

# Transcriptional regulation of the human *ST6GAL2* gene in cerebral cortex and neuronal cells

Sylvain Lehoux · Sophie Groux-Degroote · Aurélie Cazet · Claire-Marie Dhaenens · Claude-Alain Maurage · Marie-Laure Caillet-Boudin · Philippe Delannoy · Marie-Ange Krzewinski-Recchi

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**Abstract** The second human  $\beta$ -galactoside  $\alpha$ -2,6-sialyltransferase (hST6Gal II) differs from hST6Gal I, the first member of ST6Gal family, in substrate specificity and tissue expression pattern. While *ST6GAL1* gene is expressed in almost all human tissues, *ST6GAL2* shows a restricted tissue-specific pattern of expression, mostly expressed in embryonic and adult brain. In order to understand the mechanisms involved in the transcriptional regulation of *ST6GAL2*, we first characterized the transcription start sites (TSS) in SH-SY5Y neuroblastoma cells. 5' RACE experiments revealed multiple TSS located on three first alternative 5' exons, termed EX, EY and EZ, which are unusually close on the genomic sequence and are

all located more than 42 kbp upstream of the first common coding exon. Using Taqman duplex Q-PCR, we showed that the *ST6GAL2* transcripts initiated by EX or EY are mainly expressed in both brain-related cell lines and human cerebral cortex, testifying for the use of a similar transcriptional regulation *in vivo*. Furthermore, we also showed for the first time hST6Gal II protein expression in the different lobes of the human cortex. Luciferase reporter assays allowed us to define two sequences upstream EX and EY with a high and moderate promoter activity, respectively. Bioinformatics analysis and site-directed mutagenesis showed that NF- $\kappa$ B and NRSF are likely to act as transcriptional repressors, whereas neuronal-related development factors Sox5, Pur $\alpha$  and Olf1, are likely to act as transcriptional activators of *ST6GAL2*. This suggests that *ST6GAL2* transcription could be potentially activated for specific neuronal functions.

S. Lehoux · S. Groux-Degroote · A. Cazet · P. Delannoy · M.-A. Krzewinski-Recchi  
Structural and Functional Glycobiology Unit, UMR CNRS 8576, University of Sciences and Technologies of Lille, 59655 Villeneuve d'Ascq, France

C.-M. Dhaenens · C.-A. Maurage · M.-L. Caillet-Boudin  
INSERM, U837, place de Verdun, 59045 Lille, France

C.-M. Dhaenens · C.-A. Maurage · M.-L. Caillet-Boudin  
Faculté de Médecine, Institut de Médecine prédictive et de Recherche thérapeutique, Centre de Recherches Jean-Pierre Aubert, University of Lille 2, place de Verdun, 59045 Lille, France

M.-A. Krzewinski-Recchi (✉)  
Unité de Glycobiologie Structurale et Fonctionnelle, UMR CNRS n°8576, Bâtiment C9, Université des Sciences et Technologies de Lille, F-59655 Villeneuve d'Ascq, France  
e-mail: marie-ange.recchi@univ-lille1.fr

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## Abbreviations

DSiaT	drosophila sialyltransferase
Gal	$\beta$ -D-galactopyranosyl
GalNAc	$\beta$ -D-N-acetylgalactosaminyl
GlcNAc	$\beta$ -D-N-acetylglucosaminyl
HPRT	hypoxanthine phosphoribosyltransferase
LDN	LacdiNAc, GalNAc $\beta$ 1-4GlcNAc
LN	LacNAc, Gal $\beta$ 1-4GlcNAc
NF- $\kappa$ B	nuclear factor-kappa B
NRSF	neuron-restrictive silencer factor
Olf1	olfactory neuron-specific binding protein
Pur $\alpha$	purin-riche element binding protein alpha
Q-PCR	quantitative polymerase chain reaction

RACE	rapid amplification of cDNA end
Sia	sialic acid
SLDN	siaLacdiNAc, Sia $\alpha$ 2-6GalNAc $\beta$ 1-4GlcNAc
SLN	sialyl-LacNAc, Sia $\alpha$ 2-6Gal $\beta$ 1-4GlcNAc
Sox5	SRY box 5
SP1	specificity protein 1
TSS	transcriptional start site
UTR	untranslated region

## Introduction

Cell surface carbohydrate structures containing sialic acid play a crucial role in cellular interaction mechanisms such as cell-to-cell, cell-to-pathogen and cell-to-protein recognitions. Sialylated structures are expressed in a tissue-, time- and cell type-specific manner and imply that sialylation should be finely regulated. The transfer of sialic acid (Sia) from the activated donor substrate, CMP-sialic acid, to the terminal position of glycans of glycoproteins and glycolipids is catalyzed by sialyltransferases, a subset of glycosyltransferases gathered in CAZy family 29 [1]. Twenty different sialyltransferases have been identified in mammals. Each of the enzymes exhibits a specificity for acceptor substrate and for the linkage formed:  $\alpha$ 2-3 to a  $\beta$ -D-galactopyranosyl (Gal) residue;  $\alpha$ 2-6 to a Gal,  $\beta$ -D-N-acetylgalactosaminyl (GalNAc) or  $\beta$ -D-N-acetylglucosaminyl (GlcNAc) residue, or  $\alpha$ 2-8 to another sialic acid residue (for review [2–4]).

Among the 20 human sialyltransferases, only two members have been identified in the ST6Gal family. ST6Gal I was identified more than 20 years ago and is certainly one of the most studied glycosyltransferases. It has been characterized in details and its cDNA cloned from different species including rat [5], bovine [6] and Human (hST6Gal I) [7]. In contrast, the cDNA encoding the second human ST6Gal (hST6Gal II) has been cloned in 2002 and its enzymatic properties have been only characterized *in vitro* [8,9]. Both enzymes share 48 % amino acid sequence identity in the catalytic domain and transfer a sialic acid residue in  $\alpha$ 2,6 linkage to Gal $\beta$ 1-4GlcNAc (LacNAc, LN) disaccharide sequence, to form the sialyl-LacNAc (SLN) [8,9]. The corresponding genes (*ST6GAL1* & *ST6GAL2*) have a similar genomic organization of the coding region with 5 coding exons and phylogenetic analyses indicate that they have evolved from a common ancestor [4].

Despite these similarities, *in vitro* assays have underlined slight differences in their enzymatic properties. Using synthetic acceptor substrates, hST6Gal I shows a 4-fold decreased activity towards GalNAc $\beta$ 1-4GlcNAc (LacdiNAc, LDN) compared to LN, whereas hST6Gal II exhibits a 2-fold increased activity towards this motif, suggesting that hST6Gal II is the enzyme involved in Sia $\alpha$ 2-

6GalNAc $\beta$ 1-4GlcNAc (SLDN) biosynthesis [10]. Interestingly, a similar observation was also made for DSiaT, the only sialyltransferase identified in *Drosophila melanogaster*, which also prefers LDN as an acceptor substrate [11]. Moreover, sequence alignments also confirmed a close relationship of DSiaT to ST6Gal family, suggesting that mammals ST6Gal and DSiaT proceed from a common ancestral gene [2,11].

While *ST6GAL1* gene is expressed in almost all mammal tissues with a notable lower expression in brain and testis [12], *ST6GAL2* gene exhibits a restricted tissue-specific expression pattern. Expression of *ST6GAL2* has been detected mainly in adult and embryonic brains, and to a lower extent in thyroid gland, small intestine, colon, and testis [8,9]. Moreover, a macro-array analysis of gene expression levels of 140 glycosyltransferases and glycosidases in the cerebral cortex from developing and adult mice has shown an increased expression of *st6gal2* during mouse embryo development, followed by a decrease expression in adult brain. On the contrary, the expression of most of sialyltransferase genes, including *st6gal1*, remains low and unchanged during brain development [13]. Since DSiaT is only expressed during embryonic development of the central nervous system [11], we can hypothesize that *ST6GAL2* compensates the low expression of *ST6GAL1* in brain for a precise unknown function, which could be conserved from invertebrate to human. However, pituitary hormones, glycodelin and urokinase, are the only glycoproteins known to exhibit terminal SLDN motifs [14–16]. Moreover,  $\alpha$ 2,8-sialylation is the most, if not the only, type of sialylation depicted for neuronal functions such as cell surface interactions, neurogenesis or neuronal plasticity [17,18] and terminal  $\alpha$ 2,6-sialylation has not been yet reported in brain-related glycoconjugates.

Sialyltransferases are known to be expressed in a tissue- and time-specific manner [12]. The expression of these enzymes is mostly regulated at the transcriptional level through the usage of alternative untranslated 5' exons under the control of tissue-specific promoters. In particular, it is well-established that *ST6GAL1* expression is regulated by three major promoters leading to the expression of an ubiquitous transcript [19], a mature B lymphocytes-specific transcript [20] and a liver-specific mRNA [21]. In contrast, nothing is known about *ST6GAL2* transcriptional regulation, but a specific control seems to occur in brain, especially during embryonic development.

In order to clarify the mechanisms involved in the regulation of *ST6GAL2* expression, we first identified and characterized its 5' ends organization in SH-SY5Y neuroblastoma cell line. 5' RACE experiments showed that at least three *ST6GAL2* mRNAs, distinct in their 5' untranslated ends, were expressed in SH-SY5Y cells. The relative expression of these transcripts was analyzed using duplex

Taqman Q-PCR strategy in different brain-related cell lines and in different lobes of the human cortex. We showed that the *ST6GAL2* specific transcripts identified in cultured cells were also expressed in the human cortex. Moreover, the hST6Gal II protein was also detected in the different areas of the human cortex. Luciferase assays allowed us to define two promoter regions that do not contain any canonical TATA or CAAT boxes, but contain general and neuronal-related transcription factor binding sites. The role of these factors was investigated by PCR site-directed mutagenesis.

## Materials and methods

### Cell culture

Human embryonic carcinoma NT2/D1, neuroblastoma SH-SY5Y, neuroblastoma Kelly, astrocytoma/glioblastoma U118 and glioblastoma T98G cell lines were provided by Dr Luc Buée (INSERM, U837, Lille, France). The NT2/D1, SH-SY5Y, U118 and the T98G cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4500 mg/l of glucose (BioWhittaker, Lonza, Belgium). Human neuroblastoma Kelly cells were cultured in RPMI 1640 medium (BioWhittaker). Media were supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (Eurobio), 2 mM of L-glutamine, 100 U/ml penicillin and 10 µg/ml streptomycin, and incubated at 37°C under 5 % CO<sub>2</sub>.

### Amplification of 5' cDNA Ends, 5' RACE

Total RNA from SH-SY5Y cells were extracted using the NucleoSpin<sup>®</sup> RNA II kit according to the manufacturer's recommendations (Macherey-Nagel, Hoerd, France). 5' RACE amplifications were performed with the First Choice RLM-RACE kit (Ambion, Huntingdon, UK) according to the manufacturer's instructions. Initial reverse transcription was performed with random decamers oligonucleotides using 10 µg of total RNA from SH-SY5Y cells. After the synthesis of the first strand cDNA, PCR was performed with provided Outer Primer (OP) and gene-specific primers GSP1, GSP1.X, GSP1.Y or GSP1.Z (Table 1) using the following PCR conditions: 94°C for 2 min; 40 cycles of 94°C for 30 sec, 62°C for 30 sec and 72°C for 3 min. Nested-PCR were performed with provided Inner Primer (IP) and gene-specific primers GSP2.1 or GSP2.2, or exon-specific primers GSP2.X, GSP2.Y or GSP2.Z in the following PCR conditions: 94°C for 2 min; 40 cycles of 94°C for 30 sec, 72° for 30 sec and 72°C for 2 min. PCR amplifications were performed with the Taq polymerase from Taq PCR Core Kit (Qiagen GmbH, Germany) according to provider's instructions. Nested-PCR products obtained were size separated by agarose gel electrophoresis, subcloned into

pCR2.1 TOPO TA cloning (Invitrogen, Carlsbad, CA) and sequenced by Genoscreen (Lille, France).

### Human cerebral cortex control samples and RNA preparation

Brains were obtained at autopsy from 8 subjects without any brain pathologies. The brain tissues were obtained from the Lille Neurobanque (France) in accordance with the local ethics committee protocol. Post-mortem delay was <36 h for all samples. Tissue samples from cortical areas were cut off frontal, temporal, parietal, occipital regions. Total RNA was isolated using the RNeasy Total RNA Isolation System according to the manufacturer's recommendations (Promega, Madison, USA).

### SYBR<sup>®</sup> Green and duplex Taqman Q-PCR

First strand cDNA synthesis were achieved using 2 µg of total RNA from cultured cell lines (SH-SY5Y, NT2/D1, U118, T98G and Kelly) or brain samples with First-Strand cDNA Synthesis Kit (Amersham Biosciences, Freiburg, Germany), according to the manufacturer's instructions, using random hexameric primers (pd(N)<sub>6</sub>) and oligodT in a 3:1 ratio. SYBR<sup>®</sup> Green Q-PCR and subsequent analysis were performed using the MX4000 Multiplex Quantitative System (Stratagene, La Jolla, CA, USA) equipped with Version 3.0 software. The 25 µl final volume was composed of 12.5 µl of the Brilliant<sup>®</sup> SYBR<sup>®</sup> Green QPCR Master Mix Kit (Stratagene), 0.3 µM of primers *ST6GAL2* 5', *HPRT* 5' and *HPRT* 3' primers (previously described by Zhang *et al.* [22]) and 0.6 µM of *ST6GAL2* 3' primer and 4 µl of cDNA diluted 1:40. The thermal cycling profile was 95°C for 10 min, then 40 PCR cycles as follows: 95°C for 1 min, 51°C for 1 min and 72°C for 1 min. Q-PCR assays were performed in triplicate in 96-well plates. Standard curves were generated for each pair of primers using serial dilutions of cDNA. Standard curves efficiency used are: 101.3 % for *ST6GAL2* and 99.6 % for *HPRT*. Duplex Taqman Q-PCR and subsequent analysis were performed using the MX4000 Multiplex Quantitative System (Stratagene) equipped with Version 3.0 software. For the *ST6GAL2* exon-specific amplifications, forward primers specific of each exon sequence were paired with a common reverse primer, exon1-3'Taq, hybridizing within the exon 1 sequence. *HPRT* primers were designed to hybridize within the exon 1 and exon 2 sequences. Taqman probes were synthesized by Applied Biosystems. The *ST6GAL2* probes were FAM labelled and the normalizing gene *HPRT* probe was VIC labelled (Table 1). PCR reactions were performed with 12.5 µl of the QuantiTect Multiplex PCR Kit Mix (Qiagen), 0.4 µM of each primer, 0.4 µM of Taqman probe and 4 µl of cDNA diluted 1:40 in a final volume of 25 µl.

**Table 1** Nucleotide sequences of 5'-RACE adapter and primers, Q-PCR primers and Taqman probes, and of primers used for PCR site-directed mutagenesis. Oligonucleotides were synthesized by Eurogentec (Seraing, Belgium)

Primer	Sequence
5'-RACE Adapter	5'-GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA-3'
5' Outer Primer	5'-GCTGATGGCGATGAATGAACACTG-3'
5' Inner Primer	5'-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3'
GSP1	5'-CCTCTTCTCACCCGCCTC-3'
GSP1.X	5'-GGAGCCGGGCACTGGGAAGTTGGG-3'
GSP1.Y	5'-CTGGAGTCCAAGGCGTGCGTGTCA-3'
GSP1.Z	5'-TACTGAGCCACAGGGGCACAACACTG-3'
GSP2.1	5'-AGCGGGGTTGCTGTGCGGT-3'
GSP2.2	5'-AGGCGGGGAGGGCTCATG-3'
GSP2.X	5'-AGACTTGGGCTGCGTGGGGCGGTGC-3'
GSP2.Y	5'-TCCTCCTGCCACCGTGGGGTCTCG-3'
GSP2.Z	5'-CCCCAAAGCGCAAGCCGTCTCCC-3'
<i>SYBR® Green primer</i>	<i>Sequence</i>
ST6GAL2 5'	5'-ACGCTGCTGATTGACTCTTCT-3'
ST6GAL2 3'	5'-CACATACTGGCACTCATCTAA-3'
HPRT5' [22]	5'-GACCAGTCAACAGGGGACAT-3'
HPRT3' [22]	5'-AACACTTCGTGGGGTCCTTTTC-3'
<i>Taqman Primer</i>	<i>Sequence</i>
HPRT5'Taq	5'-GCCCTGGCGTCGTGATT-3'
HPRT3'Taq	5'-CTCAGCATAATGATTAGGTATGCAAAA-3'
X5'Taq	5'-GCAGCCCAACTTCCCAGTG-3'
Y5'Taq	5'-GGTGTGCTGCGCTTCGA-3'
Z5'Taq	5'-ACGGGCGGATACAGTTGTG-3'
Exon1-3'Taq	5'-TCAGCACCTTGTGTCTTAATGCA-3'
<i>Taqman probe</i>	<i>Sequence</i>
HPRT	5'-VIC-TGATGATGAACCAGGTAT-TAMRA-3'
X	5'-FAM-CCAGCTAGGTTCACTT-MGB-3'
Y	5'-FAM-AAGAAGGCTGGTTCATT-MGB-3'
Z	5'-FAM-CAGTAAAGGTTCACTTGCCA-MGB-3'
<i>Primer</i>	<i>Sequence</i>
NFκB-5'	5'-CAGGCGCCTTTACTCAATCCTGG-3'
NFκB-3'	5'-CCAGGATTGAGTAAAGGCGCCTG-3'
NRSF-5'	5'-CCGAGTGGGCGATTGGTGTCCA-3'
NRSF-3'	5'-TGGACACCAATCGCCCACTCGG-3'
Olf1-3'	5'-GCTGCCAAGCTTTCCCGTCAAATGCCGCTG-3'
Purα-5'	5'-GGATGGAGGCTTACACCGGGG-3'
Purα-3'	5'-CCCCGGTGTAAGCCTCCATCC-3'
Sox5-5'	5'-GCCCCGAGAGTATGAGGCGTGC-3'
Sox5-3'	5'-GCACGCCTCATACTCTCGGGC-3'
SP1.1-5'	5'-CTGCCGAAGAAAGGGACCCG-3'
SP1.1-3'	5'-CGGGTCCCTTTCTTCGGCAG-3'
SP1.2-5'	5'-CCCCCACTTTTCCACGCAG-3'
SP1.2-3'	5'-CTGCGTGGAAAAGTGGGG-3'
SP1.3-5'	5'-GAGCTCGGGTGTGTTGGCGC-3'
SP1.3-3'	5'-GCGCCAAACACCCGAGCTC-3'
SP1.4-5'	5'-GCCCCGCTAAATGCGCTTG-3'
SP1.4-3'	5'-CAAGCGCATTTAGCGGGGC-3'

After 95°C for 15 min, 40 PCR cycles were performed as follows: 95°C for 1 min, 60°C for 1 min (hybridization and elongation). Q-PCR assays were performed in triplicate in 96-well plates using MX4000 apparatus (Stratagene). Standard curves were generated for each trio of primers and probe of a *ST6GAL2* specific transcript in duplex with primers and probe of the normalizing gene *HPRT*, using 2-fold serial dilutions of cDNA. Standard curves efficiencies used are: 99.5 % and 97.8 % for EX specific transcript and *HPRT*, 100.4 % and 100.5 % for EY specific transcript and *HPRT* and 98.0 % for EZ specific transcript and *HPRT*.

#### SDS-PAGE and Western blotting analysis

Brain tissues were desintegrated on ice with a glass homogenizer, in RIPA Buffer (Pierce, Rockford, USA), containing CHAPS (0.5 % w/v) and a cocktail of protease and phosphatase inhibitors (Roche, Meylan, France). Subconfluent SH-SY5Y cell cultures, were harvested by adding a solution of 4 mM EDTA in PBS, pelleted and resuspended in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Triton X-100), containing a cocktail of protease and phosphatase inhibitors (Roche). The protein concentration of extracted proteins (from brain tissues or cells) was determined with the Micro BCA™ Protein Assay Reagent kit (Pierce). 40 µg of total proteins of each sample were boiled for 10 min in reducing Laemmli Sample buffer and resolved by SDS/PAGE on 8 % minigels (Bio-Rad, Richmond, USA). After transfer onto a nitrocellulose membrane (200 mA, 2 h), blocking was performed using TBS (Tris Buffer Saline) containing 0,05 % Tween 20 and 5 % (w/v) non-fat dried milk for 1 h at room temperature (RT). Primary antibody incubations with anti-hST6Gal II (Sigma, St Louis, USA) or anti-actin (Santa Cruz Biotechnology inc., Europe) antibodies were performed overnight at 4°C in TBS, 0,05 % Tween 20 and 5 % (w/v) non-fat dried milk, at 1:1000 dilution for the anti-hST6Gal II antibody or 1:10000 dilution for the anti-actin antibody. After three washing steps of 10 min in TBS, 0,05 % Tween 20, incubation of 1 h at RT with horseradish peroxidase-conjugated goat anti-rabbit IgG was performed at 1:10000 dilution in TBS, 0,05 % Tween 20 and 5 % (w/v) non-fat dried milk. Membranes were finally washed three times for 10 min in TBS, 0,05 % Tween 20 and detection was achieved using enhanced chemiluminescence (ECL+® advance Western blotting detection reagents, Amersham Biosciences, Little Chalfont, Buks, U.K.).

#### Bioinformatics analysis

*In silico* analysis were performed with Matinspector 2.2 ([www.genomatix.de](http://www.genomatix.de)) using TRANSAC matrices 4.0 [23]

with “core similarity: 0.95” and “matrices similarity: Optimized”.

#### Construction of reporter plasmids for luciferase assays

Genomic DNA of SH-SY5Y cells was extracted with the Nucleospin® Blood kit (Macherey-Nagel) following manufacturer's instructions. The -1851 / +263 genomic region upstream EX was amplified from genomic DNA of SH-SY5Y using primers 5'-CGCTTGCCACGGTACCCGGT CATT TTTGT-3' and 5'-AGACTTAAGCTTTCGTGGGGCG GTGCGGG-3'. Underlined nucleotides represent the restriction sites that were incorporated, *KpnI* and *HindIII* respectively. PCR fragments were subcloned into pGL3-Basic vector (Promega) upstream of the *Firefly* luciferase gene at *KpnI/HindIII* sites, generating the pGL3\_-1851/+263 vector. The pGL3\_-1851/+263 was submitted to 5' deletions using natural restriction enzyme sites. Digested ends were blunted with DNA Terminator End Repair Kit (Euro-medex, Mundolsheim, France) following manufacturer's instructions. Then, blunted ends were ligated with the Quick T4 DNA ligase (Biolabs, Ozyme, Saint Quentin Yvelines, France). *HindIII* and *BglII* were used to obtain pGL3\_-752/+263, *HindIII* and *XhoI* were used to obtain pGL3\_-498/+263, *HindIII* and *NheI* were used to obtain pGL3\_-121/+263 and *HindIII* and *SmaI* were used to obtain pGL3\_-92/+263. Following the same strategy, the -121 / +1452 genomic region was amplified from genomic DNA of SH-SY5Y using primers 5'-TCACCGGACTTGGCGAGCTA-3' and 5'-GCCGCCTG AGATCTACATAAG-3'. Underlined nucleotides represent the *BglII* restriction site that was incorporated. Using the *NheI* natural site of the amplified sequence and the *BglII* incorporated site, the PCR fragments were subcloned into the pGL3-Basic vector at the *NheI/BglII* sites, generating the pGL3\_-121/+1452 vector. Plasmids pGL3\_+517/+1452, pGL3\_+773/+1452, pGL3\_+465/+772, pGL3\_-121/+772, pGL3\_-121/+464 and pGL3\_-121/+91 were generated by enzymatic digestions, ends blunting and ligation. The plasmid pGL3\_+1046/+1452 was generated using primers 5'-GGAGGGTACCGGGTAAGGGTT-3' and 5'-CCGTACC TTAATAAGCTTTCTACATA-3'. Underlined nucleotides represent the restriction sites that were incorporated, *KpnI* and *HindIII* respectively. The amplified fragment was cloned into the pGL3-Basic upstream of the *Firefly* luciferase gene at the *KpnI/HindIII* sites, generating the pGL3\_-1046/+1452 vector. The different constructions were sequenced to ensure the absence of mutation (Genoscreen).

#### PCR site-directed mutagenesis

The base substitution mutations for the predicted binding sites NF-κB, NRSF, Olf1, Purα, Sox5, and the four SP1



sites, named SP1.1, SP1.2, SP1.3 and SP1.4, were introduced by PCR site-directed mutagenesis with the HotStar HiFidelity polymerase kit (Qiagen) following manufacturer's instructions. Using the pGL3<sub>-121/+772</sub> plasmid as a template, two first rounds of PCR using a common forward primer, 5'-CGATAGGTACCGAGCTCTACGCG-3' (containing a *KpnI* site), and a reverse mutated site-specific primer (See Table 1), and a common reverse primer, 5'-TGCCAAGCTTACTTAGATCGCAGATCTC-3 (containing a *HindIII* site), and a forward mutated site-specific primer (See Table 1) were performed for each mutated site in the PCR conditions: 95°C for 5 min; 30 cycles of 95°C for 1 min, 53–60°C for 1 min and 72°C for 1 min. The expected amplified PCR fragments were purified on agarose gel and used as templates with the common forward and reverse primers for the final PCR using identical conditions. Olf1 mutated site was generated with the common forward primer and the Olf1-3' primer (containing a *HindIII* site) (Table 1) using the following PCR conditions: 95°C for 5 min; 30 cycles of 95°C for 1 min, 53°C for 1 min and 72°C for 1 min. The final PCR fragments were subcloned into the pGL3-Basic vector at the *KpnI/HindIII* sites and sequenced (Genoscreen) to ensure the absence of any other mutations. The pGL3<sub>-121/+91NF-κB</sub> plasmid was obtained by enzymatic digestion at the *SmaI* site from the pGL3<sub>-121/+772NF-κB</sub> plasmid.

#### Transient transfection and luciferase assays

Transfections were performed with the Cell Line Nucleofector<sup>®</sup> Kit V (AMAXA, Europe) according to manufacturer's instruction for the SH-SY5Y cell line grown on 6 well plates. Briefly, 2.10<sup>6</sup> SH-SY5Y cells were harvested at ~70 % confluence, centrifuged 5 min at 1000 rpm and resuspended in 100 μl of Nucleofector<sup>®</sup> Kit V solution (AMAXA). The cell suspensions were mixed with 2 μg of pGL3-Basic plasmid constructions and 40 ng of control *Renilla* plasmid. Cells and plasmids were transferred in an AMAXA cuvette and submitted to nucleofection using G-004 program that has been optimized by the manufacturer for the SH-SY5Y cells. Cells were then plated on 6-well plates with 2 ml of culture media and incubated for 24 h. Cell survival and transfection efficiency with AMAXA protocol were checked by GFP expression plasmid and were 50 % and 70 %, respectively, consistent with the manufacturer's data. Cells were lysed in 250 μl of Passive Lysis Buffer (PLB, Dual Luciferase Reporter Assay System, Promega) and 20 μl were used for luciferase assay. *Firefly* and *Renilla* luciferase activities were measured using Dual Luciferase Reporter Assay System (Promega). Luminescence was measured with the Centro luminometer (Berthold Technologies)

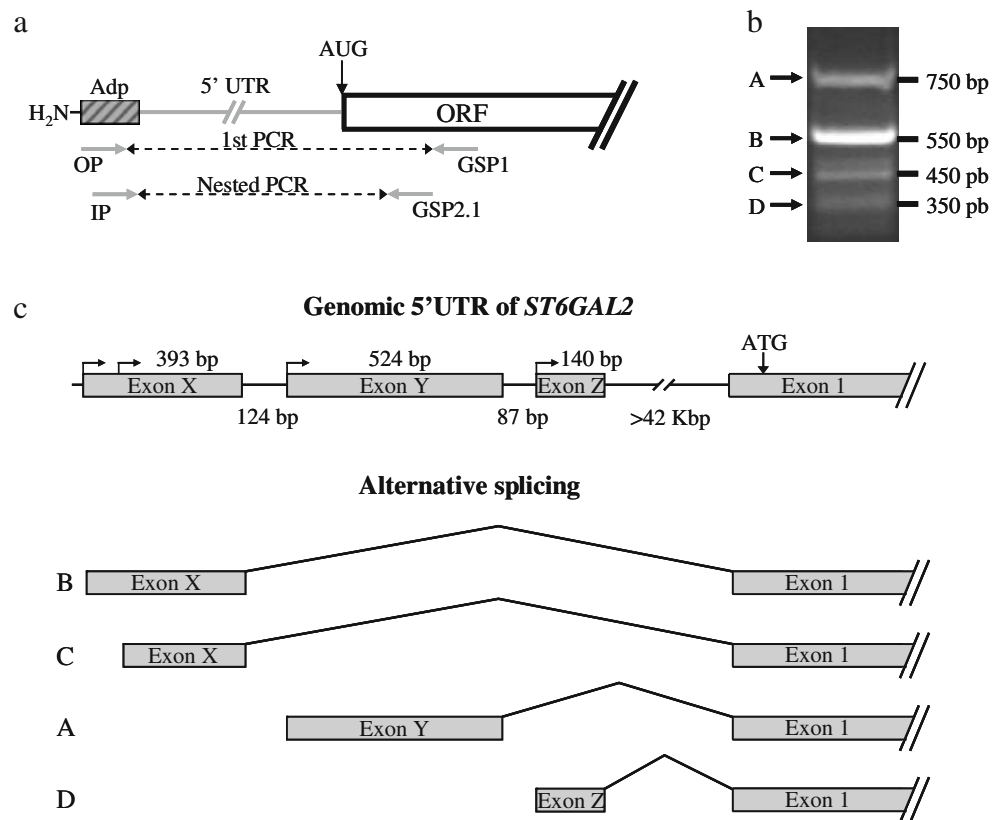
## Results

### Identification of alternative 5' UTR by 5' RACE

The coding sequence of *ST6GAL2* gene is divided in 5 exons spanned over 85 kbp on chromosome 2. Mature transcripts are more than 8 kb long including a 5 kb 3'-untranslated region (UTR) and incomplete sequences located 42 kbp upstream of the first coding exon were previously reported in 5' UTR [8,9]. To determine the transcription start sites (TSS) of the human *ST6GAL2* gene and potential alternative 5' UTR, capped mRNA 5' RACE was performed using total RNA isolated from SH-SY5Y human neuroblastoma cells. A first PCR was performed with Outer Primer and GSP1, which hybridized within the sequence of the first coding exon E1. A nested PCR was then performed with Inner Primer and GSP2.1 (Fig. 1a). Experimental conditions were optimized for both first-round and nested PCR as described in Materials & Methods section. As shown in Fig. 1b, which is representative of at least three independent amplifications, four bands from 350 to 700 bp were obtained after the nested PCR. The PCR products, named A, B, C and D, were subcloned, sequenced and the sequences were BLAST-aligned (<http://www.ncbi.nlm.nih.gov/BLAST/>) against the human genome. The amplified products were assigned to *ST6GAL2* transcripts, including at the 5'-end three alternative unique sequences termed EX, EY and EZ (GenBank accession # FJ433883, FJ433884, FJ433885), which were located on chromosome 2 more than 42 kbp upstream exon E1 and separated from each other by 124 and 87 bp, respectively (Fig. 1c). Splicing junctions between these exons and exon E1 were in agreement with the GT-AG splicing rule. The sequence of band A contained the sequence of EY, band B corresponded to the sequence of EX and band C corresponded to an 80 bp shorter 5'-truncated sequence of EX. Finally, the sequence of band D contained the sequence of EZ. Moreover, the presence of EX, EY or EZ was mutually exclusive among *ST6GAL2* 5' UTR (Fig. 1c), in agreement with the fact that the 5' ends of EY and EZ do not contain splicing acceptor sites.

Another series of 5' RACE experiments using exon-specific primer pairs were performed to determine precisely the transcription start site (TSS) of each exon. Exon-specific primers hybridizing within the 3'-end sequence of each exon (Table 1) were used in pair with Inner Primer. Results of the nested PCR are shown in Fig. 2a. For EY and EZ specific amplifications, only one band was observed, but as expected, two band sizes were revealed for EX. The amplified fragments were subcloned and sequenced. Twenty nine clones corresponding to different sizes of EX, from 315 bp to 393 bp, were sequenced (Fig. 2b), defining two groups of sequences: a first group with sizes of 315, 318, 320 and

**Fig. 1** Identification of *ST6GAL2* transcript 5' ends by 5' RACE. **(a)** Schematic representation of 5' RACE procedure. Adp, oligonucleotide adapter provided by FirstChoice® RLM-RACE kit; ORF, Open Reading Frame; OP, Outer Primer; IP, Inner Primer. **(b)** Agarose gel (2 %) analysis of nested PCR products with primer pair I.P. / GSP2.1. **(c)** Genomic organization of the 5' -end of the *ST6GAL2* gene. Oriented arrows represent the transcription start site (TSS) of each exon. Numbers on the top of exons and numbers at the bottom of the introns indicate their respective lengths in bp



326 bp, and a second group with sizes of 380, 381 and 393 bp. These two groups of sequences corresponded to the two amplified products observed (Fig. 2a, lane EX) and also to bands B and C in Fig. 1b. In parallel, 8 clones from EY were sequenced and only a one bp variation of the TSS was observed, EY length being 523 or 524 bp (Fig. 2b). Finally, among the 6 different EZ clones sequenced, no variation was observed, EZ length being 140 bp (Fig. 2b).

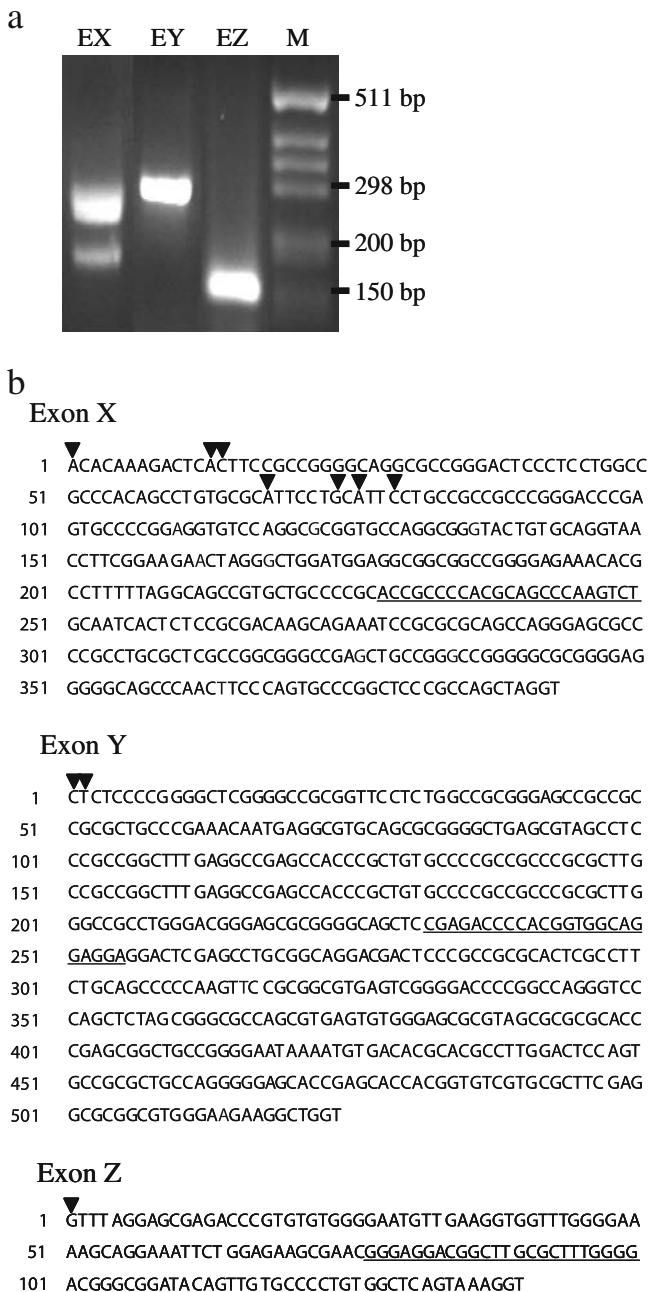
Quantification of transcripts expression by duplex Taqman Q-PCR in cultured cells

The relative expression of *ST6GAL2* exon-specific transcripts was determined by duplex Taqman Q-PCR using *HPRT* as a normalizing gene. A common reverse primer that hybridizes within the exon E1 of *ST6GAL2* cDNA sequence and three exon-specific forward primers hybridizing within EX, EY or EZ, respectively, were designed (Table 1). Taqman probes were designed within exon E1 / exon EX, EY or EZ junction sequence. For *HPRT*, primers and probe were designed to hybridize within exon E1 / exon E2 sequences (Table 1). In order to improve cDNA synthesis and to decrease threshold cycle (Ct) values for both *HPRT* and exon-specific *ST6GAL2* transcripts, a mix of random hexameric primers and oligo-dT primers was used in a ratio 3:1 as primers for retro-transcription. The relative expression of specific transcripts was determined

for several glial and neuronal cell types, including two glioblastoma U-118 and T98G cells, two neuroblastoma SH-SY5Y and Kelly cells and the neuroprogenitor cells NT2/D1. In SH-SY5Y cell line, our results clearly showed a predominant expression of transcripts containing EX or EY, while EZ specific transcript was expressed at low levels (Fig. 3). A similar pattern of expression with a predominant expression of EX and EY specific transcripts was also detected in NT2/D1 cells (Fig. 3), whereas a low level of expression of these transcripts and no expression of EZ specific transcript was observed in the Kelly cell line, and almost no expression of all transcripts in T98G and U-118 cell lines. The level of expression of the specific transcripts was consistent with the total expression of *ST6GAL2* (Data not shown), indicating that no other transcript with alternative 5' UTR seems to be expressed in these cell lines.

Quantification of total and transcript-specific *ST6GAL2* expression in human cerebral cortex

We next investigated the *in vivo* expression of *ST6GAL2* in the temporal, occipital, frontal and parietal lobes of the human cerebral cortex. For each region, up to five different samples were available for quantification. We first quantified the total expression of *ST6GAL2* (Fig. 4a). In all samples, the expression of *ST6GAL2* was in average above the level of expression in the SH-SY5Y cell line. Furthermore, *ST6GAL2*



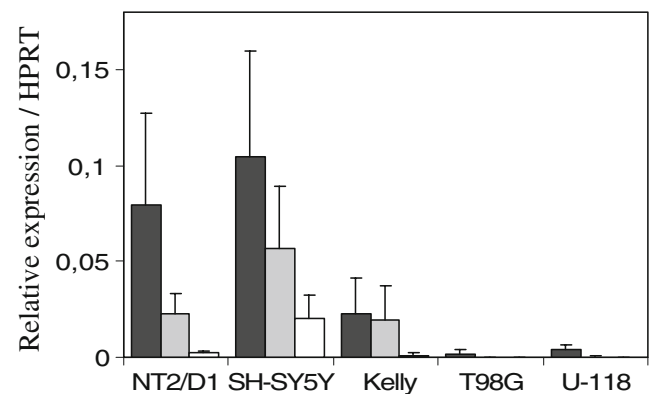
**Fig. 2** Exon-specific 5' RACE of *ST6GAL2* transcripts. **(a)** Agarose gel (2 %) analysis of 5' RACE exon-specific nested PCR products. Lanes EX, EY and EZ show amplifications with primer pairs I.P. / GSP2.X, I.P. / GSP2.Y and I.P. / GSP2.Z, respectively. M, molecular weight marker (Invitrogen 1 kb). **(b)** Nucleotide sequence of EX, EY and EZ. Arrowheads indicate the different transcription start sites identified by 5' RACE. The sequences of exon-specific primers used for the three exon-specific 5' RACE are underlined

expression was heterogeneous among the different tissue samples for each subject and among the different subjects for each cortex area. Specific transcript expression identified in cultured cells by 5' RACE was also considered. All brain samples expressed transcripts that contained EX, EY or EZ, confirming the results obtained with cultured cells. Moreover

in each sample, the level of expression of EX, EY and EZ specific transcripts corresponded to the level of *ST6GAL2* total expression suggesting that no other transcript is expressed in these samples (Fig. 4b). In cortical areas, the ratio of EZ/EX containing transcripts was increased compared with SH-SY5Y, NT2/D1 and Kelly cells. Similar variations were observed for the ratio EZ/EY. In spite of these slight differences, our results suggest that the transcriptional regulation of *ST6GAL2* in the human cerebral cortex is similar to that of the cultured cell lines.

#### hST6Gal II protein expression in the human cerebral cortex areas

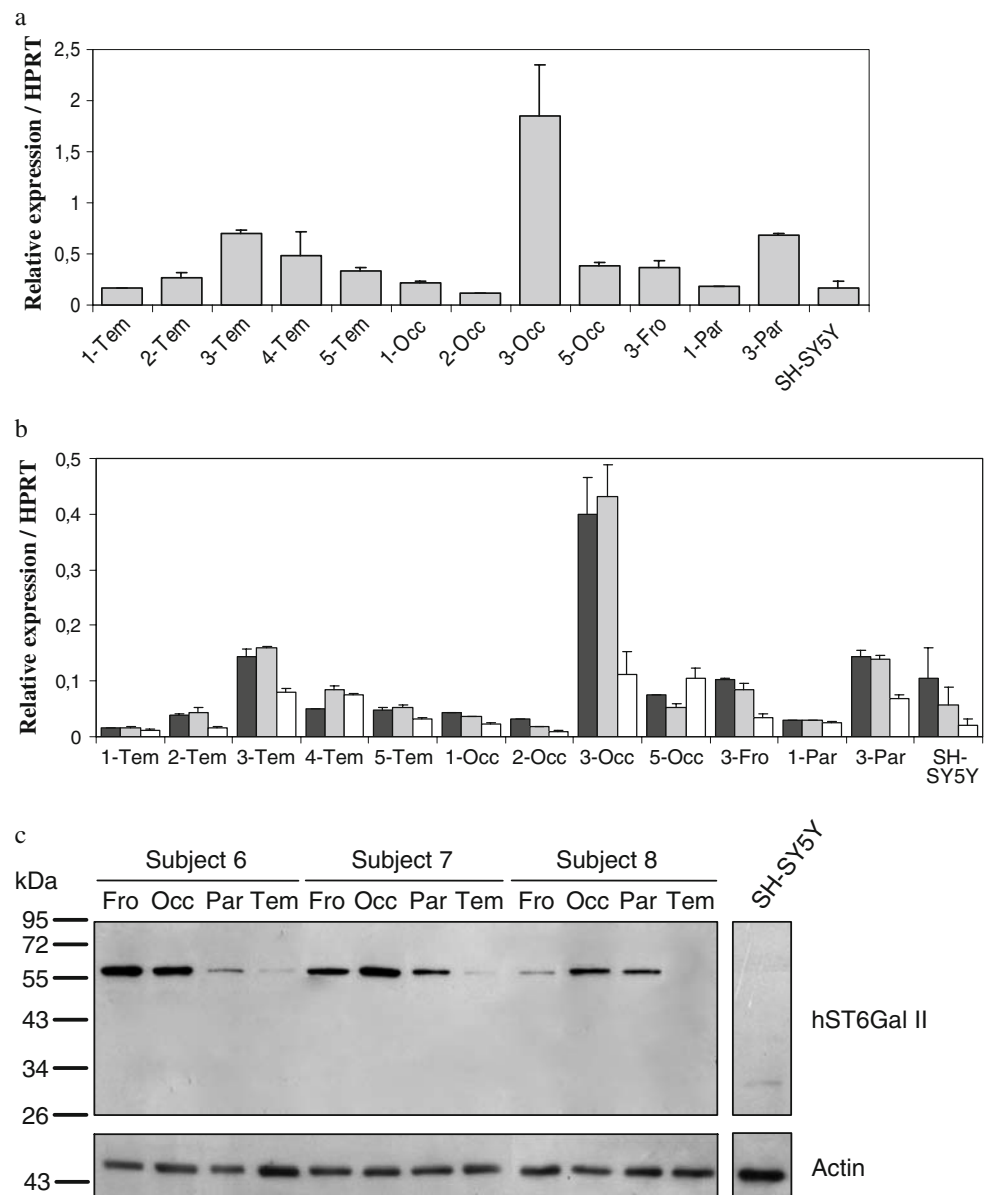
Western blotting specificity of the anti-hST6Gal II antibody was checked using protein extracts from COS-7 cells transfected with the expression plasmid p3xFLAG-CMV-10 containing either the full length sequence of hST6Gal I or hST6Gal II, or the empty plasmid. Using the anti-ST6Gal II antibody, a single band was detected only in protein extracts from cells transfected with p3xFLAG-CMV-10-hST6Gal II, whereas the anti-FLAG antibody revealed one band in protein extracts from cells transfected with p3xFLAG-CMV-10-hST6Gal II or p3xFLAG-CMV-10-hST6Gal I. The apparent molecular weights of the proteins detected with the anti-hST6Gal II and anti-FLAG antibodies were similar and consistent with the predicted molecular weight of 3xFLAG-tagged hST6Gal II (62 kDa), confirming that the anti-hST6Gal II antibody is specific for hST6Gal II (data not shown). hST6Gal II protein expression was subsequently analysed by Western blotting in the four cerebral cortex lobes of three subjects and in SH-SY5Y cells. The results presented in Fig. 4c show a single band around 60 kDa in all brain samples, in agreement with the predicted molecular weight of hST6Gal II (58 kDa). The



**Fig. 3** Duplex Taqman Q-PCR analysis of *ST6GAL2* transcripts relative expression in various human cell lines. Black bars, EX-specific transcript; grey bars, EY-specific transcript; open bars, EZ-specific transcript. Values are normalized to *HPRT* gene expression. Data are means  $\pm$  S.D. of  $n \geq 3$  experiments



**Fig. 4** Protein and mRNA expression of *ST6GAL2* in different lobes of human cerebral cortex. **(a)** Expression of total *ST6GAL2* transcripts by Q-PCR using SYBR<sup>®</sup> Green. **(b)** Expression of *ST6GAL2* exon-specific transcripts by Duplex Taqman Q-PCR. Black bars, EX-specific transcript; grey bars, EY-specific transcript; open bars, EZ-specific transcript. Values are normalized and expressed relatively to *HPRT* gene. Data are means  $\pm$  S.D. of  $n=3$  measurements. **(c)** hST6Gal II protein expression visualized by Western blotting. Numbers 1 to 8 correspond to different subjects; Tem., Occ., Fro., Par. refer to Temporal, Occipital, Frontal, Parietal regions of a given sample, respectively. Actin expression visualized by Western blotting was used as a loading control. Numbers 1 to 8 refer to different subjects. Tem, Occ, Fro, Par refer to Temporal, Occipital, Frontal and Parietal regions of a given subject, respectively



expression of the protein was heterogeneous among the different samples tested, with a lower expression in the temporal lobe for the three subjects. In addition, as observed for the gene expression, inter-individual variations were also detected (Fig. 4a). In these experimental conditions, hST6Gal II protein was not detectable in SH-SY5Y.

*In silico* analysis of the 3.6 kb sequence upstream and including EX, EY and EZ

Preliminary bioinformatics analysis predicted a 600 bp sequence upstream EX as the most probable promoter region for *ST6GAL2* ([www.genomatic.de](http://www.genomatic.de)). We submitted to further *in silico* analysis the genomic region from 1.9 kb upstream EX to 200 bp downstream EZ: -1851 to +1452,

nucleotide +1 referred as the first nucleotide of EX longest form identified by 5' RACE (Fig. 5a). The results for the 1.9 kb sequence upstream EX as well as the results for the 1.7 kb sequence overlapping the three exons are shown in Fig. 5a and b. These analyses did not reveal any canonical TATA or CAAT boxes, but several putative binding sites for general transcription factors such as SP1, NF- $\kappa$ B, GATA1 and MZF1, and also putative binding sites for transcription factors involved in neuronal functions such as NRSF, Pit1, MYT1, Pur $\alpha$ , Olf1 and Sox5 (Fig. 5a and b).

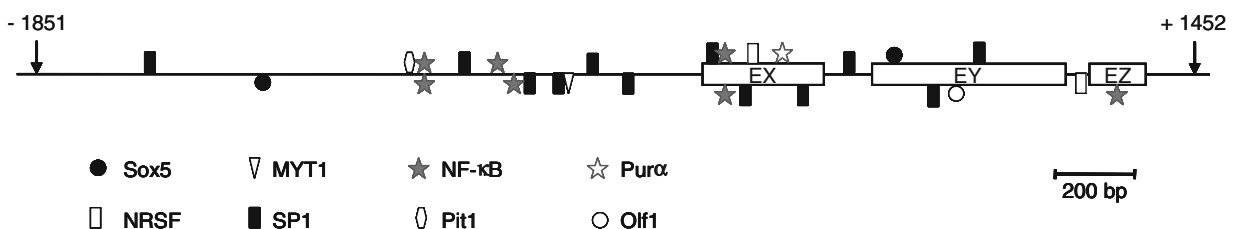
SH-SY5Y transient transfections and luciferase assays

To determine the minimal promoter region upstream EX, the sequence -1851 / +263 was subcloned in the pGL3-

a

-1851 CCCGTTTCAATTTTGTATTTTAGTAGAGACAGGTTTCAACATTATGGCCAGGCTGGTCTCGAACTCCTGACCTCGTGATCCGCCGCCT  
-1761 CGGCCTCCCAAAGTACTGGGATTACAGGTGTGAGCCACCAGCCAGGCTGAAGGTAGGTACTATTACATCCATTATACAGAAGAGAAAACA  
-1671 GAAGCCCACAGACTTGTCAACCCCTCTGCGCCCCTACTATTAAAGAGTGTAGTCTTCTGCAGTATTTCTTTCTCTATCCCCACCA  
-1581 GCTATGGGTCTCTATCAGGTGTTTTGTTTCTGTTTTGTTTTGGTCACTTTTAAACCACACTGTATGATTTTACATTTATAAAAATAAATT  
-1491 TTTCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGTAGACGGAGTCTCGCTCTGTGCCAGCCGGA→CCTGCGACTGCAGTGGCCCAAT  
-1401 CTCGGCTCACTGCAAGCTCCGCTTCCCGGGTTCACGCCATTCTCTGCCTCAGCCTCCCGAGTAGCTGGGACTACAGCGGCCTGCCACCG  
-1311 CGCCCGGCTAATTTTTGTATTTTAGTAGAGACGGGTTTACCTTGTAGCCAGGATGGTCTCGATCTCCTGACCTCATGATCCACCC  
-1221 GCCTCGGCCTCCCAAAGTCTGGGATTACAGGCGTGAGCCACCAGCCCGCCGACATAATTTTTCTTTTTTAAAAATATCTGTGTAACAC  
-1131 ATTGTTTCAATCAGGCAGTTCAGGAGATTCCAGCAATTCATGTGTGACAGATAATTAATACAAATGGGGAAACTAGTAGTAAGCGTTTTCCGCCACA  
-1041 CTTTTGTTTGAACACAGTGCATTATACCCACTCGATTTAGTAAATCCCGTGCTGTTTACATTTCTGTAACCTTTTGAGGGAGTTC  
-951 TGGGCGCCACTGTCTAGCTCAGAGTTCAGGCTAAGGTAGGCACTAATGAATGCTGAATGAATTGGGTAGCCAGTTAATTGCTTTTTTCC  
-861 CCCTAAAAAAAAGTCTAAATTAAGTAAATAAAGTATTTCGGGATTTTTTAAATAATACATTTAATGGCAGATCTGTCTCCAAAACG  
-771 TGTGTCCTCTAGCAGTCTGAGGGTGTGGGGTGGGGTGGGGTGACTAACAGTCCCCCTGAGTGGGCCAACCTTAGCAATATTATCC  
-681 ATCTACCTGGGATCCCCTACACCTGAACAGCTCTTGGGTCCGCGAGAGAACCAGCGGGTGGGGCACCCAATGCCAGGAGTGCAGCGC  
-591 GAGAGACACGCTGAAGTTCACAGGAGATTTACAGCAAAACAAGGGCGGC→TTGGGGTCTAGGACCAGTGCAGCCGCTCTCG  
-501 AGAAAACGCAAGGGCGCGGTGACAAGTGGACATTCCGACGCGACCAAGCCGACACTCGATGGCACCTCCCGCGGCCCGCGCAG  
-411 GCTCCAGCTGCCAGCTCGCAGCGCCACTGGCTCCGGAGGCTGCTTCAACCACTTGTCCCGCGGCTTCTACCCCAAGAACTTTCCGT  
-321 CCCTCTGGGGTGGGGACCGGAGTGTGGAGGGCGGGCAGTGGGGGTCTCCAGGGAGCACCTGACCGGACTTGGCGAGCTACCCCT  
-231 CCCAAATGAAAGTGGCCCGCTCCCAAGTCCATCCCATATCCTTAGAGAAGCTCTGGGAGCGAGGGCCAGCGGAGTGGCGAGCCTGGT  
-141 TGAGCGAGATGAACAAAGGAGCTAGCGAGCGGGGAGGATGCTGCGCTCCCTGCGCCCAACCCAGCCGGCTGCCAGCGCTGCCAGGC  
-51 GCGCAGTAGCCCGCCTTCCGCGTGGCGGGGGAGTGTGGACAGGGTACACAAAGACTCACTTCCCGGGGCGAGGCGCGGGACT  
+41 CCTCTCTGGCGCCACAGCCTGTGCGCATTCTGCTTCTGCGCCCGCCGGGACCCGAGTGCCTCCCGGAGGTGTCCAGGCGCGTGGC  
+131 AGGCGGTACTGTGAGGTAACCTTCGGAAGAACTAGGGCTGGATGGAGCGCGCGGGGGAGAAAACACGCCCTTTTAGGCAGCCGTGC  
+221 TGCCCGCACCCGCCAGCAGCCCAAGTCTGCAGTCACTCTCCGCGACAAGCAGAAATCCGCGCGAGCCAGGAGCGCCCGCCTGCG  
+311 CTCGCGCGCGGGCGAGCTGCCGGCCGGGGCGGGGAGGGGGAGCCCACTTCCCAGTGCCCGGCTCCCGCCAGTCTAGGTGAGCCT  
+401 GGAGCGGAAGGATGAAAGGGATCGCGGAGAGGGAGGGGGCTCTGGGGGAGCGGAGCTCGGGGGCGGGGCGCGCGCAGCCTCGTGCG  
+491 TGGGCGGGAAACCGCGCTCTCCCCGGGCTCGGGGCGCGGTTCTCTGCGCGGGAGCGCGCGCGGCTGCCGAACAATGAGG  
+581 CGTGACGCGGGGCTGAGCGTAGCCTCCCGCGGCTTTGAGGCGGAGCCACCGTGTGCCCAGCGCGCGCTTGACAGTGCAGCGCG  
+671 GCGCGAGCAGCGCTGCGCCCGGAGAGTGATCCCGCAGGCCCTGGGACGGGAGCGGGGCGAGCTCCGAGACCCACGGTGGCAGGA  
+761 GGAGGACTCGAGCTCGCGCAGGACGACTCCCGCGCGCACTCGCCTTCTGCAGCCCCAAGTTCGCGGCGTGAGTGGGGACCCCGG  
+851 CAGGTCACAGCTCTAGCGGGCGCCAGCGTGTGAGTGGGAGCGGTAGCGCGCACCCGAGCGGCTCCGGGAATAAAATGTGACACG  
+941 CACGCCCTGGACTCCAGTGCCCGCTGCCAGGGGAGCACCGAGCACCGGTGTCTGCGCTTCGAGGCGCGCGTGGGAAGAAGGCTG  
+1031 GTAAGGAGGGACTGGTAAAGGTTGGCGACGCTTTCTTGGTGGCGCTGCAGCTCCGCGTGTGCGGCGGAGTTACACTCCGTGT  
+1121 TTAGGAGCAGACCCGTGTGGGGAATGTTGAAGTGGTTTGGGAAAAGCAGGAAATTTGGAGAAAGCGAACGGGAGGAGCGCTTGGC  
+1211 CTTTGGGACGGCGGATACAGTTGCCCCCTGTGGCTCAGTAAAGTGATCTTTTATTTGGCTCTTTGGCTCTGGAAGGGAGTGAAG  
+1301 ATTTTGTCTTTCTCAGCCAACCCCGTACAAAACCTGCACTTCTTAAAGGTTAAAGTGTGTTCTGTTTCTGTTTCTGTGGCTA  
+1391 TAATGTTTGGGAGTAGTAGGATCTTTAGAATCCTAAATACCCGAGTGTAAATTCTTATGTAG

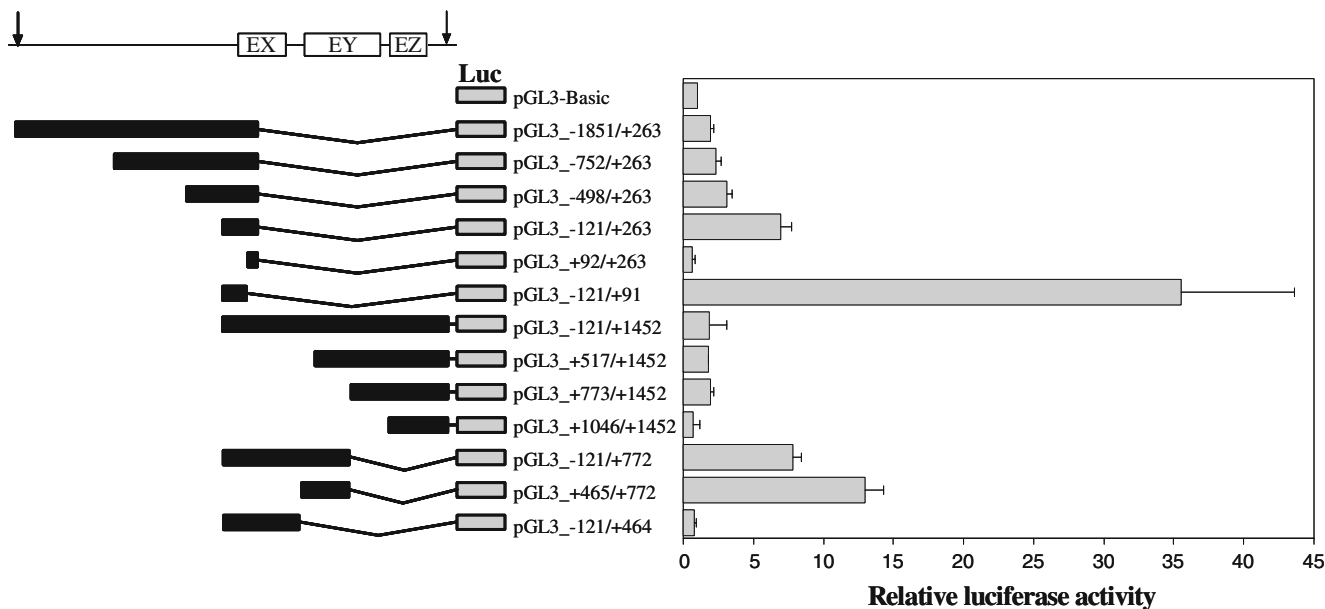
b



**Fig. 5** *In silico* analysis of the nucleotide sequence  $-1851 / +1452$ . (a) Nucleotides are numbered with the nucleotide +1 as the first TSS. Arrowheads indicate the different TSS of EX, open triangles indicate the different TSS of EY and the asterisk indicates the TSS of EZ. Potential transcription factor binding sites are underlined with arrows, left arrow when the binding site is on the  $-$  strand and right arrow when the binding site is on the  $+$  strand. (b) Schematic diagram of the  $-1851 / +1452$  region. Upper transcription factors are located on the  $+$  strand and bottom transcription factors are located on the  $-$  strand

Basic vector upstream the luciferase gene and named pGL3<sub>-1851/+263</sub>. This plasmid or 5' deleted constructions: pGL3<sub>-752/+263</sub>, pGL3<sub>-498/+263</sub>, pGL3<sub>-121/+263</sub> and pGL3<sub>+92/+263</sub> were transfected into SH-SY5Y cells for luciferase assays. The results presented in Fig. 6 show a 7-fold increase of luciferase activity for the plasmid pGL3<sub>-121/+263</sub> compared to pGL3-Basic, whereas the luciferase activity for constructs pGL3<sub>-1851/+263</sub>, pGL3<sub>-752/+263</sub> and pGL3<sub>-498/+263</sub> only show a 2- to 3-fold increased activity. Luciferase activity for the plasmid pGL3<sub>+92/+263</sub> is even lower than the activity for the pGL3-Basic vector. These data strongly suggest the existence of a promoter region within the sequence  $-121 / +92$ . We also analyzed the  $-121 / +1452$  region encompassing EX, EY and EZ. The plasmid pGL3<sub>-121/+1452</sub> and its truncated forms: pGL3<sub>+517/+1452</sub>, pGL3<sub>-121/+772</sub>, pGL3<sub>+773/+1452</sub>, pGL3<sub>+1046/+1452</sub>, pGL3<sub>-121/+464</sub>, pGL3<sub>+465/+772</sub> and pGL3<sub>-121/+91</sub> were generated. The 5' end of the genomic sequence inserted into the plasmid

pGL3<sub>+517/+1452</sub> starts 8 nucleotides downstream from the first TSS of EY. The genomic sequence inserted into the plasmid pGL3<sub>-121/+91</sub> corresponds to the  $-121 / +91$  sequence. This sequence ends 12 nucleotides downstream of the last identified TSS of EX. These different plasmids were transfected into SH-SY5Y cells and luciferase activity was determined. The plasmids pGL3<sub>-121/+1452</sub>, pGL3<sub>+517/+1452</sub>, pGL3<sub>+773/+1452</sub>, pGL3<sub>+1046/+1452</sub> and pGL3<sub>-121/+464</sub> did not show any significant increase in luciferase activity (Fig. 6) whereas pGL3<sub>-121/+772</sub> and pGL3<sub>+465/+772</sub> showed a 8- and 13-fold increased activity compared to pGL3-Basic, respectively (Fig. 6), suggesting that the sequence  $+465 / +772$  corresponds to a promoter region. Moreover, the plasmid pGL3<sub>-121/+91</sub>, corresponding to the previous pGL3<sub>-121/+263</sub> construct deleted of 181 bp at the 3' end, showed a dramatic 35-fold increase of luciferase activity, indicating a high promoter activity within this 212 bp sequence upstream EX. Finally, the sequence  $+92 / +464$  is likely to contain negative regulatory elements since luciferase activity decreased for plasmids pGL3<sub>-121/+772</sub> and pGL3<sub>-121/+464</sub> compared to pGL3<sub>+465/+772</sub> and pGL3<sub>-121/+91</sub>. Altogether, the results presented in Fig. 6 suggest a series of positive and negative regulatory elements within a 900 bp sequence including EX and EY. This region starts with a strong promoter sequence ( $-121 / +91$ ) followed by a region containing negative regulatory elements ( $+92 / +464$ ) and then a second promoter sequence ( $+465 / +772$ ).



**Fig. 6** Luciferase assays after transfections of different plasmid constructs in SH-SY5Y neuroblastoma cells. On the left, a schematic representation of the different constructs inserted in pGL3-Basic reporter vector upstream of the luciferase gene. On the right: the

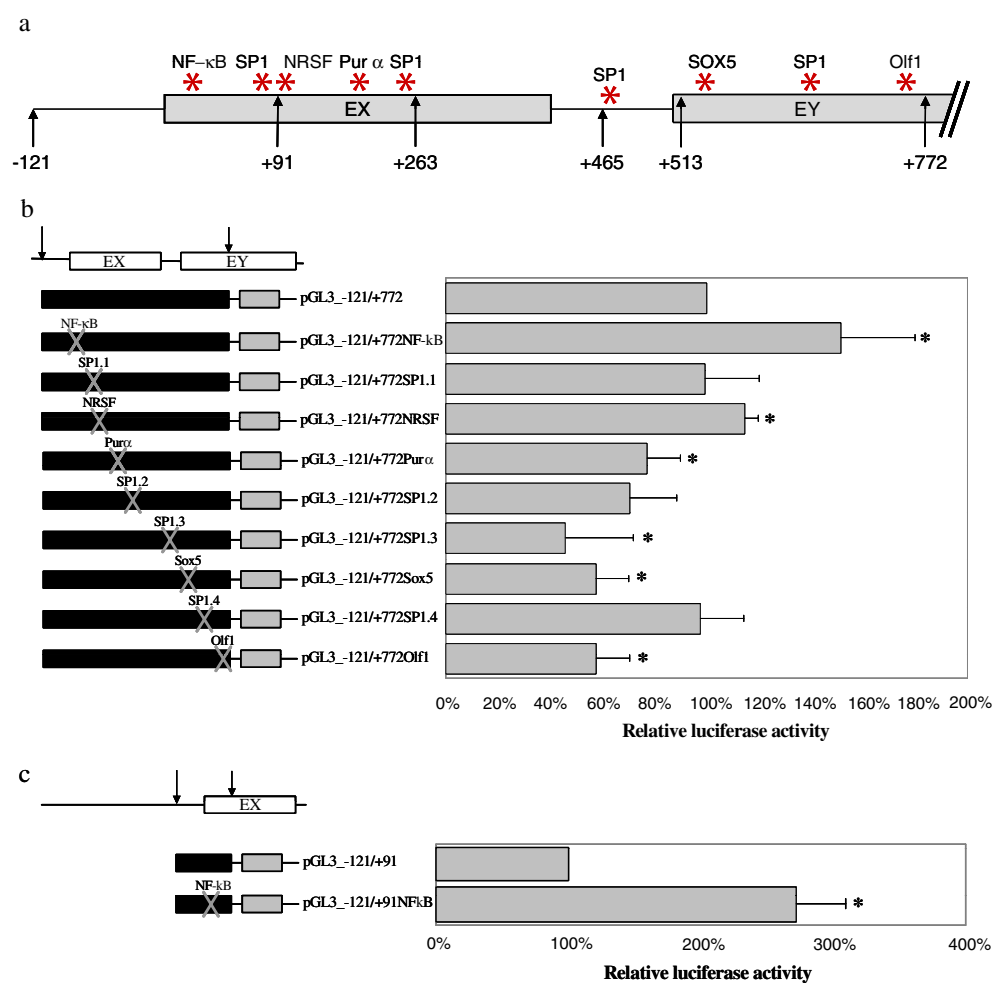
results of luciferase assays. Transfection efficiencies are normalized with the co-transfected plasmid expressing *Renilla* luciferase and luciferase activities are expressed compared to pGL3-Basic vector activity. The data are means  $\pm$  S.D. of  $n \geq 3$  experiments

## Identification of transcription factor binding sites by PCR site-directed mutagenesis

According to the results shown in Fig. 6, the genomic sequence -121 / +772 is likely to include positive and negative regulatory elements involved in *ST6GAL2* transcriptional regulation. Considering both general and brain-related transcription factor binding sites predicted by bioinformatics analysis, we mutated by PCR site-directed mutagenesis the NF- $\kappa$ B, NRSF, Olf1, Pur $\alpha$ , Sox5, SP1.1, SP1.2, SP1.3 and SP1.4 binding sites (Fig. 7a). Thus, plasmids pGL3<sub>-121/+772</sub>NF- $\kappa$ B, pGL3<sub>-121/+772</sub>NRSF, pGL3<sub>-121/+772</sub>Olf1, pGL3<sub>-121/+772</sub>Pur $\alpha$ , pGL3<sub>-121/+772</sub>Sox5, pGL3<sub>-121/+772</sub>SP1.1, pGL3<sub>-121/+772</sub>SP1.2, pGL3<sub>-121/+772</sub>SP1.3 and pGL3<sub>-121/+772</sub>SP1.4 were obtained by substitution mutation of the plasmid pGL3<sub>-121/+772</sub>. Mutated plasmids were transfected into SH-SY5Y cells and luciferase activity was compared to the activity of the pGL3<sub>-121/+772</sub> transfected cells (Fig. 7b). The pGL3<sub>-121/+772</sub>SP1.1, pGL3<sub>-121/+772</sub>SP1.2 and pGL3<sub>-121/+772</sub>SP1.4 constructs showed no significant

difference of luciferase activity with the plasmid pGL3<sub>-121/+772</sub> (Fig. 7b), indicating that these SP1 predicted binding sites do not play a role in *ST6GAL2* gene regulation in SH-SY5Y cells. Luciferase activity of the plasmids pGL3<sub>-121/+772</sub>NRSF and pGL3<sub>-121/+772</sub>NF- $\kappa$ B showed a 15 % and 50 % increase, respectively, compared to the pGL3<sub>-121/+772</sub> plasmid (Fig. 7b), suggesting a repressive role for both factors. The plasmids pGL3<sub>-121/+772</sub>Pur $\alpha$ , pGL3<sub>-121/+772</sub>Sox5, pGL3<sub>-121/+772</sub>Olf1 and pGL3<sub>-121/+772</sub>SP1.3 showed a decrease in luciferase activity compared to the non-mutated plasmid, suggesting that Pur $\alpha$ , Sox5, Olf1 and the third SP1 binding sites and the corresponding transcription factors play an activator role in this gene regulation. Considering the result obtained with the mutated plasmid pGL3<sub>-121/+772</sub>NF- $\kappa$ B and since the plasmid pGL3<sub>-121/+91</sub> includes the NF- $\kappa$ B site, we constructed the mutated plasmid pGL3<sub>-121/+91</sub>NF- $\kappa$ B and tested it for luciferase assays. The result exposed in Fig. 7c showed a strong increase of luciferase activity of the mutated construct compared to control, confirming that NF- $\kappa$ B would act as an inhibitory factor.

**Fig. 7** Luciferase assays after transfections of the mutated plasmids in SH-SY5Y neuroblastoma cells. **(a)** Schematic representation of the -121 / +772 nucleotide sequence. Asterisks indicate the transcription factor binding sites that were mutated by PCR site-directed mutagenesis. Arrows indicate the position of restriction sites used for plasmid constructions. **(b)** On the left: a schematic representation of the different plasmid constructs. On the right: the results of luciferase assays. **(c)** On the left: a schematic representation of the different plasmid constructs. On the right: the results of luciferase assays. Transfection efficiencies are normalized with the co-transfected plasmid expressing *Renilla* luciferase and luciferase activities are expressed as a percentage of the pGL3<sub>-121/+772</sub> (b) or pGL3<sub>-121/+91</sub> (c) vector activity. The data are mean  $\pm$  S.D. of  $n \geq 3$  experiments. \*,  $P < 0.05$  vs. pGL3<sub>-121/+772</sub> (b) or pGL3<sub>-121/+91</sub> (c)





## Discussion

### 5' UTR of *ST6GAL2* mRNA in SH-SY5Y cells

In this study, we identified the 5' UTR of *ST6GAL2* mRNA in the SH-SY5Y neuroblastoma cell line. 5' RACE experiments showed that transcription leads to three different forms of mRNA regarding to their 5' ends. Each transcript contains one different and unique non-coding exon named EX, EY and EZ. The three exons are separated from the first common coding exon E1 by more than 42 kbp, which is, on one hand, a common feature for sialyltransferase genes [24–26]. On the other hand, these three exons are unusually close on the genomic sequence since EX and EY are separated by 124 bp and EY and EZ are separated by only 87 bp (Fig. 1c). We accurately defined the transcription start sites of EX, EY and EZ with a series of exon-specific 5' RACE experiments. While the TSS of EY and EZ are almost not submissive to variation, we showed that the transcription of EX starts in at least seven TSS over 78 bp (Fig. 2b), which is a common attribute for TATA-less promoter [27]. Moreover, the sequences of EX, EY and EZ that we have identified by 5' RACE match with partial sequences in the EST databank (GenBank accession # AB058780, AB059555, BC008680, BE612797 and BX353569). Alternative promoters and alternative 5' non-coding exons are usual for sialyltransferase genes and reflect the need for tissue- and/or time-specific expression [4,12,28], but such a closeness for the first alternative untranslated exons is rather unusual and raises the question of the biological significance of this genomic organisation. A promoter model based on more than 1.7 million human full-length cDNA 5'-end sequences, established that alternative TSS separated by at least 500 bp are theoretically sufficient to constitute mutually alternative promoters and brain was classified as the second-richest origin of putative tissue-specific alternative promoters [29]. The EX cluster of TSS being separated by 427 bp from the EY TSS cluster, itself separated by 608 bp from the EZ TSS, independent promoters could be expected. However, analysis of specific transcripts in cell lines and brain samples is not in favour of a specific regulation by independent promoters but rather indicates that alternative TSS are driven from the same large promoter region, sharing most of the regulatory cis-elements.

### Expression of EX, EY and EZ containing transcripts in brain-derived cell lines and in cerebral cortex regions

The relative expression of transcripts containing either EX, EY or EZ was determined using duplex Taqman Q-PCR with the *HPRT* normalizing gene. In SH-SY5Y cells, EX and EY containing transcripts were more expressed than the

EZ containing transcript (Fig. 3). Consistent with the total expression of *ST6GAL2* gene (Data not shown), duplex Q-PCR also revealed that the highest expression of these three transcripts was detected in neuroblastoma SH-SY5Y cells and in proneuronal NT2/D1 cells, whereas their expression was low in the neuroblastoma Kelly cells and was hardly detected in the T98G and U118 glioblastoma cells. We have also shown that the three *ST6GAL2* specific transcripts were expressed in the different lobes of human cerebral cortex (Fig. 4b). In spite of differences in the expression level of *ST6GAL2* in the samples that we examined, the relative expression of *ST6GAL2* exon-specific transcripts was similar to that of neuronal cell lines (with EX>EY>EZ) suggesting a similar transcriptional regulation of *ST6GAL2* in human cerebral cortex and cultured cell lines, which is in favour of a common promoter region controlling the three transcription start sites. These results also confirm that the SH-SY5Y cell line is an adequate and relevant model for the analysis of *ST6GAL2* gene brain-specific transcriptional regulation. Previous studies reported that *ST6GAL2* is mostly expressed in brain with a low expression in the different lobes of the cerebral cortex, but no data are available concerning its expression in the different cell types [8,9]. Prior the identification of *ST6GAL2*, it had been reported that normal human neurons and glial cells did not express *ST6GAL1* [30,31]. Considering the highest expression of *ST6GAL2* in SH-SY5Y and NT2/D1 among the brain-related cell lines tested, our data suggest that *ST6GAL2* would be expressed preferentially in neurons *in vivo* and could compensate the low expression of *ST6GAL1* for the synthesis of specific  $\alpha$ 2,6-sialylated glycoconjugates. However, glycoconjugates exhibiting SLDN or SLN terminal structures have not been yet reported in brain tissues. Further studies with cultured primary neurons and glial cells would be helpful to shed light on *ST6GAL2* cell-type specific expression. In addition, *ST6GAL2* transcripts were found to be expressed in the embryonic carcinoma NT2/D1 cells. Human and murine fetal tissues also express *ST6GAL2* [8,9]. The NT2/D1 and SH-SY5Y cell lines are well-known models for studying neuronal differentiation under retinoic acid exposition [32,33]. In such a context, it could be interesting to monitor variations in  $\alpha$ 2,6-sialylation and in particular the presence of SLDN motif, as well as the expression of both *ST6GAL1* and *ST6GAL2* genes through neuronal differentiation. An alternative approach to answer this question would be to use murine primary neurons in culture.

### Expression of hST6Gal II in brain-derived cell lines and in cerebral cortex regions

In this study, we demonstrated for the first time that hST6Gal II protein is expressed in the different lobes of the

cerebral cortex. Independently of inter-individual variations, we also showed that hST6Gal II is less expressed in the temporal lobe compared to the other cortex areas (Fig. 4c). However, the correlation between *ST6GAL2* mRNA expression and hST6Gal II protein expression remains to be established since Western blotting and Q-PCR analysis could not be performed on the same brain samples. In parallel, the protein was almost not detectable in SH-SY5Y cell line, suggesting that the level of expression of hST6Gal II in cultured cells is lower compared to human brain. Two of our previous studies concerning glycosyltransferase expression have already underlined the inter- and intra-individual variations of mRNA expression observed in this study [34,35]. Human cerebral cortex is divided into areas that are different from one to another in terms of cytoarchitecture and chemoarchitecture, functions and patterns of gene and protein expressions [36,37]. Therefore, variations of expression between the different cerebral cortex samples could be expected. Moreover, genetic variations including male-female differences, epigenetic mechanisms and aging are known to influence mRNA production [38–40] and may also explain inter-individual variations observed in our study.

#### Promoter regions and transcription factors

Three alternative 5' non-coding exons suggest three alternative promoters. However, taking into account the unexpected closeness of EX, EY and EZ, this would imply that regulatory elements lie within EX and/or EY sequence. Using luciferase reporter assays in SH-SY5Y cells, we showed that two regions (-121 / +91 and +465 / +772) upstream EX and within the 5' part of EY, respectively, possess a promoter activity. The first region showed the highest luciferase activity when transfected into SH-SY5Y cells. This result is consistent with Q-PCR results, which showed that EX was more expressed than EY and EZ. The second sequence is more controversial since it is partially within EY sequence. A negative regulatory region is likely to reside between these two positive regulatory regions, since the luciferase assays with plasmids containing parts or total region +92 to +464 constantly showed a decreased enzyme activity compared to plasmids without parts of this sequence: pGL3<sub>-121/+91</sub> vs. pGL3<sub>-121/+263</sub> or pGL3<sub>-121/+464</sub> and pGL3<sub>+465/+772</sub> vs. pGL3<sub>-121/+772</sub> (Fig. 6). We were not able to show evidence for a promoter region leading to exon EZ transcription with luciferase assays. This may partially reflect the very low endogenous expression of EZ in SH-SY5Y cells. Concerning the potential transcription factors involved in *ST6GAL2* regulation, our investigations suggest that NRSF and NF-κB repress the transcription while Olf1, Sox5, Purα and SP1

would act as activators for the transcription of *ST6GAL2*. The SP1, Sox5 and Olf1 transcription factors are thought to act as transcription activators and are EY-related, whereas NF-κB seems to repress *ST6GAL2* transcription in a sequence upstream EX. Between the two identified promoter regions, NRSF and Purα appear to act respectively as repressor and activator of *ST6GAL2* transcription. In the central nervous system, the NF-κB family of transcription factors are involved in processes such as neuronal plasticity, myelin formation, synaptic transmission and inflammation [41]. The potential NF-κB site in the EX promoter sequence is predicted to bind homodimers of p50 proteins, which do not contain a transactivator domain, and so act as a transcription repressor [42–44]. The mutagenesis data strongly suggest that the homodimer p50/p50 is involved in the inhibition of *ST6GAL2* transcription in SH-SY5Y cells. Nevertheless, transcriptional activator elements involved in the high promoter activity of the sequence -121 / +91 (Fig. 6) remain to be identified. The neuron-restrictive silencer factor (NRSF) essentially represses expression of neuron-specific genes in non neuronal cells or tissues [45,46]. According to our results, NRSF would repress the expression of *ST6GAL2*, suggesting that the expression in SH-SY5Y cells could be disinhibited for precise neuronal-related function(s). Olf1, Sox5 and Purα are transcription factors that have been shown to be involved in neurogenesis and general neuronal functions [19,47–50] and would argue for a specific neuronal expression of *ST6GAL2*. SP1 is generally an activator factor that binds GC-rich sequences and four putative SP1 binding sites lie within the two identified *ST6GAL2* promoter sequences. Our data suggest that only the third site (SP1.3) is significantly implicated (Fig. 7b).

In the SH-SY5Y context, the expression of *ST6GAL2* is likely to be repressed by NRSF and NF-κB factors and at the same time enhanced by Sox5, Olf1, Purα and SP1 factors. As for NRSF, the transcriptional repression of NF-κB p50/p50 could potentially be removed to allow hST6Gal II to fulfil precise functions. In the light of transcription factors potentially involved in *ST6GAL2* regulation, one can hypothesize that hST6Gal II is required during brain development and/or for selective neuronal functions in adult brain. Interestingly, we previously showed that *ST6GAL2* expression is dramatically increased after IL-6 and IL-8 cytokines stimulation in the human bronchial mucosa. In this situation, neither *ST6GAL1* nor other sialyltransferase genes studied showed such an increase in their transcriptional expression [35]. This suggests that hST6Gal II would play a pivotal role that was not previously highlighted in the inflammation process in airways. In the present study, we did not find potential response elements for transcription factors involved in IL-6 and/or IL-8 response pathways. Nevertheless, five potential

NF- $\kappa$ B binding sites have been identified (nucleotides -675; -485; -439; +32 and +1173). Two of these sites are supposed to bind the repressor homodimer p50/p50, and the three other sites are predicted to bind p50, c-rel and p65, the two latter known to be transcriptional activators in the context of inflammation [51].

## Conclusion

In this study, we identified and characterized the 5' UTR and promoter regions of *ST6GAL2* in neuroblastoma SH-SY5Y cell line. Furthermore, we showed that *ST6GAL2* and its different transcripts are expressed in human cerebral cortex. The protein hST6Gal II was also detected in the four areas of the cerebral cortex. In the light of transcription factors potentially involved in *ST6GAL2* regulation, one can hypothesize that hST6Gal II could be required during brain development, for cell-type selective functions in adult brain and possibly in pathological conditions. Indeed, it has been previously shown that the genetic locus linked to autosomal dominant acute necrotizing encephalopathy (ADANE) contains four genes including *ST6GAL2* [52]. Although no mutation was found in the coding regions of these genes, the authors do not exclude mutations in the promoter sequences that could alter genes expression. Although variations of  $\alpha$ 2,6-sialylation have not been investigated in ADANE, incorrect sialylation caused by deregulation of *ST6GAL2* expression may not be excluded. This work, along with further experiments, will be helpful to understand mechanisms that finely regulate *ST6GAL2* gene expression.

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