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# Multiplex RT-PCR method for the analysis of the expression of human sialyltransferases: application to breast cancer cells

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**In many cases of human cancer, the appearance of hypersialylated glycan structures is related to a precise stage of the disease; this may depend on altered regulation of one or more sialyltransferases genes. Since several distinct sialyltransferase enzymes arising from different unique genes transfer sialic acid residues in the same linkage onto the same acceptor, it is impossible to precisely determine which enzyme is involved in the observed phenotype based on enzymatic assays. We have developed a very sensitive and highly reproducible multiplex reverse transcriptase-polymerase chain reaction technique in order to monitor the expression of four human sialyltransferases genes *ST6Gal I, ST3Gal I, ST3Gal III* and *ST3Gal IV* in small cell samples. Multiplex PCR amplification using specific primers for each sialyltransferase and detection of amplification products by polyacrylamide gel electrophoresis is a method that is fast and easy to handle and has proven to be useful for establishing sialyltransferase patterns of expression in breast immortalized cell line HBL100 as well as in breast cancer cell lines MCF-7/6, MCF-7/AZ and MDA.**

***Keywords*: sialyltransferases, breast cancer cells, multiplex RT-PCR, glycosyltransferases**

***Abbreviations*: bp, base pair; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kb, kilobase; PNA, Peanut (*Arachis hypogaea*) agglutinin; RT-PCR, reverse transcription-polymerase chain reaction; TBE, Tris base 0.13 M, boric acid 45 mM; $Na_2EDTA$ 2.55 mM, pH 8.8 buffer; Tm, melting temperature**

***Enzymes*: the nomenclature of sialyltransferases is based on that of Tsuji *et al.* [36]: ST3Gal I: CMP-NeuAc, Gal$\beta$1-3GalNAc $\alpha$2,3-sialyltransferase, EC 2.4.99.4; ST3Gal III: CMP-NeuAc, Gal$\beta$1-3/4GlcNAc $\alpha$2,3-sialyltransferase, EC 2.4.99.6; ST3Gal IV: CMP-NeuAc, Gal$\beta$1-4GlcNAc or Gal$\beta$1-3GalNAc $\alpha$2,3-sialyltransferase, EC 2.4.99.-; ST6Gal I: CMP-NeuAc, Gal$\beta$1-4GlcNAc $\alpha$2,6-sialyltransferase, EC 2.4.99.1.**

## Introduction

It is now well established that cell surface glycoconjugates play essential roles in maintaining the function as well as the structure of the living cells. The glycan moiety of glycoconjugates is characteristic of a cell type, and one of the most important findings in the past decade is that the oligosaccharide sequences are strictly controlled during development and differentiation of cells [1–5]. Several examples have shown that these changes in glycan structures result from a fine control of glycosyltransferase expression [6–8]. In pathological states such as cell transformation, the misregulation of glycosyltransferase expression leads to aberrant glycosylated cell surface glycoconjugates [9, 10], and the higher amount of sialic acids as well as sialyltransferase expression have been associated for a long time with the tumourigenicity and the metastatic potential of tumour cells [11–13].

Sialic acids are a family of sugars found at the non-reducing terminal position of glycan chains of glycoproteins and glycolipids [14]. Because of this terminal position, sialylated oligosaccharide sequences of mammalian cell surface glycoconjugates are involved in many biological processes including cell–cell recognition. The selectin family is the best documented example of cell adhesion molecules at the cell-surface that are able to recognize sialylated ligands such as sialyl-Lewis$^x$ (SLe$^x$, NeuAc$\alpha$2-3Gal$\beta$1-4[Fuc$\alpha$1-3]GlcNAc$\beta$1-*R*), and that mediate the adhesion of leukocytes [15–17]. Aberrant expression of Lewis type antigens has been reported for many cancers including those of lung, colon, stomach and kidney [10]. In particular, carcinoma and leukemia cells are enriched in SLe$^x$ structures [10, 18].

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**Table 1.** Human sialyltransferases cDNA cloned.

| *Systemic name*[a] | *EC no.* | *Acceptor(s)* | *Origin* | *GenBank accession no.* | *Reference(s)* |
|---|---|---|---|---|---|
| ST6Gal I | 2.4.99.1 | Gal$\beta$1-4GlcNAc | placenta | X17247 | 26 |
| ST3Gal I | 2.4.99.4 | Gal$\beta$1-3GalNAc | placenta | L29555 | 27 |
| ST3Gal III | 2.4.99.6 | Gal$\beta$1-3(4)GlcNAc | placenta | L23768 | 28 |
| ST3Gal IV | 2.4.99.- | Gal$\beta$1-4GlcNAc and Gal$\beta$1-3GalNAc | placenta | L23767; X74570 | 29–30 |
| ST8Sia I | 2.4.99.8 | NeuAc$\alpha$2-3Gal$\beta$1-4Glc-Cer | melanoma cells | X77922; D26360; L32867; L43494 | 31–34 |
| ST8Sia II | 2.4.99.- | (NeuAc$\alpha$2-8)$_n$ NeuAc$\alpha$2-3-*N*-Glycans | placenta | L29556 | 27 |
| ST8Sia IV | 2.4.99.- | (NeuAc$\alpha$2-8)$_n$NeuAc$\alpha$2-3-*N*-Glycans | embryonic brain | L41680 | 35 |

[a] The abbreviations for sialyltransferases used in this paper are according to the new systematic nomenclature proposed by Tsuji *et al.* [36].

Carcinoma cells are also enriched in sialyl-Lewis[a] structures (SLe[a], NeuAc$\alpha$2-3Gal$\beta$1-3[Fuc$\alpha$1-4]GlcNAc$\beta$1-*R*) [19, 20] which is also a ligand for E-selectin [21, 22]. It is, thus, an attractive hypothesis that tumour cells would use the same selectin-carbohydrate interaction to adhere to endothelial cells during metastasis [23].

The biosynthesis of the sialylated oligosaccharide sequences is catalysed by a family of enzymes named sialyltransferases which use CMP-sialic acid as the activated sugar donor. More than fifteen different sialyltransferases acting on glycoproteins and/or glycolipids have been characterized to date, thirteen of them have been cloned from mammalian and avian sources [24, 25] and seven have been cloned from human tissues or cells (Table 1).

Changes in sialylated structures at the cell surface have been observed during development, differentiation or disease processes. These changes depend on altered regulation of the expression of one or more of the sialyltransferase genes, although other mechanisms such as competition between different enzymes acting on the same acceptor substrate, different enzyme localization in the Golgi apparatus, variations in the concentration of activated sugar donor cannot be excluded. As an example, maturation of thymocytes has been correlated to an increase of the Gal$\beta$1-3GalNAc $\alpha$2,3-sialyltransferase (ST3Gal I[‡]; EC 2.4.99.4) transcript enhancing sialylation of the T-antigen (Gal$\beta$1-3GalNAc$\alpha$1-*O*-Ser) and leading to a change from $PNA^+$ to $PNA^-$ phenotype in medullar thymocytes [37]. Human colon cancer cells have been shown to express higher levels of Gal$\beta$1-4GlcNAc $\alpha$2,6-sialyltransferase (ST6Gal I; EC 2.4.99.1) when compared to normal mucosa [38] which results in a higher level of sialylated N-glycan structures at the surface of neoplastic mucosa [39]. Finally, fibroblasts transfected with an activated *ras* oncogene result in the enhancement of ST6Gal I enzymatic activity due to higher level of ST6Gal I mRNA [40], and in an increased invasive potential of the cells [41].

Changes of the glycosylation of O-linked oligosaccharidic chains also occurred in cancer cells in which a higher proportion of short truncated highly sialylated glycans are observed. Sialyl-Tn antigen (NeuAc$\alpha$2-6GalNAc$\alpha$1-*O*-Ser) which is almost absent from normal cells, is up regulated in tumours and may be considered as a good marker for tumourigenicity [9, 42]. On the other hand, the polymorphic epithelial mucin MUC1, expressed in various breast cancer cells, was shown to be aberrantly glycosylated [43] leading to several abnormally sialylated O-glycoforms [44]. These alterations are due to a several-fold increase of the enzymatic transfer activity of sialic acid residues on to Gal$\beta$1-3GalNAc-*R* acceptor substrates in breast cancer cells [43]. This sialyltransferase activity may encompass the enzymatic activities of at least three different enzymes: ST3Gal I, ST3Gal II and ST3Gal IV that are encoded by different genes located on separate chromosomes [45]. Thus, these enzymatic activities cannot be distinguished in *in vitro* assays. Northern blot of mRNAs isolated from different breast cancer cell lines hybridized with a bovine ST3Gal I cDNA probe, but the relative levels of expression of ST3Gal I mRNA did not parallel the increased activities observed previously [43].

Human cancer related oligosaccharide structural changes are quite well documented [9] but there is still little information available concerning the regulated expression of sialyltransferase activities in human breast cancer cells. These studies are greatly hindered due to the fact that sialyltransferase proteins are weakly expressed and may have identical transfer activity towards the same acceptor [45].

In this paper, we report for the first time the use of a fast and sensitive multiplex RT-PCR technique in order to monitor the expression of four cloned human sialyltransferases: ST6Gal I, ST3Gal I, ST3Gal III, ST3Gal IV (Table 1) in the human breast cell lines: HBL-100, MCF-7/AZ, MCF-7/6 and MDA-MB-231.

[‡] The abbreviations for sialyltransferases used in this paper are according to the new systematic nomenclature proposed by Tsuji *et al.* [36].

## Materials and methods

### Materials

All reagents were of analytical or molecular biology grade. dATP-[alpha$^{35}$S] (37 TBq mmol$^{-1}$) was purchased from ICN (USA). First-Strand cDNA Synthesis kit was from Pharmacia Biotech (USA), TA Cloning™ kit was obtained from Invitrogen (USA) and Sequenase™ Version 2.0 DNA Sequencing kit was from USB (USA). Hi-Taq™ DNA polymerase and acrylamide 2X bis-acrylamide (29/1) was purchased from Bioprobe (France). 1 kb DNA Ladder, culture media, and fetal calf serum were from Gibco-BRL (France). Jet Start kit was obtained from Genomed Inc. (USA).

### Cell culture and isolation of cellular RNA

Human breast epithelial cell line HBL 100 (ATCC HTB-124) [46] was a gift from Dr M. Crépin (Paris, France). MCF-7/AZ and MCF-7/6 [47] both derived from the original MCF-7 breast cancer cells (ATCC HTB-22) [48], and MDA-MB-231 cells (ATCC HTB-26) [49] were a generous gift of Professor M.M. Mareel (Laboratory of Experimental Cancerology, University Hospital, Ghent, Belgium). They were grown in Eagle's minimal essential medium Earle's Salt supplemented with 10% fetal calf serum, 1% non-essential amino acids, 2 mM glutamine, 5 μg ml$^{-1}$ insulin, and antibiotics (40 μg ml$^{-1}$ streptomycin; 40 U ml$^{-1}$ penicillin). HepG2 cells from Dr B. Laine (Institut Pasteur, Lille, France) were from ATCC (HB 8065) and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1% non-essential amino acids, 2 mM glutamine, and antibiotics. All cells were grown at 37 °C in a humidified atmosphere of 5% $CO_2$.

Total RNA was extracted using the guanidium thiocyanate method described by Sambrook *et al.* [50] and cellular RNA was quantitated by spectrophotometry at 260 nm.

### Reverse transcription and polymerase chain reaction

Five μg of total cellular RNA were heated at 65 °C for 10 min and placed on ice for 2 min. Reverse transcription into cDNA was achieved using the First-Stand cDNA Synthesis kit according to the manufacturer protocol with oligo-d(T) as initiation primer in a final reaction volume of 33 μl. Two μl of the retrotranscription reaction were subjected to PCR amplification using sialyltransferases or GAPDH specific primers. cDNA sequences for human sialyltransferases and mouse GAPDH were obtained from the Genbank™/EMBL Data Bank (Table 1) and used to design primer pairs. The oligonucleotide primers, their combination and amplification product lengths are shown in Table 2. Primer pairs have been carefully selected through computer analysis using *Primer Premier* Version 3.1 software (Biosoft International, Palo Alto, USA). The oligonucleotides were synthesized and purified by Eurogentec s.a. (Belgium).

The 23 μl PCR mixture consisted of 0.6 U Hi-Taq DNA polymerase (or 1 U for multiplex PCR), 10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.2 mM dNTP and 0.6 μM of each primer. Samples were overlaid with two drops of mineral oil (Sigma Chem. Corp., St Louis, USA). Reactions were run in a PTC-100™ thermal cycler (MJ Research, Watertown, USA) using the following conditions: 1 min at 94 °C, 1 min at 58 °C and 1.5 min at 72 °C for 36 cycles. Thirty PCR cycles were performed for specific GAPDH fragment amplification. In all experiments, negative control reactions were done in which cDNA templates were replaced with sterile water, to check the absence of contaminants.

Ten μl aliquots of the PCR reaction were size-separated on a 10% polyacrylamide gel equilibrated in TBE. Gels were stained with ethidium bromide (1 μg ml$^{-1}$), photographed using Polaroid film under UV light and analysed by computerized densitometric scanning of the image using a Hewlett-Packard ScanJet 4c Scanner, Deskcan II software and Quantiscan program (Biosoft, USA). Sizes of the generated fragments were estimated according to the migration of a 1 kb DNA ladder. Amplification products were subcloned using the TA Cloning™ kit, subsequent purification of plasmid DNA obtained was performed using Jet Star kit and sequenced using the Sequenase Version 2.0 DNA Sequencing kit and dATP-[alpha$^{35}$S].

## Results and discussion

The methodological aim of this work was to determine the RT-PCR conditions to monitor the differential expression of sialyltransferase genes simultaneously, in one tube reaction in a small sample of a given cell type. cDNA templates were generated by reverse-transcription of total RNA harvested from various human cell lines.

### Primer selection

The first point was to determine a specific primer pair for each sialyltransferase and we have focused on four cloned human sialyltransferase cDNAs ST6Gal I, ST3Gal I, ST3Gal III, ST3Gal IV (see Table 1). The primer selection was governed by the following considerations: (i) primer pairs should allow discrimination between all sialyltransferases and GAPDH; (ii) they had to be designed such that they could be used under identical PCR conditions; and (iii) amplified products had to be of various lengths in order to be distinguishable in multiplex PCR experiments. To fulfil these criteria, five sets of 22-mer primer pairs have been designed and analysed using the *Primer Premier* Version 3.1 software.

In a first step of selection, the primer GC content was selected to achieve an elevated melting temperature of 69 °C and above (see Table 2) and their Tm values were very close (69–72 °C) to avoid differential yields of the amplified

**Table 2.** 22-mer oligonucleotide primer pairs used for PCR amplification of sialyltransferases and GAPDH cDNA sequences.

| *Target cDNA* | *Primers set*[a] | *GC content (%)* | *Tm (°C)* | *ΔG (Kcal mol$^{-1}$)* | *PCR product size (bp)* |
|---|---|---|---|---|---|
| ST6Gal I | 5′-$_{849}$TATCGTAAGCTGCACCCCAATC-3′ | 50 | 69.1 | − 42.6 | 371 |
| | 3′-$_{1199}$GAAGGCCTGGTAAGTGACGATT-5′ | 50 | 69.1 | − 42.1 | |
| ST3Gal I | 5′-$_{361}$TCAGAGTGGTGCCTGGGAATGT-3′ | 55 | 71.2 | − 41.9 | 537 |
| | 3′-$_{877}$CGTTTCCCTTGACCGTGGTGAT-5′ | 55 | 71.2 | − 43.2 | |
| ST3Gal III | 5′-$_{752}$CGGATGGCTTCTGGAAATCTGT-3′ | 50 | 70.3 | − 42.9 | 300 |
| | 3′-$_{1031}$AGTTTCTCAGGACCTGCGTGTT-5′ | 50 | 70.2 | − 41.3 | |
| ST3Gal IV | 5′-$_{322}$CCCAAGAACATCCAGAGCCTCA-3′ | 55 | 71 | − 41.3 | 458 |
| | 3′-$_{759}$CTAATTCGTCTTCGGGTGGTGC-5′ | 55 | 71 | − 43.6 | |
| GAPDH | 5′-$_{278}$TGGAGTCCACTGGCGTCTTCAC-3′ | 59 | 70 | − 41.9 | 698 |
| | 3′-$_{964}$GTTGTCCCACCACCTGGAGTAC-5′ | 59 | 70 | − 41.9 | |

[a] The number on the left side of each sequence indicates the position of the first nucleotide in the coding sequence of each cDNA.

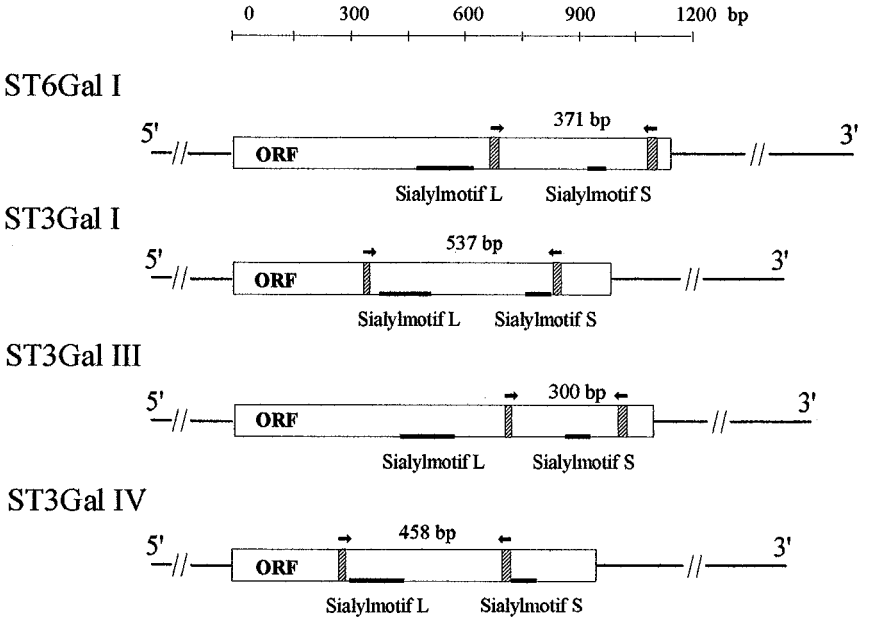


**Figure 1.** Schematic representation of the four sialyltransferases (ST6Gal I, ST3Gal I, ST3Gal III and ST3Gal IV) mRNA showing the open reading frame (ORF) and the position of the specific primer sets (hatching and arrows). The size of PCR products obtained are indicated in bp, above the ORF and sialylmotifs L and S are positioned with black lanes.

products. The internal stability (ΔG) for each primer was also chosen in the same range, being less than − 41.5 Kcal mol$^{-1}$. Secondly, primers for which dimerization within and between primers was stabilized by more than three bonds in the 3′ end were rejected. We also have discarded primers which folded back on themselves to form secondary structure loops stems stabilized by more than four bonds. Third, the selected pair of primers still had the potential to bind to other regions of the sialyltransferase sequences with high similarities leading to the production of secondary bands. In order to avoid false priming, primers showing more than three bonds at the 3′ end were also rejected.

Despite a very low overall sequence similarity (less than 50%), a comparison of cloned sialyltransferase amino acid sequences has shown highly conserved regions that have been termed sialylmotif L and S [51, 52]. These two regions are implicated in the binding of the sialyltransferase substrate CMP-NeuAc [53]. In order to be highly specific for each sialyltransferase cDNA, primer pairs were designed in the 3′ region of the open reading frame, outside these two sialylmotifs and they were located on distinct exons (Figure 1).

## RT-PCR is specific for each of the four human sialyltransferases

HepG2 cell line is a human hepatocarcinoma cell that is known to produce ST6Gal I mRNA [54], but also small amounts of ST3Gal I, ST3Gal III, ST3Gal IV mRNAs [45]. First strand cDNA were synthesized from HepG2 total RNA rather than poly(A)$^+$ mRNA to minimize the risk of losing the weakly expressed sialyltransferase mRNAs. Two µl of reverse transcription reaction (which represents 300 ng of total RNA) were used in conjunction with the various primer pairs designed previously in a positive control to assess the reliability of the RT-PCR method to amplify a specific cDNA fragment. Thirty-six cycles of amplification of this RT reaction gave rise to unique amplified PCR products of the expected length as was observed on ethidium bromide stained polyacrylamide gel shown in Figure 2 (lanes 2, 4, 6, and 8). No amplified fragment could be detected in the absence of cDNA templates (Figure 2; lanes 1, 3, 5 and 7). These different PCR fragments were subcloned in the pCR™ II vector and sequenced. The results of the sequencing reactions (data not shown) have indicated that the sequence of the different PCR fragments corresponded perfectly to the expected cDNA sequences, showing undoubtedly the specificity of the amplification technique. In addition, ST3Gal I, ST3Gal III, ST3Gal IV mRNAs are usually barely detectable by conventional hybridization methods such as Northern analysis [45] which requires large amounts of target RNAs. Here, this low level of expressed transcripts was specifically detected and amplified by RT-PCR from a small number of cultured cells.

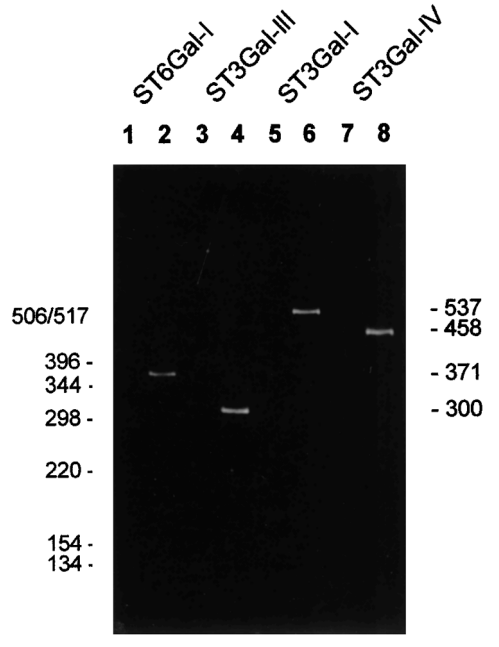


**Figure 2.** Ethidium bromide stained polyacrylamide gel of one-half of final products from separate RT-PCR amplification of four sialyltransferase fragments. The target cDNA was reverse transcribed from total cellular RNA derived from HepG2 cells (2 µg) using ST6Gal I (lanes 1 and 2), ST3Gal III (lanes 3 and 4), ST3Gal I (lanes 5 and 6), ST3Gal IV (lanes 7 and 8) specific primer pairs. Lanes 1, 3, 5, and 7 are the negative controls with no cDNA. Size markers (bp) appear on the left side of the gel picture.

We next sought to determine the optimal number of cycles required to visualize all PCR products on ethidium bromide stained polyacrylamide gel and to remain within the exponential phase of the amplification curve using a constant amount of input cDNA and identical reaction conditions for each independent tube. Densitometric analysis and integration of the sialyltransferase signals obtained for 26–36 cycles of amplification are shown in Figure 3 and clearly indicate that the four sialyltransferases are differentially expressed in HepG2 cells. The signal for ST6Gal I was of the highest intensity compared to those obtained for the other sialyltransferases, and was detectable after 22 cycles of amplification (data not shown). Saturation of the amplification reaction was reached after 30 cycles, after which PCR product increases at an unknown rate. Concerning the other sialyltransferases, amplification fragments could not be detected before 26 cycles and signals were not saturated up to 34 cycles of amplification. ST3Gal III specific signal was more intense than these of ST3Gal I and ST3Gal IV, this latter sialyltransferase mRNA being less expressed in HepG2 cells. Thus, the relative intensity of the various PCR signals obtained after 28 cycles of amplification truly reflects the initial abundance of the corresponding transcripts since PCR amplifications are proceeding in the exponential phase. These signals were analysed by computerized densitometric scanning of the picture obtained and our data indicated that ST6Gal I, ST3Gal I, ST3Gal III, and ST3Gal IV were expressed in a ratio of 5/2/3/1 relative to one another, in HepG2 cells.

## The specificity of primer pairs allows multiplex PCR

In PCR experiments, degradation of samples and pipetting errors can lead to variations from one tube to another. Therefore, to avoid intertube variability, we have developed a multiplex PCR method with the four primer pairs previously selected to obtain in a single reaction tube, a pattern of the expression of these enzymes, in a given cell type. We adapted the PCR conditions by increasing gradually the amount of Taq polymerase to remain in the exponential phase of the reaction, using as a control, the signal obtained for ST6Gal I from HepG2 cDNA. The prerequisite determination of an exponential range of amplification for all sialyltransferase sequences at once, was achieved by terminating the PCR at sequential cycles (data not shown). We found that 36 PCR cycles were necessary to co-amplify the four specific sialyltransferase fragments in a relative proportion comparable to that previously obtained in individual assays. Figure 4 lane 10 shows the electrophoretic profile of

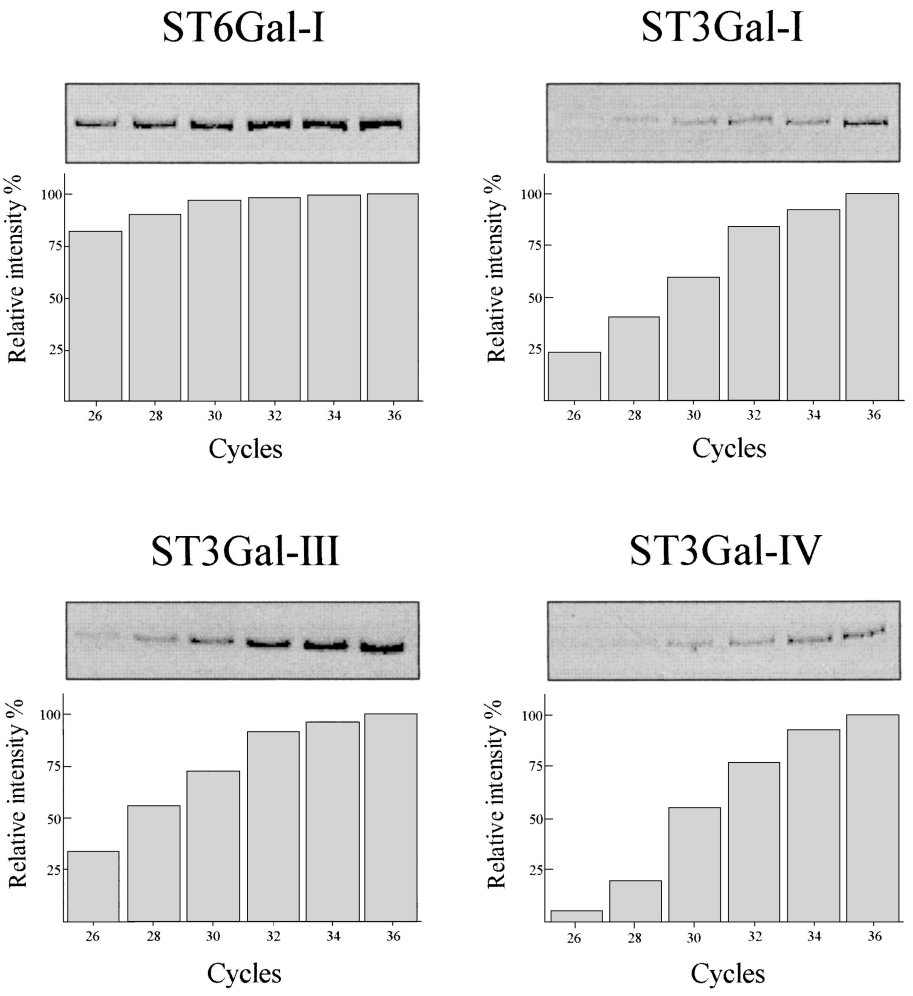


**Figure 3.** Kinetics of the amplification of the various sialyltransferase mRNAs isolated from the HepG2 cells. PCR products were run in 10% polyacrylamide gel, ethidium bromide stained and signals obtained were integrated by Quantiscan software (Biosoft International, Palo Alto, USA) at cycles 26, 28, 30, 32, 34 and 36.

the amplified products obtained for the simultaneous PCR amplification of the four sialyltransferase mRNA sequences present in HepG2 total RNAs. The initial step of reverse transcription of RNAs from various HepG2 sources could also be a source of variability hampering the standardization of inter-assay results. HepG2 total RNA samples that have undergone reverse transcription separately gave similar results (data not shown).

We chose glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which is a housekeeping gene as an endogenous internal control. Unfortunately, the simultaneous amplification of mRNAs initially present at widely different levels such as GAPDH and sialyltransferase mRNAs cannot provide the optimal condition of PCR linearity. Nevertheless, in experiments where specific fragments were co-amplified together with GAPDH, we observed that the fragments corresponding to the four sialyltransferases were obtained in the same ratio with, as expected, a large amount of GAPDH amplification fragment. So, this experimental approach in which multiple sialyltransferase cDNAs were simultaneously co-amplified allowed the precise monitoring of changes in mRNA levels relative to one another.

## Differential expression of sialyltransferase mRNAs in human breast cancer cells

An extensive literature describes the variations in sialyltransferase activities as one of the biochemical events accompanying physiological modifications such as cancer and invasive properties of cancer cells [9, 41, 43]. Although the exact mechanisms underlying control of sialyltransferase expression have not yet been characterized, there is a large body of evidence that their regulated expression is mainly controlled at the transcriptional level [for review see 24].

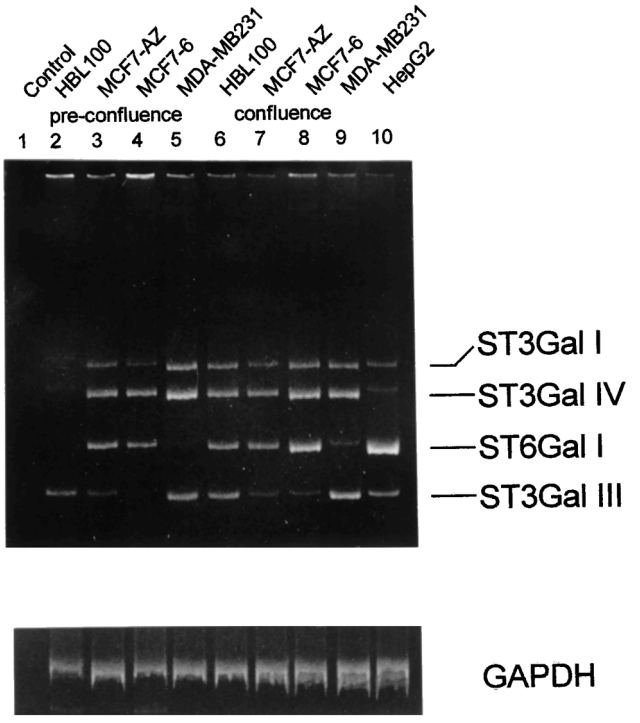


**Figure 4.** RT-PCR co-amplification of the four sialyltransferase cDNAs: ST6Gal I, ST3Gal I, ST3Gal III, ST3Gal IV, from the breast cells mRNA: HBL100 (lanes 2 and 6), MCF-7/AZ (lanes 3 and 7), MCF-7/6 (lanes 4 and 8), and MDA-MB-231 (lanes 5 and 9) at pre-confluence (lanes 2–5) and at confluence (lanes 6–9); and from HepG2 cell mRNA (lane 10). The products of 36 cycles of amplification performed according to Materials and Methods were run on 10% polyacrylamide gel and ethidium bromide-stained. The localisation of specific fragments for ST6Gal I (371 bp), ST3Gal III (300 bp), ST3Gal I (537 bp) and ST3Gal IV (458 bp) are indicated on the right side of the figure. Lane 1 corresponds to the negative control. The products of 30 cycles of amplification of the unregulated gene *GAPDH* are shown at the bottom of the Figure.

These changes in the expression of sialyltransferase genes can be detected with multiplex-PCR, a powerful tool providing useful diagnostic or prognostic information and eventually, may be related to a precise stage of the disease. We have thus used this simple, fast and accurate technique to survey sialyltransferase mRNA levels in a panel of human breast cell lines in which only ST6Gal I was barely detected by Northern analysis (data not shown). HBL100 is an epithelial cell line found to contain a tandemly integrated SV40 virus genome. Although there was no evidence of breast lesion in the cell donor, these cells should not be considered as normal cells [46]. MCF-7/AZ and MCF-7/6 clones were both derived from the breast adenocarcinoma cell line MCF-7 obtained from a pleural effusion [48] and differ in their invasiveness [47]. The breast adenocarcinoma MDA-MB-231 cell line was also isolated from a pleural effusion [49]. We have performed *in vitro* assays for cell–cell aggregation for these four cell lines according to Bracke *et al.* [55] (data not shown) which reflect their order of invasiveness relative to each other. Their invasiveness ranking could be stated as follows: MDA-MB-231 > MCF-7/6 > MCF-7/AZ > HBL100.

The expression pattern of the four sialyltransferases ST6Gal I, ST3Gal I, ST3Gal III and ST3Gal IV was analysed in total RNA extracted from these cultured cells at pre-confluence (70–80% of cell density) and at confluence. The multiplex PCR assays were performed in triplicate without significant changes in the amplification profiles, which demonstrates also the reliability of the method. One significant profile of the signals obtained after polyacrylamide gel is shown in Figure 4 and the relative intensities obtained after amplification of the transcripts of the different sialyltransferases are summarized in Table 3. The ratio obtained between the different sialyltransferase mRNAs detected are similar before or at confluence in all cell types, with the exception of HBL100 cells (see Figure 4 and Table 3). These latter cells express only ST3Gal III mRNA before confluence and produce equally the four sialyltransferase mRNAs at confluence (Figure 4, lanes 2 and 6). The two clones of MCF-7 (MCF-7/AZ and MCF-7/6) which differ in invasiveness capacity [47] present similar patterns of expression of sialyltransferases (Figure 4 and Table 3) and cannot therefore be distinguished on these criteria. The pattern of expression of sialyltransferases differs greatly between MCF-7 clones and MDA, even if these cell lines are all invasive. Finally, sialyltransferase cDNA levels detected at confluence are higher in MCF-7/AZ, MCF-7/6 and MDA than at pre-confluence.

These cultured breast cancer cells have proven to be a useful *in vitro* model in this study, although it is obvious that surveying larger numbers of different cancer cell lines

**Table 3.** Relative intensities obtained after multiplex RT-PCR analysis of sialyltransferases in human breast cell lines (Figure 4).

| | *Pre-confluence* | | | | *Confluence* | | | |
|---|---|---|---|---|---|---|---|---|
| | *HBL100* | *MCF-7/AZ* | *MCF-7/6* | *MDA-MB231* | *HBL100* | *MCF-7/AZ* | *MCF-7/6* | *MDA-MB231* |
| ST3Gal I | − | + | ± | +++ | ++ | + | +++ | +++ |
| ST3Gal IV | − | +++ | +++ | +++++ | +++ | +++ | +++++ | +++++ |
| ST6Gal I | − | +++ | +++ | − | +++ | +++ | +++++ | ± |
| ST3Gal III | + | ± | − | +++ | +++ | ± | ± | +++++ |

and breast cancer tissue samples will be necessary to establish good correlation between variations in sialyltransferase mRNA levels and their invasiveness.

In spite of the significant changes of the levels of sialyltransferase mRNA detected in these cells, no good correlation could be established either with sialylation patterns previously observed in these cells, nor with the sialyltransferase enzymatic activities. This is due in part to the fact that available data on the sialylation of breast cancer cell glycans are still fragmentary and based most of the time on the binding of lectins and antibodies [44, 56, 57]. One of the best documented examples is the abnormal occurrence of short sialylated glycan structures (sialyl-Tn, sialyl-T and disialyl-T antigens) on the cell-surface mucin MUC1 in cancer cells [44, 58]. These alterations have been correlated with a higher Gal$\beta$1-3GalNAc $\alpha$2,3-sialyltransferase activity [43]. This unique activity could be supported by at least three different enzymes: ST3Gal I, ST3Gal II and ST3Gal IV, which are not distinguishable based on enzymatic assays. Northern blot analysis showed no increased ST3Gal I mRNA level [43] and our multiplex RT-PCR data showed that, at confluence, ST3Gal I and ST3Gal IV were amplified at similar levels in the different cell lines. ST3Gal II has been recently cloned from different species [59–61] and could also be responsible for that increased activity.

Lewis type antigens $SLe^x$ and $SLe^a$ have been observed in MUC1 isolated from MDA-MB-231 [44]. More generally, $SLe^x$ is considered as a tumour associated antigen in breast cancer [62]. This could be associated with the high level of expression of ST3Gal III and ST3Gal IV observed by RT-PCR in this cell line, enzymes that are implicated in the biosynthesis of $SLe^a$ and $SLe^x$ respectively.

## Concluding comments

We have reported in this paper the use of a sensitive RT-PCR method to detect and specifically amplify cDNAs of various cloned $\alpha$2,3- and $\alpha$2,6-sialyltransferases implicated in the biosynthesis of human cancer related sialylated epitopes found on N- and O-glycosylproteins.

The method we have developed is particularly interesting because: (i) it will provide a new alternative to measure precisely sialyltransferase mRNA levels in very small samples of human breast carcinoma cells or biopsies. These appear as potential early indicators of the changes occurring in human cancers. (ii) It will be easily extended to other human sialyltransferase cDNAs already cloned such as $\alpha$2,8-sialyltransferases involved in $G_{D3}$ biosynthesis (ST8Sia I) or in $\alpha$2,8-polysialylated chains (ST8Sia II and ST8Sia IV). High levels of expression of the ST8Sia I gene as well as $G_{D3}$ have been described in human melanoma cell lines and melanoma tissues [63], whereas normal melanocytes express $G_{D3}$ at minimal levels [64]. In mammals, polysialic acid (PSA) occurs almost uniquely on N-linked chains of the neuronal cell adhesion molecule (N-CAM) and on the $\alpha$-subunit of sodium channels in the brain. However recently, PSA has been observed on proteins in rat basophilic leukemia cells and in MCF-7 human breast cancer cells [65]. $\alpha$2,8-sialylation of $G_{D3}$ and/or N-glycosylproteins in these cancer cells, could be due to the oncogenic expression of the $\alpha$2,8-sialyltransferases and serve as good markers of malignancy.

Changes in O-glycan sialylation also occur in human pathogenesis. Sialyl-Tn antigen (NeuAc$\alpha$2-6GalNAc$\alpha$1-*O*-Ser/Thr) and Sialyl-T antigen (NeuAc$\alpha$2-3Gal$\beta$1-3GalNAc$\alpha$1-*O*-Ser/Thr) expressions have been shown to be up-regulated in many transformed cells [9, 42], and serve as markers of malignancy in breast cancer [66]. As yet, no human cDNA sequence has been obtained for ST6GalNAc I, the enzyme which has been proposed to be responsible for the synthesis of Sialyl-Tn antigen. On the other hand, three different enzymes could participate in the biosynthesis of sialyl-T antigen: ST3Gal I, ST3Gal II, and to a lesser extent, ST3Gal IV. Previously cloned human ST3Gal I and ST3Gal IV sequences have been used in this study, whereas a human ST3Gal II cDNA clone was not available during the time course of this study. We expect that, in the near future, these human sialyltransferase cDNA sequences will be available and we are currently working on refining this RT-PCR method to allow assessment of mRNA levels for sialyltransferases of interest in clinical samples of breast cancer.

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