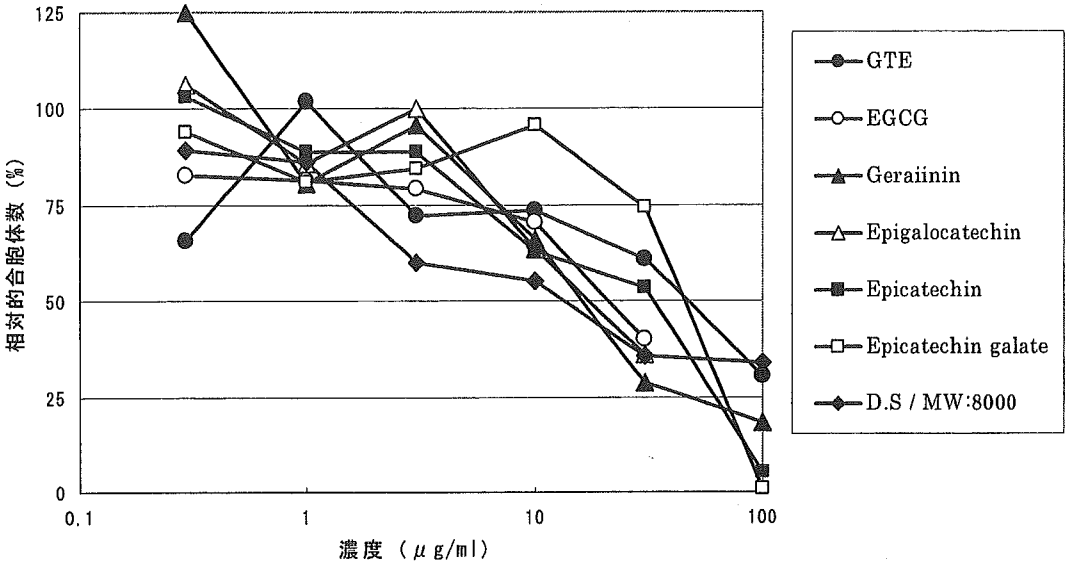
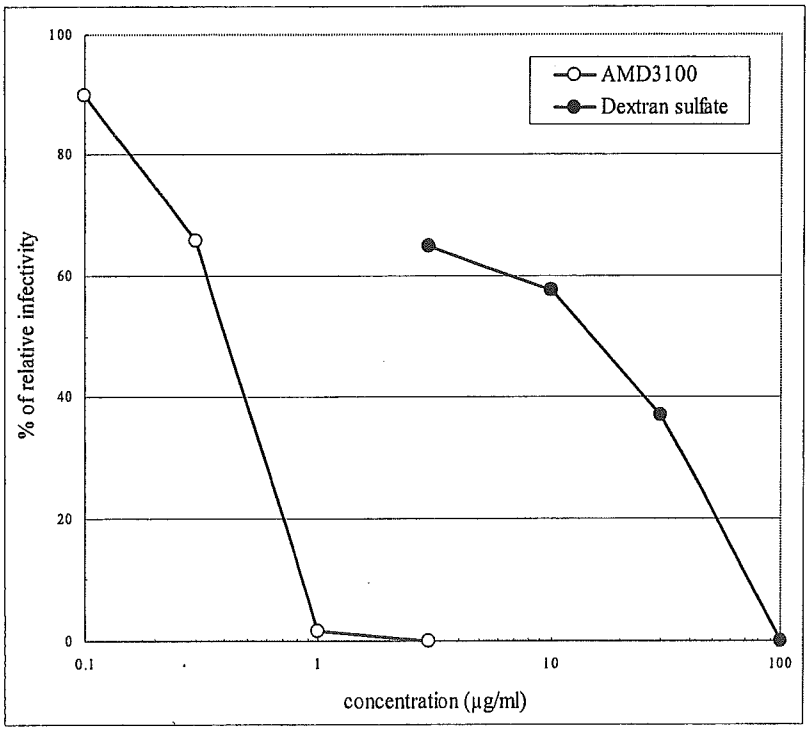


図5 合胞体形成抑制試験



別紙　5

研究成果の刊行に関する一覧表

課題番号	H16-創薬-008
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著者（発表者）氏名　論文タイトル名　発表誌名　巻号，ページ，出版年

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特許出願

[発明の名称]　新規な抗ウイルス剤
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JOURNAL OF EXPERIMENTAL ZOOLOGY (MOL DEV EVOL) 306B:59-69 (2006)

Isolation of the Feline α1,3-Galactosyltransferase Gene, Expression in Transfected Human Cells and its Phylogenetic Analysis

BIBHUTI BHUSAN ROY, ATSUSHI JINNO-OUE, MASAHIKO SHINAGAWA, AKIRA SHIMIZU, KAZUSHI TAMURA, NOBUAKI SHIMIZU, ATSUSHI TANAKA, AND HIROO HOSHINO*

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ABSTRACT The enzyme alpha 1,3-galactosyltransferase (α1,3-GT), which catalyzes synthesis of terminal α-galactosyl epitopes (Gal α1,3Gal β1-4GlcNAc-R), is produced in non-primate mammals, prosimians and new-world monkeys, but not in old-world monkeys, apes and humans. We cloned and sequenced a cDNA that contains the coding sequence of the feline α1,3-GT gene. Flow cytometric analysis demonstrated that the α-galactosyl epitope was expressed on the surface of a human cell line transduced with an expression vector containing this cDNA, and this α-galactosyl epitope expression subsided by α-galactosidase treatment. The open reading frame of the feline α1,3-GT cDNA is 1,113 base pairs in length and encodes 371 amino acids. The nucleotide sequence and its deduced amino acid sequence of the feline α1,3-GT gene are 88–90% and 85–87%, respectively, similar to the reported sequences of the bovine, porcine, marmoset and cebus monkey α1,3-GT genes, while they are 88% and 82–83%, respectively, similar to those of the orangutan and human α1,3-GT pseudogenes, and 81% and 77%, respectively, similar to the murine α1,3-GT gene. Thus, the α1, 3-GT genes and pseudogenes of mammals are highly similar. Ratios of non-synonymous nucleotide changes among the primate pseudogenes as well as the primate genes are still higher than the ratios of non-primates, suggesting that the primate α1,3-GT genes tend to be divergent. *J. Exp. Zool. (Mol. Dev. Evol.) 306B:59–69, 2006.*

Alpha 1,3-galactosyltransferase (α1,3-GT) (EC 2.4.1.151) is responsible for the synthesis of terminal α-galactosyl epitopes (Gal α1,3Gal β1-4GlcNAc-R) in sugar chains. These epitopes are produced by non-primate mammals, prosimians and new-world monkeys and are found on the cell surface ($>10^6$ epitopes/cell) as well as in secreted glycoproteins. The full-length α1,3-GT sequences of human and orangutan (Koike et al., 2002) and some partial sequences, similar to α1,3-GT genes of non-primate mammals, have been detected in humans and higher primates (Larsen et al., '90; Joziasse et al., '91). These sequences have been judged to be pseudogenes because of the generation of premature stop codons due to multiple base deletions (Larsen et al., '90; Joziasse et al., '91; Koike et al., 2002). The expression of α1,3-GT protein is not detected in humans and other catarrhines (Spiro and Bhoyroo, '84; Galili et al., '87, '88; Thall and Galili, '90), thus resulting in the production of large amounts of a natural antibody against the α-galactosyl epitope (Galili et al., '84, '87).

The α1,3-GT is a Golgi membrane-bound enzyme that catalyzes the addition of α-galactosyl epitopes to existing β-galactose terminals accord-

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ing to the following reaction (Basu and Basu, '73; Blanken and Van den Eijnden, '85; Elices et al., '86): UDP galactose+β-D-galactosyl-1,4-*N*-acetyl-D-glucosaminyl-R→ UDP+α-D-galactosyl-1,3-β-D-galactosyl-1,4-*N*-acetyl-D-glucosaminyl-R, in which R may be a glycoprotein or a glycolipid. α1,3-Galactose and less abundant α1,6-linked galactose can be cleaved by the enzyme α-galactosidase (EC 3.2.1.22).

The whole mRNA for α1,3-GT has been found in cells of non-primate mammals and marmoset (Joziasse et al., '89; Larsen et al., '89; Henion et al., '94; Strahan et al., '95; Koike et al., 2002), but only partial mRNAs have been detected in those of old-world monkeys (OWM) (rhesus monkey, green monkey and patas monkey) or human cells (Joziasse et al., '89; Joziasse, '92), indicating that the regulatory sequences can still be functional. Several homologs of the α1,3-GT gene have been described in the human genome, but all of them contain several frame-shift mutations that lead to the generation of premature internal stop codons (Larsen et al., '90; Joziasse et al., '91). One homolog is thought to correspond to the original α1,3-GT gene because it contains intronic sequences (Joziasse et al., '91) as well as one exonic sequence corresponding to the largest part of the catalytic domain of the enzyme (Larsen et al., '90), and this gene has been localized on chromosome 9. Another one, which does not contain any intronic sequences and is located on chromosome 12, corresponds to a copy of the α1,3-GT gene (Larsen et al., '90; Joziasse et al., '91). This pseudogene has also been found in apes and OWM (Galili and Swanson, '91).

By Northern blot analysis, 3.6–3.9 kb α1,3-GT transcripts are detected in bovine and marmoset cells, but not detected in human and OWN cells (Joziasse et al., '89). For the first time, Koike et al. (2002) have detected the full-length sequences of α1,3-GT of orangutans and humans using sensitive PCR-based methods. Comparison of the deduced amino acid sequences with the marmoset gene sequence has revealed that in the human α1,3-GT pseudogene sequence, three single-nucleotide deletions are present at the site corresponding to the amino acid positions 81, 256 and 284 of marmoset α1,3-GT protein, and result in the appearance of the stop codons at positions 268 and 362. Thus, only non-functional proteins are made even when the full-length mRNA is overexpressed and translated in human cells.

A study on the murine α1,3-GT gene has revealed that it is distributed over nine exons that span at least 35 kb of the genomic sequence (Joziasse, '92). Transcription of this gene results in the production of four distinct mRNAs that are generated by the alternative splicing. Translation of these individual mRNAs produces four related isoforms of α1,3-GT consisting of 337, 349, 359 and 371 amino acids.

In this study, we have described the isolation and characterization of a cDNA clone comprising the complete coding sequence of feline (cat) α1, 3-GT that is capable of catalyzing the synthesis of α-galactosyl epitopes on human cells. Phylogenetic analysis was performed to evaluate the evolutionary relationship among the six different full coding sequences of α1,3-GT genes and two pseudogenes.

MATERIALS AND METHODS

Cell culture

8C feline kidney cells (Fischinger et al., '73) were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum (FCS) at 37°C under 5% CO_2 in humidified air. A human osteosarcoma cell line, HOS, infected with a Melanesian strain of human T-cell leukemia virus type I (HTLV-I), termed HOS/HTLV-I_{MEL5}, was cultured in Dulbecco's modified EMEM (Hoshino et al., '93).

Reverse transcriptase-polymerase chain reaction

When 8C cells became confluent, they were collected in an Eppendorf tube using a scraper. Total RNA was isolated from 8C cells using an RNA extraction kit, SepaGene (Sanko-Junyaku Co. Ltd., Tokyo, Japan), according to the manufacturer's protocol. cDNA was synthesized using total cellular RNA as the template, using the SuperScript™ Preamplification System for First-Strand cDNA Synthesis kit (GibcoBRL, Life Technologies™, Carlsbad, CA) in accordance with the manufacturer's protocol. PCR was used to amplify the α1,3galactosyltransferase gene in cDNA preparation. Two sets of primers were used for PCR. One set was made according to the porcine α1,3-GT sequence as follows: 5′-ATGAATGTCAAAGGAAGAGTGGTTCTGTCA-3′ as a forward primer and 5′-TCAGATGTTATTTCTAACCAAATTATACTC-3′ as a reverse primer. The other set was designed using DNA sequences of α1,3-GT of pig (Strahan et al., '95), mouse (Larsen et al., '89), cow (Joziasse et al., '89) and

marmoset (Galili et al., '88). Namely, the regions where the nucleotide sequences of α1,3-GT are well conserved were selected and used to make PCR primers as follows: 5′-GGAGAAAATAAT GAATGTCAA-3′ as a forward primer and 5′-TCAGATGTTATTTCTAACCAAATT-3′ as a reverse primer. The initiation and stop codons in the primers are underlined.

Cloning of the feline α1,3-GT and sequencing

The PCR product was cloned into a TA cloning vector, pCR2.1 (Invitrogen, Carlsbad, CA), and the ligated product was used to transform DH5α *E. coli* strain. *E. coli* colonies were tested for mini-scale plasmid preparation, and colonies containing a 1.1 kb insert after multiple enzyme digestion were selected. Clones containing inserts were sequenced using Texas Red-labeled M13 forward and reverse primers for sequencing DNA. A Hitachi SQ-5500 automated DNA sequencer and software (Hitachi, Tokyo, Japan) were used to determine DNA sequences as described previously (Jinno et al., '98). The plasmid vector pCR2.1 containing the insert and the expression vector pcDNA3 (Clontech Co., Palo Alto, CA) were digested with *BamH*I and *Not*I and purified. The insert in the pCR2.1 vectors was then re-ligated to the digested pcDNA3 vector DNA. After transformation of competent cells, DH5α, by this ligation product, plasmids purified from colonies contained an insert of about 1.1 kb. It was confirmed by enzyme digestion of the plasmid DNA with *Stu*I, *Spe*I, *BamH*I and *Stu*I that the inserts were in a correct direction (data not shown).

Transfection of the feline α1,3-GT gene and detection of its expression

The mammalian expression vector pcDNA3 harboring the gene of the feline α1,3-GT was transfected into a clonal line of HTLV-I-infected HOS cells using LipofectAMINE (GibcoBRL) to examine the effects of α1,3-GT expression on HTLV-I infection, and neomycin-resistant cells were selected as described elsewhere (Jinno et al., '98). In short, the cells were seeded at 4×10^5 cells/ml/well into 12-well plates and incubated overnight. The cells in nine wells were independently transfected with pcDNA3 DNA harboring the gene of the feline α1,3-GT or pcDNA3 DNA alone using LipofectAMINE (GibcoBRL). The cells were selected with neomycin after 24 hr incubation and maintained for 3 weeks. The expression of α-galactosyl epitope on the surface of HOS and HOS/HTLV-I cells and feline cells was detected by flow cytometry (FCM) (Cyto ACE-100, Auto cell screener, Japan Spectroscopic Co., Ltd.) after treatment with fluorescent isothiocyanate labeled *Bandeiraea simplicifolia* Isolectin B_4 (FITC/BS-IB_4) (Sigma Chemical Co., St. Louis, MO) (Wood et al., '79; Azimzadeh et al., '97; Bracy et al., '98). Briefly, cells were collected into Eppendorf tubes from cultured plates, washed with washing solution (cold PBS with 1% FCS and 0.01% NaN3), pelleted by centrifugation at 5,000 rpm for 5 min and treated for 1 hr with diluted FITC/BS-IB_4 (3.3 pg/ml) on ice. The cells were washed and fixed with 1% paraformaldehyde and analyzed by FCM. In seven (#1–#7) out of nine wells seeded with the feline α1,3-GT-transduced cells, 20–45% of cells were positively stained with FITC/BS-IB_4 (Table 1). These cells were cloned using 96-well plates by seeding 1 cell/well and thus obtained clones were screened again by FCM.

α-Galactosidase treatment

Cells were treated with α-galactosidase as described by Bracy et al. ('98). Briefly, cells were seeded at 4×10^5 cells/well into 6-well plates in 2 ml and incubated overnight. The culture medium was replaced with fresh medium and the cells were incubated for another day. The cells were collected into two Eppendorf tubes from each cultured plate, washed with the washing solution

TABLE 1. Flow cytometry of the cells transfected with the feline α1,3-GT gene

Cell	BS-IB4-positive cells (%)
HOS/HTLV-I	4
HOS/HTLV-I/pcDNA3	5
HOS/HTLV-I/fGT	
#1	45
#2	44
#3	38
#4	32
#5	27
#6	27
#7	22
#8	4
#9	4

Human HOS/HTLV-I cells in nine culture wells were transfected with the feline α1,3-GT gene independently. Neomycin-resistant cells were selected. These HOS cells were processed for FCM using FITC/BS-IB4 lectin and examined for the expression of α-galactosyl epitope on the cell surface. HOS/HTLV-I/pcDNA3 cells were used as a control negative for terminal α1,3-galactose. The cut-off value for FCM was set at the fluorescence intensity that gave 5% positive for the control cells as shown in Figs. 1 and 2.

and pelleted by centrifugation at 5,000 rpm for 5 min. One of them was treated with α-galactosidase dissolved in sodium citrate/phosphate buffer, and the other tube was treated only with buffer for 1 hr at 37 °C. The cells were washed with the washing solution and treated for 1 hr with FITC/BS-IB_4 on ice. The cells were washed and fixed with 1% paraformaldehyde and analyzed by FCM.

Synonymous (K_S) and non-synonymous (K_A) nucleotide substitutions per site

The number of synonymous substitutions per possible synonymous site (K_S) and the number of non-synonymous substitutions per possible non-synonymous site (K_A) (Li et al., '85; Li, '93) were calculated for codons of full-length α1,3-GT gene and pseudogene sequences using MEGA 2.1 (Kumar et al., 2001). K_A/K_S ratios less than 1.0 are generally considered as evidence that the proteins have evolved under negative or purifying selection; pairwise comparisons between active genes show this pattern (Wolfe and Sharp, '93; Endo et al., '96). K_A/K_S ratios over 1.0 suggest positive or directional selection.

Phylogenetic trees for the α1,3-GT isolates

Two phylogenetic trees using the amino acid sequences and nucleotide sequences of the α1, 3-GT cDNAs were constructed using the N–J method (Saitou and Nei, '87), and the reliability of the clusters obtained was evaluated by means of 1,000 bootstrap replicates. The MEGA 2.1 software was used to make the trees.

RESULTS

Isolation of feline α1,3-GT cDNA

A feline kidney cell line, 8C, was used to make α1,3-GT cDNA. Cellular RNA was isolated and reverse transcribed. PCR with the two sets of primers described above was used to amplify cDNA. The PCR product was then ligated with the cloning vector pCR2.1 and the construct was used to transform *E. coli* cells. Plasmid DNAs were isolated from *E. coli* colonies that harbored DNA with the expected length of the insert and used for DNA sequencing. We sequenced seven different clones, and six out of seven clones gave overlapping nucleotide sequences. The full-length coding sequence of the feline α1,3-GT gene was 1,113-bp long (GenBank accession number AY167024) and the deduced amino acid sequence contained 371 amino acids. Its molecular mass was calculated to be 43,568 Da.

Expression of α-galactosyl epitope on the cell surface

The expression vector pcDNA3 with the feline α1,3-GT insert was made and transfected into a cloned human osteosarcoma cell line, HOS, infected with HTLV-I, i.e., HOS/HTLV-I (Hoshino et al., '93) in nine culture wells, and neomycin selection was done. The cells were maintained about 3 weeks in neomycin-containing medium and examined by FCM using fluorescent isothiocyanate-labeled *B. simplicifolia* Isolectin B_4 (FITC/BS-IB_4). In the first screening, FCM results showed that 20–45% cells derived from seven (#1–#7) out of nine wells containing the feline α1,3-GT plasmid-transfected cells were positively stained with FITC/BS-IB_4 (Table 1). The cells derived from wells #1 and #2 were single-cell cloned by seeding them into 96-well plates at a density of one cell per well, and thus obtained two of 19 clones were about 98% positive by FCM (Fig. 1). The clones derived from initial #1 and #2 wells were designated as HOS/HTLV-I/fGT#1-a and #2-a. The surface expression of α-galactosyl epitopes was stable as it was detected by FCM even after 20 cell passages (data not shown). In contrast, the surface expression of α-galactosyl epitopes was not detected upon FCM of untransfected or only pcDNA3 vector-transfected cells. When the feline α1,3-GT-transduced HOS/HTLV-I cells were treated with α-galactosidase, specific reduction of α-galactosyl epitope expression was detected (Fig. 2) by staining with FITC/BS-IB_4 as compared with results of these cells treated with buffer alone. These findings indicated that the cDNA cloned from 8C cells really coded for the feline α1,3-GT. The cells for highly positive α-galactosyl epitopes grew as well as untransfected HOS/HTLV-I cells, and their morphology was indistinguishable from that of the untransfected HOS/HTLV-I cells.

Analysis of the feline α1,3-GT gene sequence

We aligned the deduced amino acid sequences of the α1,3-GT genes of feline, porcine, murine, bovine, cebus and marmoset origins and the human and orangutan α1,3-GT pseudogenes using software for multiple alignments, Clustal W (Thompson et al., '94) (Fig. 3). In our alignment of the amino acid sequences of the eight α1,3-GT genes, the feline sequence was 85–87% similar to

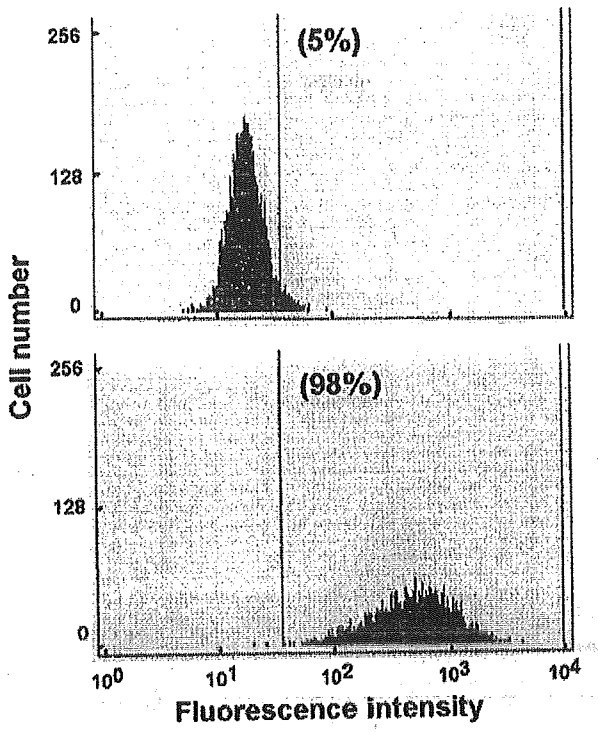

Fig. 1. Detection of α-galactosyl epitope expression. The pcDNA3 control vector-transfected HOS/HTLV-I cells (A) and the feline α1,3-GT gene-transfected HOS/HTLV-I cells (B) were processed for FCM using FITC/BS-IB_4 and examined for the expression of α-galactosyl epitope on the cell surface. The cut-off value for fluorescence intensity was set as 5% of control cells were scored positive (A); the cells that showed stronger intensity than the cut-off value were considered to be positive (B).

those of bovine, porcine, cebus and marmoset origins; the human and orangutan sequences were 82–83% and the murine sequence was 77% similar to the feline sequence. The nucleotide sequences of the bovine, porcine, marmoset and cebus α1, 3-GT genes and of the orangutan and human α1, 3-GT pseudogenes showed a high similarity (88–90%) to the feline sequence, while the murine sequence was only 81% similar to it (Table 2).

Figure 3 shows that there is an especially high similarity among all eight α1,3-GT sequences after amino acid number 84 (feline), 85 (porcine), 85 (murine), 81 (bovine), 89 (human), 89 (cebus), 89 (orangutan) and 90 (marmoset): there was 82–91% similarity in the domain corresponding to amino acid numbers 84–370 of the feline sequence, while in the domain 1–83 the similarity was much lower and between 65% and 72% (Table 2). The highly similar domain has been thought to encode catalytic activities (Henion et al., '94). Although the human and orangutan α1,3-GT genes are inactive, we still noticed the high similarity in their nucleotide sequences with those of other animal α1,3-GT genes. The murine sequence showed a slightly lower similarity: 82% in the 84–370 domain and 62% in the 1–89 domain (Table 2).

To study the evolutionary forces that have been operated among α1,3-GT genes, we analyzed their sequences for synonymous substitution per site (K_S) and non-synonymous substitution per site (K_A) between paired species using the modified Nei–Gojobori method (Kumar et al., 2001). The total number of possible synonymous sites in an individual sequence among the eight α1,3-GT gene sequences was between 287 and 296 (standard error between 6 and 8) with an average of 292, and the total number of possible non-synonymous sites was between 795 and 810 (standard error between 7 and 16) with an average of 803 (data not shown). The total number of possible synonymous and non-synonymous sites in an individual sequence was between 1,082 and 1,104 with an average of 1,095. The total synonymous-site differences between paired samples were between 8 and 121 (standard error between 3 and 9) with a mean of 65 (Table 3), and the non-synonymous-site differences were between 12 and 129 (standard error between 3 and 12) with a mean of 69 (Table 4). We found that the range of the paired samples K_S (calculated using MEGA 2.1 software) was 0.028–0.410 (standard error between 0.009 and 0.028) with a mean of 0.223, and the K_A range was 0.015–0.161 (standard error between 0.006 and 0.015) with a mean of 0.086 (data not shown). The K_S values are significantly higher than the K_A values ($P < 0.0005$, according to Student's t-test). Table 5 shows the K_A/K_S ratios where all the values are within a range of 0.219–0.763, indicating that the α1,3-GT genes are in a direction from negative, purifying selection to neutrality. The ratios between the species that express the active enzyme are around 0.2–0.4 and those of primates, including cebus, orangutan and human but not marmoset, are around 0.7–0.8 irrespective of whether the enzyme is active (cebus) or inactive (orangutan and human). The marmoset α1,3-GT gene shows an intermediate type: 0.273 with that of cebus and about 0.7–0.8 with that of orangutan and human (Table 5).

Phylogenetic trees of the α1,3-GT genes

To analyze the evolutionary distance among α1,3-GT genes of feline, murine, bovine, porcine,

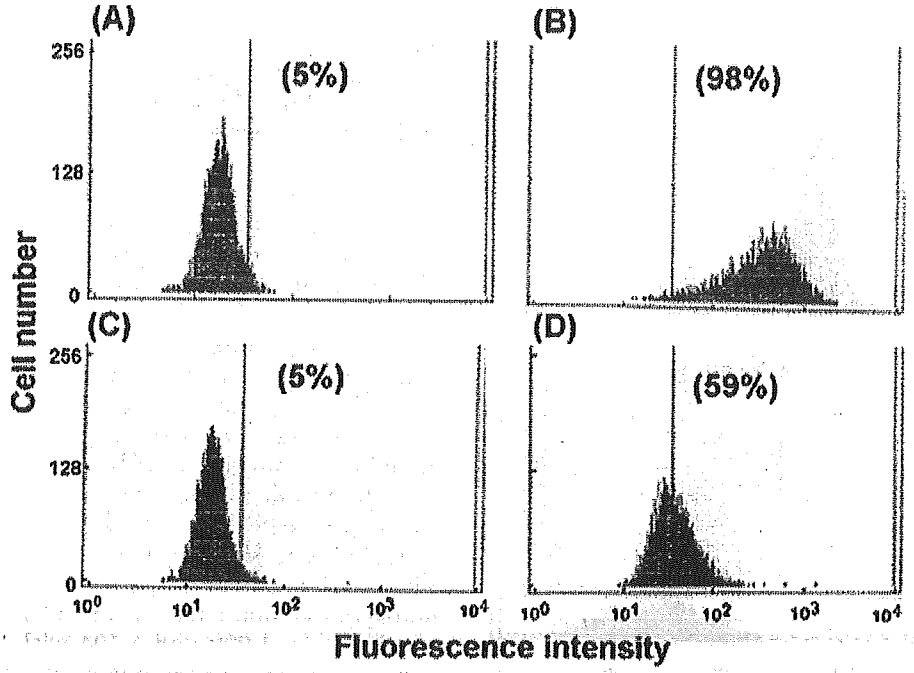

Fig. 2. Detection of α-galactosyl epitope expression. The pcDNA3 vector-transfected HOS/HTLV-I and the feline α1,3-GT plasmid-transfected HOS/HTLV-I cells were initially treated with buffer (**A** and **B**) alone or α-galactosidase (**C** and **D**). The cells were then processed for FCM (as described in Fig. 1) and examined for the expression of α-galactosyl epitope on the cell surface.

cebus and marmoset origins and the α1,3-GT pseudogenes in orangutan and human species, we constructed phylogenetic trees for these α1,3-GT genes and pseudogenes by the N–J method (Saitou and Nei, '87) using the total coding nucleotide sequences (date not shown) and the amino acid sequences (Fig. 4). When the feline α1,3-GT nucleotide sequence was aligned with the other α1,3-GT nucleotide sequences and a phylogenetic tree was made with Poisson correction, it was closely related with the other mammalian α1,3-GT nucleotide sequences. The phylogenetic tree showed that the feline α1,3-GT gene clustered with all other mammalian α1,3-GT genes, especially with the porcine and bovine genes, with a bootstrap support of 93% (date not shown).

The phylogenetic tree constructed using the deduced amino acid sequences shown in Fig. 4 reveals a markedly similar pattern to that constructed using the nucleotide sequences. We also aligned 1–83 and 84–370 amino acid sequences of the feline α1,3-GT with all other α1,3-GT amino acid sequences to make two other phylogenetic trees (data not shown). Both of the phylogenetic trees show almost the same pattern as shown in Fig. 4.

DISCUSSION

When HOS/HTLV-I human cells were transfected with the cDNA encoding the feline α1, 3-GT, they became positive for the expression of α-galactosyl epitopes. In addition, α-galactosidase treatment of HOS/HTLV-I cells transfected with the feline α1,3-GT led to specific reduction of α-galactosyl epitope expression (Fig. 2) as detected by staining with FITC/BS-IB_4. The enzymatic removal of α-galactosyl epitopes was, however, incomplete, probably because the reaction was performed at sub-optimal pH to preserve the cell viability as described by Bracy et al. ('98). Thus, newly cloned human cells showed the stable expression of the feline α1,3-GT (Fig. 1), which catalyzes the addition of α-galactosyl epitopes to existing carbohydrate side chains.

According to the general topology of glycosyltransferases (Paulson and Colley, '89; Joziasse et al., '92), it has been reported that there are three domains in the sequences of glycosyltransferases: a cytoplasmic domain, a transmembrane domain and a luminal domain. Henion et al. ('94) have shown that the 67 amino acids from positions

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Feline      MNVKGRVVLSMLVVSTVIVVFWEYINSPEGSFLWIYHSKNPEVGDSSTQKGWWFPSWFNN : 60
Murine      .....K.I.L..I....V......V...D.........T.I....ENRW..D.......K. : 60
Bovine      .....K.I.................H.....LF..NP.R.....G..I.....L.R.... : 60
Porcine     ............L....M.............LF...Q.......-..A.R.......... : 59
Marmoset    .....K.I.....................................D...A..D....G.... : 60
Cebus       .....K.I.....................................D.-.A.......G.... : 59
Orangutan   .....K.I............I......T................D....A.....L..... : 60
Human       .....K.I................F...T................D...A......L..... : 60

Feline      RTHSYPEEEAVD----EGDEQRKENSE--ELQLSDWFNPQKRPDVVTVTEWKAPVVWEGT :114
Murine      G....Q.DNVEG-----RR.KGRNGDRIE.P..W.....KN....L...P....I..... :115
Bovine      ---G.H..DGDI----NEEKEQRNED.-SK.K.......F...E...M.K.......... :112
Porcine     G....H...DAI----GNEKEQRKEDNRG..P.V.....E...E...I.R.......... :115
Marmoset    GI.N.QQ..EDTDKEKGRE.EQ.KEDDTT..R.W.....K...E.M...Q.......... :120
Cebus       GI.N.QQ..EDIDKEKGRE.EQRKEDDTT....W.....K...E.....K.......... :119
Orangutan   GI.N.QQR.EDIDKEKGRE.-QRKENDTT..R.W.....K...K.....R.......... :119
Human       GI.N.QQG.EDIDKEKGRE.-QRKENDTT..R.W.....K.H.E.....R.......... :119

Feline      YNKAILENYYARQKITVGLTVFAVGRYIEHYLEEFLISANRYFMVGHKVIFYIMVDDVSK :174
Murine      .DT.L..K...T..L.........K.......D..E..DM......R....V.I..T.R :175
Bovine      ..R.V.D....K.............................KH.....P..........R :172
Porcine     ..R.V.D....K.............................T..............I.R :175
Marmoset    ...........K...........I...........VT..............V....... :180
Cebus       ...........K...........I...........VT..............V....... :179
Orangutan   F.....G....K.......M...I...NG......IT....................... :179
Human       F.....G....K.....R.M...I...ND......IT....................... :179

Feline      MPLIELGPLRSFKVFEIKPEKRWQDISMMRMKIIGEHIVAHIQHEVDFLFCMDVDQVFQD :234
Murine      ..VVH.N..H.LQ....RS.............T.....L..................... :235
Bovine      ..............K..............T.......................... :232
Porcine     ..................S.............T.....L.................N :235
Marmoset    A.F.............V..............T.....L..................... :240
Cebus       V.F.............V..............T.....L..................... :239
Orangutan   V.F......H......V..............T....L..................... :239
Human       L.F......H...M..V..............T....L..................... :239

Feline      SFGVETLGQSVAQLQAWWYKADPDEFTYERRKESAAYIPFGEGDFYYHAAIFGGTPTQVL :294
Murine      N........L...........S.EK......EL.....................HI. :295
Bovine      K.......E.............ND.................................. :292
Porcine     N.....................H.....................Q.............. :295
Marmoset    H.....................D.....................Q.............I... :300
Cebus       H.....................D......R.........Q........V.....I... :299
Orangutan   H.............R......D..*...E.........Q.-.................. :298
Human       H.............-.R.....YD..*..W....G.....Q.-........S....I... :297

Feline      NITQECFKGILQDKKNDIEAEWHDESHLNKYFLLNKPTKILSPEYCWDYHIGLPSDIKIV :354
Murine      .L.R..........H....Q............F..............Q........S. :355
Bovine      ..........K........Q.................................A...L. :352
Porcine     ............E.....................................MSV..R.. :355
Marmoset    ...........L.........................S.....................T. :360
Cebus       ...........L.........................S.....................T. :359
Orangutan   ...R...N...L.....V.....................S....LK.............T. :358
Human       ...........L........K.................S....LK.............T. :357

Feline      KISWQTKEYNLVRNNI :370
Murine      .VA...........V :371
Bovine      .M........V....V :368
Porcine     ..A..K.......... :371
Marmoset    .L...........K.V :376
Cebus       .L.............. :375
Orangutan   .R.R...........V :374
Human       .*............V :373
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Fig. 3. Alignment of deduced amino acid sequences of different α1,3-GT genes. The amino acid sequences of the feline, porcine, murine, bovine, cebus and marmoset α1,3-GT genes and human and orangutan α1,3-GT pseudogenes are aligned using CLUSTAL W algorithm. Numbers at the right side indicate amino acid positions. Dots (.) represent amino acids identical to those in the feline gene sequence. Amino acid deletions in each gene are indicated by dash marks (-). In the human sequence, the star marks (*) indicate premature stop codons.

TABLE 2. Nucleotide and amino acid similarity between the feline and other α1,3-GT genes

Species	Nucleotide similarity (%)	Amino acid sequence				
		1–83 domain		84–370 domain		Total
		Similarity (%)	Gap	Similarity (%)	Gap	Similarity (%)
Murine	81	62	1[1]	82	0	77
Bovine	88	65	3	90	0	85
Porcine	90	72	1	91	0	87
Marmoset	89	72	0	91	0	87
Cebus	89	72	1	91	0	87
Orangutan	88	70	1	86	1	83
Human	88	70	1	86	2	82

The total coding sequences of the α1,3-GT genes were aligned, including gaps. Identical nucleotides and amino acids at each position, excluding gaps, were counted and a similarity rate (%) was calculated between the feline α1,3-GT gene and each α1,3-GT gene or pseudogene.
[1]Number of amino acid gaps placed when compared with the 1–83 or 84–370 domains of the feline α1,3-GT gene.

TABLE 3. Total number of synonymous differences between paired species among eight different α1,3-GTs

Species	Species							
	Feline	Murine	Bovine	Porcine	Marmoset	Cebus	Orangutan	Human
Feline	—							
Murine	107	—						
Bovine	79	120	—					
Porcine	64	113	61	—				
Marmoset	76	95	74	63	—			
Cebus	71	94	77	61	16	—		
Orangutan	71	87	68	56	17	15	—	
Human	72	91	73	59	21	17	8	—

Total possible synonymous sites were between 287 and 296 with an average of 292.

TABLE 4. Total number of non-synonymous differences between paired species among eight different α1,3-GTs

Species	Species							
	Feline	Murine	Bovine	Porcine	Marmoset	Cebus	Orangutan	Human
Feline	—							
Murine	100	—						
Bovine	62	123	—					
Porcine	51	110	65	—				
Marmoset	48	107	68	64	—			
Cebus	43	106	63	60	12	—		
Orangutan	60	123	83	79	37	34	—	
Human	60	129	83	79	40	36	18	—

Total possible non-synonymous sites were between 795 and 810 with an average of 803.

23 to 89 in the marmoset α1,3-GT gene, which they called a stem region, have little effect on enzymatic activity, but the sequence between 90 and 376 can show an almost full catalytic activity. The presence of stop codons in human and orangutan α1,3-GT genes at the site corresponding to the 268th amino acid of the marmoset gene (Fig. 3) will lead to the loss of about 100 amino acids at the C-terminus. This loss is expected to be sufficient to lose the entire catalytic activity

TABLE 5. K_A/K_S ratios of paired samples among eight different α1,3-GTs

Species	Species							
	Feline	Murine	Bovine	Porcine	Marmoset	Cebus	Orangutan	Human
Feline	—							
Murine	0.343[1]	—						
Bovine	0.284	0.374	—					
Porcine	0.289	0.355	0.386	—				
Marmoset	0.228	0.411	0.335	0.367	—			
Cebus	0.220	0.414	0.298	0.354	0.273	—		
Orangutan	0.306	0.517	0.445	0.510	0.780	0.808	—	
Human	0.304	0.516	0.411	0.485	0.694	0.763	0.786	—

[1]K_A/K_S ratios calculated using MEGA 2.1 software (Kumar et al., 2001).

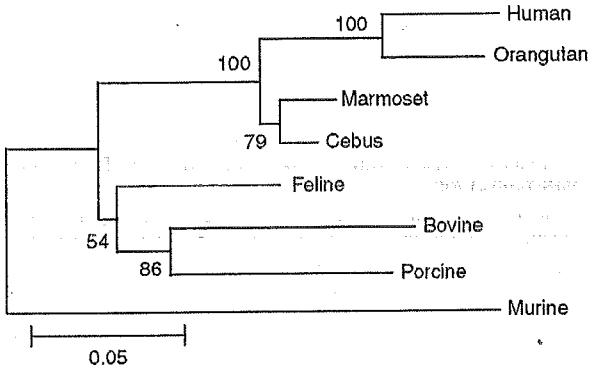

Fig. 4. A phylogenetic tree of the α1,3-GT enzyme. A phylogenetic tree of the amino acid sequences deduced from the nucleotide sequences for murine, feline, porcine, borine, marmoset and cebus α1,3-GT genes and the human and orangutan α1,3-GT pseudogenes was constructed by the N–J method using the MEGA 2.1 software. The phylogenetic relationships of the amino acid sequences are represented as unrooted cladograms. The numbers at branch nodes indicate the bootstrap support level (BSL), which is the percentage of how often each branch presents exactly the same topology in all the resampled trees. The scale bars indicate the number of substitutions per site.

even when α1,3-GT mRNA of human or orangutan is translated.

In our sequence alignment, it appeared that the amino acid residues 61–83 in the feline α1,3-GT gene form a highly variable region and the residues between 84 and 371 form a highly conserved region. This finding also suggests that the catalytic domain of the feline α1,3-GT gene is located in the highly conserved 84–370 domain. In the corresponding domains of animal sequences, including the human and orangutan pseudogenes, the functional constraints appear to have restricted mutations throughout evolution by Wilson et al. ('77). Since most of the amino acid gaps were located between positions 45 and 84 of the feline α1,3-GT sequence, this portion is unlikely to contain catalytic activity.

The α1,3-GT gene is transcribed in a small amount but the enzyme encoded is not active in higher primates (humans, apes and OWM) (Koike et al., 2002): the reason for loss of this enzyme activity in higher primates is unknown. There are several reports on production of α1,3-GT gene-deficient mice (knock-out mice). These mice can grow, live and age normally (Tearle et al., '96; Pearse et al., '98), indicating that the α1,3-GT gene can be dispensable for rodents, although this gene has been evolutionarily conserved as shown in Table 2. On the contrary, the expression of the α1,3-GT gene in human cells did not affect the growth and morphology of human cells, as we could isolate HOS/HTLV-I cell lines highly expressing α1,3-GT (Table 1 and Fig. 1). Thus, its expression did not exert adverse effects on human cells at least in tissue culture. It is intriguing for us that the α1,3-GT genes of marmoset and cebus and the pseudogenes of orangutan and human show similar degrees of substitution rates in their nucleotide sequences as well as in their amino acid sequences to the murine, bovine and porcine genes.

The rate of synonymous substitution (K_S) is usually much higher than that of non-synonymous substitution (K_A) for a normally functioning gene. Synonymous substitution may be used as a molecular clock for dating the evolutionary time of closely related species (Kafatos et al., '77; Kimura, '77; Miyata and Yasunaga, '80; Perler et al., '80). K_A/K_S ratios less than 1.0 are generally taken as negative or purifying selection, and, conversely, K_A/K_S ratios significantly greater than 1.0 are considered to be proper evidence of

directional or positive selection for amino acid replacement (Li et al., '85; Li, '93). That is, when the K_A/K_S value for a given gene is less than 1.0, the encoded protein sequence has been conserved during evolution. For example, the histone H4 gene family protein sequences are under purifying selection (Piontkivska et al., 2002) and some lineages of the primate lysozyme protein sequences are under directional selection (Messier and Stewart, '97). Among the α1,3-GT genes, the synonymous substitution rate is significantly higher than the non-synonymous substitution rate between the paired sequences ($P<0.0005$). Generally, the values of possible non-synonymous substitutions are much higher than the values of possible synonymous substitutions. Among the α1,3-GT genes we examined, the ranges of possible non-synonymous and synonymous substitution sites were 795–810 and 287–296, respectively.

Pairwise K_A/K_S ratios are high (0.7–0.8) in the species where the enzyme had lost its activity (human or orangutan), but are much lower (0.2–0.4) in the species where the enzyme is active. Although the α1,3-GT gene in marmoset and cebus is active and the K_A/K_S value between them is 0.273, their pairwise K_A/K_S values with human and orangutan pseudogenes are as high as 0.7–0.8. High K_A/K_S values (among human, orangutan and cebus) may indicate that these genes have evolved rapidly at the protein level or tend to evolve free of constraint, although their pairwise K_A/K_S ratios are not greater than 1. It is noteworthy that most of the non-synonymous substitutions are concentrated in a region that locates outside of the catalytic domain of the enzyme (Fig. 3).

Humans are known to carry natural antibodies against α1,3-galactosyl epitope at high titers and these antibodies are also known to markedly affect transplantation of xenografts derived from porcine organs and will lead to rejection (Sandrin and McKenzie, '94). A pig strain with knock-out of this gene has already been made (Dor et al., 2004). These antibodies should, however, be beneficial for survival of higher primates. These natural antibodies have been thought to exert prophylactic effects on development of cancer or infection with certain pathogens bearing α-galactosyl epitope (Gollogly and Castronovo, '96; Welsh et al., '98). It remains to be elucidated why α1,3-GT genes should have been inactivated in humans, apes and OWM and why the inactivated gene sequences still have a high degree of similarity to those of the active genes.

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