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Quantitative Analysis of Expression of Mouse Sialyltransferase Genes by Competitive PCR[1]

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The present paper describes a rapid and systematic method for semi-quantitative analysis of the expression of sialyltransferase genes. So far, fifteen sialyltransferase cDNAs have been cloned from mice. Most of these genes are expressed in developmental stage-dependent and/or tissue-specific manners, and the expression levels of some of them are too low to detect on Northern blot analysis. To resolve how each sialyltransferase contribute to synthesize sialylglycoconjugates, it is necessary to establish the method for quantification of gene expression levels of these fifteen sialyltransferases. Therefore, we developed a competitive PCR-based method for analyzing the quantitative relationship of the gene expression of fifteen sialyltransferases. Using this method, we can investigate the levels of gene expression of sialyltransferases in various cell lines and various tissues of mice, and can accurately determine their expression levels.

Key Words: **competitive PCR; sialyltransferase.**

Sialic acids and their derivatives are ubiquitous at the terminal positions of oligosaccharides of glycoproteins and glycolipids in tissues of various animal species, and they play important roles in a variety of biological processes, such as cell-cell communication, cell-matrix interaction, adhesion, and protein targeting. The transfer of the sialic acid from CMP-sialic acid to an acceptor carbohydrate is catalyzed by a family of glycosyltransferases called sialyltransferases (3). Each sialyltransferase exhibits strict specificity as to acceptor substrates and linkages. So far, PCR-based cDNA cloning of sialyltransferases has been widely performed (4–8), and 15 sialyltransferase cDNAs have been cloned from mouse. These cloned sialyltransferases are classified into four families according to the carbohydrate linkages they synthesize, that is, the ST3Gal-, ST6Gal-, ST6GalNAc-, and ST8Sia-families. The primary enzyme structures deduced from all the cloned sialyltransferase cDNAs suggest a putative domain structure with a type II transmembrane topology. There are no significant amino acid sequence similarities among these sialyltransferases, except for in two sialyl motifs, L and S, which are proposed to be the CMP-sialic acid recognition and/or catalytic sites (9). Northern blot analysis revealed the developmental stage-dependent and/or tissue-specific expression of most of these sialyltransferase genes. However, expression of some of these sialyltransferase genes was too low to detect on Northern blot analysis. Also the quantitative relationship of the gene expression of these 15 sialyltransferase genes has not been investigated systematically. To determine contribution of each sialyltransferase to the biosynthsis of carbohydrates containing sialic acids, it is very important to examine the quantitative relationship of the gene expression of these 15 sialyltransferase genes as well as the developmental stage-dependent and tissue-specific expression. Competitive PCR (10) has the advantage of being capable of more accurate determination of the quantitative relationship than simple reverse-transcriptase (RT) PCR. Here we describe a systematic

[1] The accession numbers of the GeneBank/EMBL Data Banks of the sialyltransferases reported here are X73523 (ST3Gal I), X76989 (ST3Gal II), X84234 (ST3Gal III), X95809 (ST3Gal IV), Y15003 (ST3Gal V), D16106 (ST6Gal I), D16106 (ST6GalNAc I), X93999 (ST6GalNAc II), Y11342 (ST6GalNAc III), Y15779-80 (ST6GalNAc IV), X84235 (ST8Sia I), X83562 (ST8Sia II), X80502 (ST8Sia III), X86000 (ST8Sia IV), and X98014 (ST8Sia V).

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Abbreviations used: The nomenclature for gangliosides follows the system of Svennerholm (1). The abbreviated nomenclature for cloned sialyltransferases follows the system of Tsuji *et al.* (2). CMP, cytidine 5′-monophosphate; G3PDH, glyceraldehyde 3-phosphate dehydrogenase gene; PCR, polymerase chain reaction; RT, reverse transcriptase.

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TABLE 1

Primers Used for Competitive PCR

Gene	Strand	Primer	Position	Amplified fragment (bp) Target	Competitor
ST3Gal I	sense	5′-ATGAGGAGGAAGACCCTCAAG-3′	1 to 21	403	248
	antisense	5′-CCACCAGCCTCTTGTTCAAC-3′	384 to 403		
ST3Gal II	sense	5′-GATGAAGTGCTCTCTTCGGG-3′	−1 to 19	410	240
	antisense	5′-CAGGCACGATCTGGAACAGT-3′	390 to 409		
ST3Gal III	sense	5′-GTGAAGATGGGACTCTTGGT-3′	−6 to 14	661	478
	antisense	5′-ATTGCTCAGGTCGCTGCATG-3′	646 to 655		
ST3Gal IV	sense	5′-AGCCATGCTTCCAGGGTGAG-3′	131 to 150	450	272
	antisense	5′-CCTTGAAAGCTACCAGGACC-3′	561 to 580		
ST3Gal V	sense	5′-TCAGAGCTATGCTCAGGAAGTCTTGCAGAAG-3′	240 to 270	349	250
	antisense	5′-ACTGTTCAACCTTATTACCACATCGAACTG-3′	559 to 588		
ST6Gal I	sense	5′-ATGATTCATACCAACTTGAAG-3′	1 to 21	635	530
	antisense	5′-GGTGCCCCATTAAACCTCAG-3′	616 to 635		
ST6GalNAc I	sense	5′-CATGACGAGATATTGCAGAGG-3′	−1 to 20	356	269
	antisense	5′-CTGCCTTGCTCTGAGGATTC-3′	326 to 355		
ST6GalNAc II	sense	5′-AGACCCAGGTTCCCGCCAGG-3′	104 to 123	481	287
	antisense	5′-AAGGAGGTCTTAGTGCCCAC-3′	565 to 584		
ST6GalNAc III	sense	5′-ATGGATACATAAATGTGAGGACC-3′	185 to 207	515	315
	antisense	5′-GTGGATACTGTAGCAGGCATCCA-3′	677 to 699		
ST6GalNAc IV	sense	5′-GTGGTCTACGGGATGGTCAG-3′	684 to 703	678	542
	antisense	5′-GAGAGGCTGAGGCTCAAAGG-3′	1343 to 1362		
ST8Sia I	sense	5′-GACAAATGGAAGACTGCTGTG-3′	236 to 256	845	667
	antisense	5′-CTTCTGCAGTCCCTAGGAAG-3′	1061 to 1080		
ST8Sia II	sense	5′-TGAAGAATAAGCATTTCCAGACTTGTGCC-3′	446 to 474	502	346
	antisense	5′-CAGAAGCCATAAAGGTAGATCTGAT-3′	923 to 947		
ST8Sia III	sense	5′-AGCCCGGGATGAGAAATTGC-3′	−8 to 12	474	262
	antisense	5′-TCATAATGGGCGACACATCT-3′	447 to 466		
ST8Sia IV	sense	5′-CACCCAAGATGCGCTCAATT-3′	−8 to 12	408	354
	antisense	5′-TTGGTGAAACTTCAGGCAGG-3′	381 to 400		
ST8Sia V	sense	5′-CAGGATGCGCTACGCAGACC-3′	−4 to 16	834	625
	antisense	5′-TTCTCGTACCTCTGCAGCAC-3′	811 to 830		
G3PDH	sense	5′-GGATCCACCACAGTCCATGCCATCAC-3′	536 to 555	464	—
	antisense	5′-AAGCTTTCCACCACCCTGTTGCTGTA-3′			

Note. Nucleotide positions are numbered from the adenosine of the start codon taken as +1.

method for quantifying the expression of 15 sialyltransferase genes by means of quantitative competitive PCR.

MATERIALS AND METHODS

Construction of competitor DNAs. PCR was performed using specific primer set shown in Table 1 through 30 cycles of a step program (94°C, 60 s; 55°C, 60 s; 72°C, 90 s) with each sialyltransferase cDNA as the template. For constructions of competitor DNAs for all sialyltransferases, PCR-amplified fragments were ligated into the *Eco*RV site, or *Sma*I site of pBluscript SK (+) vectors expect for those of ST6GalNAc II, ST6GalNAc III. These two were directly ligated into pKF18k vector. Then internal portions of these fragments were deleted from the resulting plasmids directly (ST3Gal 111, ST6GaI I, ST6GalNAc 1V, ST8Sia I, and ST8Sia V), after transferring to pUC118 (ST3Gal I and ST8Sia II (11)), after subjecting to site-directed mutagenesis to make the appropriate restriction site using Mutan-Super Express Km Kit (Takara, Japan) and pKF18k Vector (ST3Gal II, ST3Gal 1V, ST3Gal V, ST6GalNAc II, ST6GalNAc III, and ST8Sia III). The resulting fragments were blunted and self-ligated. In ST6GalNAc I and ST8Sia IV cases, the *Pma*CI-*Bgl*II and *Eco*RI-*Sal*I fragments were deleted from the vector portion, respectively. Then *Hind*III-*Nde*I and *Hinc*II-*Bsp*1407I fragments were deleted, respectively. The remaining fragments were blunt-ended and self-ligated. Each primer used for site-directed mutagenesis was as follows. ST3Gal II, 5′-AATGAACACCTGCAGGAATGC-3′ (nucleotide positions 37 to 57, the *Pst*I linker being underlined). ST3Gal 1V, 5′-GAGACAGGCCTCGAGGATTTTTGGCAAC-3′ (nucleotide positions 156 to 183, the *Xho*I linker being underlined). ST3Gal V, 5′-TGAACACTTGAGCTCCAAGGCCTGCA-3′ (nucleotide positions 468 to 493, the *Sac*I linker being underlined). ST6GalNAc II, 5′-ACTCTGGAATAATCGATGCCAGCACAAAGTC-3′ (nucleotide positions 297 to 327, the *Cla*I linker being underlined). ST6GalNAc III, 5′-TGAGGAAGATCTCGGCTACATG-3′ (nucleotide positions 345 to 366, the *Bgl*II linker being underlined). ST8Sia III, 5′-ATTGCTAATCTAGAAGACATAT-3′ (nucleotide positions 405 to 426, the *Xba*I linker being underlined). The resulting competitors were listed in Table 2.

Preparation of RNA and synthesis of cDNA. NIH3T3 (fibroblast), Neuro2a (Neurobalstoma), B16 (melanoma), P19 (embryonal carcinoma), and FM3A (mammary gland) cells were seeded onto 100-mm diameter dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and then cultivated to confluency, respectively. The neural differentiation of P19 cells were examined as described previously (12). These cells were harvested and used to

TABLE 2

Competitors Used in Competitive PCR

Gene	Vector	Inserted sites	Deleted sites	Length of insert (bp)
ST3Gal I	pUC118	*Eco*RI/*Hind*III	*Aor*51H1-*BamHI*	248
ST3Gal II	pKF18k	*Eco*RI/*Hind*III	*Pst*I	240
ST3Gal II	pBluescript SK(+)	*Eco*RV	*Nhe*I	478
ST3Gal IV	pKF18k	*Eco*RI/*Hind*III	*Xho*I	272
ST3Gal V	pKF18k	*Eco*RI/*Hind*III	*Sac*I	250
ST6Gal I	pBluescript SK(+)	*Eco*RV	*Pma*CI-*Bgl*II	530
ST6GalNAc I	pBluescript SK(+) w/o *Eco*RI-*Sal*I	*Sma*I	*Hind*III-*Nde*I	269
ST6GalNAc II	pKF18k	*Sma*I	*Cla*I	287
ST6GalNAc III	pKF18k	*Sma*I	*Bgl*II	315
ST6GalNAc IV	pBluescript SK(+)	*Eco*RV	*Aat*I	542
ST8Sia I	pBluescript SK(+)	*Eco*RV	*Aor51*H1-*Bgl*II	667
ST8Sia II	pUC118	*Eco*RI/*Hind*III	*Sma*I	346
ST8Sia III	pKF18k	*BamHI*/*Hind*III	*Xba*I	262
ST8Sia IV	pBluescript SK(+) w/o *Eco*RI-*Sal*I	*Sma*I	*Hinc*II-*Bsp*1407I	354
ST8Sia V	pBluescript SK(+)	*Eco*RV	*Bgl*II-*Nco*I	464

Note. w/o *Eco*RI-*Sal*I means *Eco*RI-*Sal*I fragment was deleted in the vector region.

isolate poly(A)+ RNAs, using a QuickPrep Micro mRNA purification Kit (Pharmacia). Total RNAs from various ICR mouse tissues (8-week-old) and 8-day embryos were extracted by the guanidium isothiocyanate method. Poly(A)+ RNAs was purified with Oligotex-dT30 (Takara, Japan) from each total RNA. The cDNA complementary to poly(A)+ RNA was synthesized with Superscript II (GIBCO BRL) using oligo(dT) (Pharmacia) as a primer.

Quantitative competitive PCR. Competitive PCR (h) was performed using the specific primer set (40 pmol, each) shown in Table 1 through 30–50 cycles of a step program (94°C, 60 s; 55°C, 60 s; 72°C, 90 s) with cDNA mixed with 0.01-1 pg of a competitive DNA as the template. The PCR products were electrophoresed on a 2.5–3% agarose gel, stained with ethidium bromide, and then visualized under UV light.

RESULTS AND DISCUSSION

Construction of competitor DNAs. There are no significant amino acid sequence similarities among so far cloned sialyltransferases except for in three motifs called sialyl motifs L, S and VS, which are highly conserved among these sialyltransferases. The nucleotide sequences encoding these motifs have been used for the PCR-based cloning strategy because of their high conservativeness (4). In competitive PCR experiments, we avoid using these regions for primer design as much as possible, because misannealing is possible

TABLE 3

Summary of the Gene Expression of Mouse Sialyltransferases in Various Cell Lines

	Cell type					
Gene	NIH3T3	Neuro2a	B16	P19	P19(dif.)	FM3A
ST3Gal I	+++	−	+++	−	+	++
ST3Gal II	+++	+	±	+	++	++
ST3Gal III	+	+	+	+	+	±
ST3Gal IV	++	++	++	−	−	++
ST3Gal V	−	±	++	−	−	−
ST6Gal I	+	++	++	+	+	−
ST6GalNAc I	−	−	−	−	−	−
ST6GalNAc II	−	−	−	+	±	−
ST6GalNAc III	−	+	+	±	±	−
ST6GalNAc IV	+++	+++	++	−	−	+++
ST8Sia I	−	−	−	±	++	−
ST8Sia II	−	−	−	±	++	−
ST8Sia III	−	+++	−	++	++	−
ST8Sia IV	−	−	−	±	+	−
ST8Sia V	−	−	−	++	++	−

Note. +++, high level expression; ++, intermediate level expression; +, low level expression; ±, very low level expression; −, not detected.

if these regions are used. Thus we selected appropriate regions to design specific primers for each sialyltransferase cDNA. The amplified products of each sialyltransferase cDNA with these specific primers were sub-cloned into appropriate cloning vectors and used for competitor DNA construction. Competitor DNAs were basically constructed by deleting a portion from the original amplified fragment, DNA fragments of different being thus amplified with the same primer set.

Competitive PCR analysis of the gene expression of sialyltransferases in various cell lines. The transcription levels of 15 sialyltransferase genes *in vivo* have not been investigated simultaneously. To evaluate the effectiveness of the competitive PCR method for determining the transcription levels of 15 sialyltransferase genes, we applied this method to the gene expression of sialyltransferases in various cell lines at first. Poly(A)+ RNAs from various cultured cells were reverse-transcribed to cDNAs, and the amounts of these cDNAs were adjusted by dilution and checked by RT-PCR using glyceraldehyde 3-phosphate dehydrogenase gene (G3PDH, 13), specific primers as controls. Competitive PCR was performed using above cDNAs and successively diluted competitor DNAs for the sialyltransferase cDNAs as templates with the specific primers shown in Table 1. The PCR products were separated by electrophoresis, and then the quantitative relationship between the target and competitor DNA fragments was analyzed by comparing their amounts (Fig. 1A). With the exception of the ST6GalNAc I gene, the gene expression of the sialyltransferases was more or less observed in various cell lines. The expression of the ST6GalNAc I gene seems to be very restricted, and therefore, we could not detect its expression in the cultured cells employed. To compare the expression levels of the sialyltransferase genes, the results were normalized as to cycling parameters and the amounts of competitor DNAs, and summarized in Table 3. The expression of each sialyltransferase gene was basically different in these cultured cells. However, it is interesting that the gene expression of the ST8Sia-family was mainly restricted to non- and the neural-differentiated P19 cells, with the exception that the ST8Sia III gene was expressed in Neuro2a cells. The expression of the ST8Sia I and II genes were increased during the neural differentiation of P19 cells, suggesting that these sialyltransferases are related to the neuronal development.

Although there are some differences between the results obtained on competitive PCR and Northern hybridization, or RT-PCR (data not shown), because detection sensitivity of each method was different, we judged these results are basically consistent with each other, and that this competitive PCR method is applicable for other experiments. Therefore, we applied this competitive PCR method to determine the expression levels of each sialyltransferase gene in various tissues.

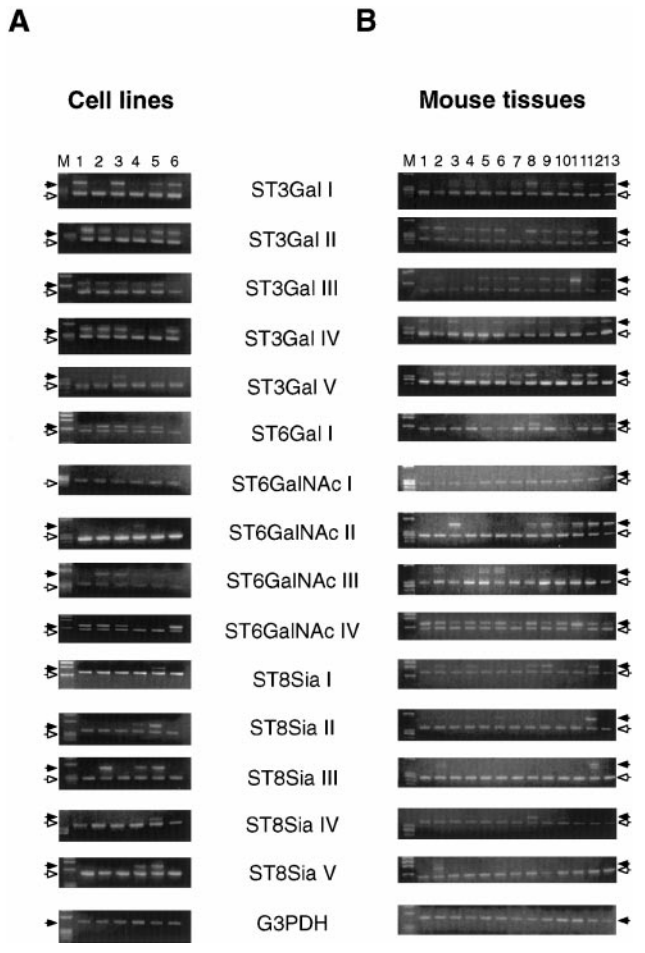

FIG. 1. The gene expressions of sialyltransferases. (A) Competitive PCR analysis of the gene expression of sialyltransferases in various cell lines and various tissues. Lane M, molecular size marker (ϕX174/*Hae*III); lane 1, NIH3T3 cells; lane 2, Neuro2a cells; lane 3, B16 cells; lane 4, P19 cells; lane 5, neural differentiated P19 cells; lane 6, FM3A cells. (B) Competitive PCR analysis of the gene expression of sialyltransferases in various tissues. Lane M, molecular size marker (ϕX174/*Hae*III); lane 1, embryo (8 days); lane 2, brain; lane 3, salivary gland; lane 4, thymus; lane 5, heart; lane 6, lung; lane 7, liver; lane 8, spleen; lane 9, kidney; lane 10, small intestine; lane 11, colon; lane 12, testis; lane 13, mammary gland. Black and white arrowheads indicate target and competitor DNA fragments, respectively.

Competitive PCR analysis of the gene expression of sialyltransferases in various tissues. Total RNAs were extracted from various mouse tissues, and further purified to poly(A)+ RNAs, and then reverse-transcribed to cDNAs. Competitive PCR with these cDNAs was performed basically as described above, and the PCR products were analyzed by agarose gel electrophoresis (Fig. 1B). The gene expression of 15

TABLE 4

Summary of the Gene Expression of Mouse Sialyltransferases in Various Tissues

	Tissue												
Gene	E8	Br	SG	Th	He	Lu	Li	Sp	Ki	In	Co	Te	MG
ST3Gal I	−	−	±	±	−	−	±	++	±	±	+	±	++
ST3Gal II	++	+++	±	++	++	++	−	+++	++	++	++	++	+
ST3Gal III	−	±	−	±	++	+	++	+	++	+	+++	±	+
ST3Gal IV	++	+	+++	±	+	++	++	++	++	−	++	++	+++
ST3Gal V	−	++	+	−	+	+	+	++	−	−	++	++	−
ST6Gal I	±	+	±	+	±	+	+	+++	+	+	++	+	+++
ST6GalNAc I	−	−	±	−	−	−	−	−	−	−	+	−	+
ST6GalNAc II	−	−	++	±	±	±	−	+	++	±	++	++	+++
ST6GalNAc III	−	±	−	−	±	±	−	±	±	−	−	−	±
ST6GalNAc IV	+++	++	+	++	++	++	+	++	+	++	+++	++	+
ST8Sia I	−	+	−	+	−	−	−	+	++	−	−	++	−
ST8Sia II	−	−	−	−	−	−	−	−	−	−	−	+++	−
ST8Sia III	−	±	−	−	−	−	−	−	−	−	−	++	−
ST8Sia IV	−	−	−	++	+	+	−	++	−	±	+	±	−
ST8Sia V	−	++	−	−	−	−	−	−	−	−	−	−	−

Note. +++, high level expression; ++, intermediate level expression; +, low level expression; ±, very low level expression; −, not detected. E8, embryo (8 days); Br, brain; SG, salivary gland; Th, thymus; He, heart; Lu, lung; Li, liver; Sp, spleen; Ki, kidney; In, small intestine; Co, colon; Te, testis; MG, mammary gland.

sialyltransferases was more or less observed in various tissues. To compare the expression levels of the sialyltransferase gene, the results were normalized as to cycling parameters and the amounts of competitor DNAs, and summarized in Table 4. The ST3Gal II-V, ST6Gal I, and ST6GalNAc IV genes are expressed ubiquitously, whereas the other genes are expressed in tissue-specific manners. In this table, we can see which sialyltransferases contribute to the biosynthsis of tissue-specific carbohydrates containing sialic acids. We investigated the tissue-specific expression of sialyltransferase genes in this experiment, and this method also seems to be applicable to investigation of the developmental stage-specific expression of these genes.

This competitive PCR method has the advantage of the rapid detection of transcripts and the quantitative analysis, although it does not discriminate the different transcript species of a gene. This method seems to be applicable to many biological phenomena related to the expression of carbohydrates containing sialic acids, and will provide useful information on the mechanisms of expression of such carbohydrates.

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