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Regulation of expression of β-galactoside α2,6-sialyltransferase in a rat tumor, Zajdela ascitic hepatoma

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Tumor cell surface sialic acid levels determine a number of important properties governing cellular interactions and cell–cell communication. Towards understanding the mechanism of regulation of sialic acid levels upon cellular transformation, we have studied the regulation of expression of β-galactoside α2,6-sialyltransferase in a rat tumor, the Zajdela ascitic hepatoma. We demonstrate distinct differences in the regulation of expression of the enzyme in the tumor cells as compared to normal liver cells. The expression of sialyltransferase is regulated both at the transcriptional and post-transcriptional level in a tissue-specific manner.

β-Galactoside α2,6-sialyltransferase; Zajdela ascitic hepatoma; Regulation of expression; Sialic acid level

1. INTRODUCTION

Sialic acids play an important role in a variety of biological processes like cell–cell communication, cell–substrate interaction, adhesion, maintenance of serum glycoproteins in circulation, and protein targeting [1–6]. Cell surface sialic acid levels change in a regulated manner during development, differentiation, and oncogenic transformation [7–9]. In oncogenic transformation, cell surface sialic acids play an important role in determining interactions between tumor cells and host cells, tumor cell immunogenicity and metastatic potential of the tumor cells [10–13].

Very little is known about the regulation of sialic acid levels on the cell surface consequent to cellular transformation. Sialic acids occur at the terminal position on the carbohydrate groups of glycoproteins and glycolipids. The transfer of sialic acid to these positions occurs post-translationally, catalyzed by a family of glycosyltransferases called sialyltransferases [14,15]. More than 10–12 different sialyltransferases are required to synthesize all the known sialo-oligosaccharide structures [7,16]. It has been suggested that terminal glycosylation sequences present in a cell are determined by the specificity of glycosyltransferases which are present in the Golgi apparatus of the cell [17]. There are only a few studies that have been made on the regulation of sialyltransferase levels. Only a few sialyltransferases have been purified and only one enzyme has been cloned [7]. The cloning of the β-galactoside α2,6-sialyltransferase from rat liver [16] has now made it possible to examine the regulation of this enzyme upon cellular transformation.

In the present study, we have used a chemically induced rat tumor cell line, Zajdela ascitic hepatoma (ZAH), to study the regulation of expression of β-galactoside α2,6-sialyltransferase. Here we report our observations on the regulation of mRNA levels of this sialyltransferase in ZAH cells as compared to that in normal liver cells.

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Abbreviations: ZAH, Zajdela ascitic hepatoma; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.

2. MATERIALS AND METHODS

2.1. *Materials*

Cycloheximide, Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were obtained from Sigma Chemical Co., restriction enzymes from New England Biolabs and Bethesda Research Laboratories, and Zetaprobe membrane from Bio-Rad. [α^{32}P]dATP and [α^{32}P]UTP were from Jonaki, BARC, India, and [^{14}C]CMP-sialic acid was from Amersham.

2.2. *Tumor cell line*

The ZAH cell lines were obtained from Dr. F. Zajdela, Institute de Radium, Orsay, France [18]. The tumor was originally induced with dimethylaminoazobenzene [19]. Tumors were maintained as ascites by serial transplantation of 0.5 ml of the ascitic fluid taken from a five-day-old tumor. Two- to three-months-old CFY rats were used throughout this study and the colony was propagated by sibling mating. The results reported throughout this paper are on ZAH C, the cell line that was induced and maintained in female rats. The ZAH D cell line, induced and maintained in male rats, was also used.

2.3. *Cell culture conditions*

ZAH cells were harvested from a five-day-old tumor, washed with

PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, pH 7.2), and cultured in DMEM containing 2.5% FCS at a density of one million cells/ml.

Primary hepatocytes were prepared from two-month-old rats by the collagenase perfusion method [20] with slight modifications. Briefly, liver was perfused in situ with 0.05% collagenase in Ca-PBS. Free hepatocytes were released by gently shaking the perfused liver in the same buffer on a rotatory shaker. The cell viability was more than 95% as determined by the Trypan blue exclusion assay. The cells were cultured in DMEM containing 2.5% FCS.

2.4. *Assay of enzyme activity*

β-Galactoside α2,6-sialyltransferase activity was estimated by the method of Wang et al. [21] with slight modifications. The only differences were that assays were carried out in 100 μl reaction mixtures using 50 nCi [^{14}C]CMP-sialic acid (262 mCi/mmol) and sodium phosphate buffer (0.25 mM, pH 7.0). One unit of the enzyme activity is defined as one pmol of [^{14}C]sialic acid transferred per hour per mg of protein. The protein concentration was determined by Folin's procedure as modified by Peterson [22].

2.5. *Sialyltransferase probe preparation*

The pST1 plasmid having the sialyltransferase clone was a kind gift from Dr. James C. Paulson. The plasmid contains a 717 bp insert from near the 5′ end of the cDNA clone of rat liver β-galactoside α2,6-sialyltransferase in pBR322 [16]. For DNA and RNA analysis the plasmid was radio-labeled by nick translation using [α-^{32}P]dATP to a specific activity of 10^8 cpm/μg using a nick translation kit from Amersham according to the manufacturer's instructions.

2.6. *Nucleic acid isolation and analysis*

DNA was isolated, restricted, and subjected to Southern blot analysis according to standard procedures [23].

RNA was isolated according to the method of Chomczynski and Sacchi [24]. Poly(A)+ RNA was prepared by affinity purification on an oligo-dT-cellulose column [23]. The RNA was size fractionated on formaldehyde agarose gels [23], blotted onto Zetaprobe membranes and probed according to the manufacturer's instructions. The final washing of the blots was done in 0.1 × SSPE (1 × SSPE is 180 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.7) at 65°C. The blots were exposed to X-ray film with an intensifying screen at −70°C.

For dot blots, aliquots containing 5, 2.5 and 1.25 μg of total RNA were spotted onto Zetaprobe membranes after denaturation with an equal volume of a solution containing 20 × SSPE and formaldehyde in the ratio of 60:40 at 65°C for 15 min. The blots were processed as described above.

Densitometric scanning was done using a Molecular Dynamics Computing Densitometer and intensities of spots were determined by volume integration using Image-Quant software.

2.7. *Cycloheximide treatment*

For in vivo cycloheximide treatment, rats were injected with 50 mg cycloheximide per kg body weight [25]. For in vitro cultured cells, the cells were allowed to adapt to the culture medium for 48 h following which cycloheximide was added to a concentration of 10 μg/ml [21].

2.8. *Nuclear run-on transcription assays*

The nuclear run-on transcription assays were carried out essentially as described by Greenberg [26]. Each assay mixture contained 400 μCi of [α-^{32}P]UTP (3000 Ci/mmol). The plasmid DNA samples were linearized by using appropriate restriction enzymes. After denaturation 10 μg aliquots of DNA were spotted per slot. Hybridization and washings were done at 65°C.

3. RESULTS

3.1. *Expression of β-galactoside α2,6-sialyltransferase*

Determination of the sialyltransferase activity in ZAH and normal liver cells showed that while normal liver cells contained 13.3 ± 2.1 units of activity, the tumor cell lines had no detectable enzyme activity. Northern blot analysis of mRNA samples from the different cell types showed that the transcript size in ZAH cells (Fig. 1a) was slightly larger than in normal liver cells. Furthermore, densitometric scanning showed that levels of the sialyltransferase mRNA from ZAH cells was 14.8-fold less when compared to normal liver cells (Fig. 1b).

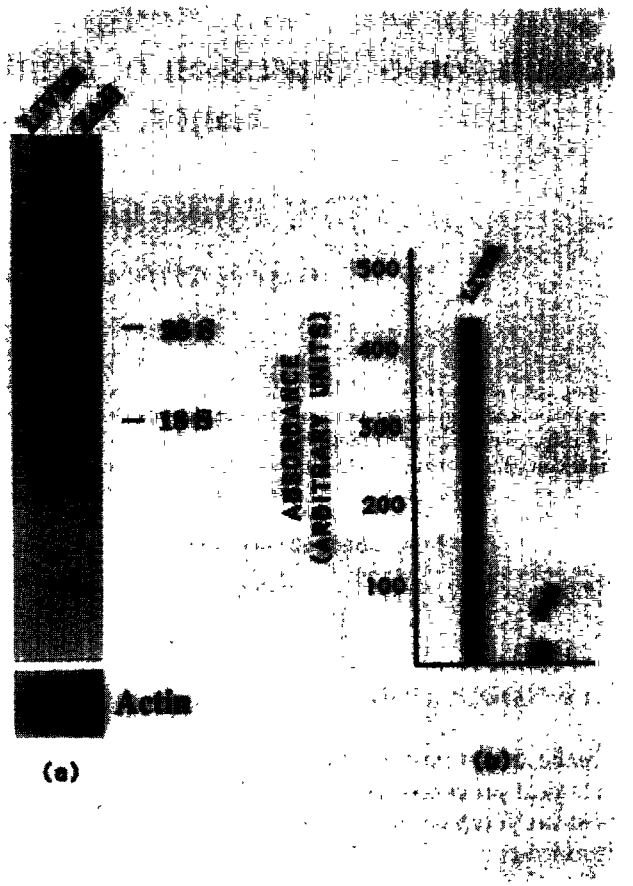

Fig. 1. (a) Northern blot showing the level and size of the sialyltransferase transcript in normal liver and ZAH cells. 10 μg of poly(A)+ RNA was loaded in each lane and probed with the pST1 partial cDNA clone (for details see section 2). (b) Densitometric scan of the blot shown in Fig. a.

3.2. *Southern blot analysis*

DNA from normal liver and ZAH cells was restricted with *Eco*RI and *Hin*dIII enzymes and subjected to Southern blot analysis using the sialyltransferase probe. No difference was observed in the blots obtained using DNA from liver cells or from the tumor cell lines (Fig. 2). This observation indicates that no alteration in the gene, like translocation or amplification, is responsible for the observed difference found in the sialyltransferase mRNA levels in ZAH and in liver cells.

3.3. *Nuclear run-on transcriptional analysis*

In order to examine if the sialyltransferase expression is regulated at the transcriptional level, we carried out nuclear run-on transcription assays on the nuclei isolated from ZAH and liver cells (Fig. 3a). Densitometric scanning of the hybridization intensity showed that the transcription rate in liver cells was 3.6-fold higher than in ZAH cells (Fig. 3b), suggesting that the difference in the mRNA population in the two cell types could be a consequence of transcriptional regulation. The control hybridization with the actin plasmid showed no major difference in the transcription rate of the actin gene between the two cell types though there are large differences in the level of mRNA (cf. Fig. 1a). These results are similar to those reported earlier [27,28].

3.4. *Effect of cycloheximide on the levels of β-galactoside α2,6-sialyltransferase mRNA*

Dot blot experiments to determine the sialyltransferase mRNA levels in liver cells, and in ZAH cells under in vivo and in vitro conditions in the presence of cycloheximide were carried out (Fig. 4). The results show dramatic differences in the levels of mRNAs. The in vivo experiments showed a 3-fold decrease in the mRNA level in liver cells as a consequence of cycloheximide treatment while there was a 16-to-17-fold increase in the level of mRNA in ZAH cells. When ZAH cells were isolated from the ascitic tumor, washed and kept in culture for 48 h, the sialyltransferase mRNA levels showed an increase (Fig. 4) as compared to the mRNA level in freshly harvested cells. When ZAH cells in culture were treated with cycloheximide, unlike in the case of in vivo experiments, there was a 3-fold decrease in the sialyltransferase mRNA, much the same as in the case of liver tissues. The liver cells maintained in culture as primary hepatocytes for 48 h did not show any change in mRNA levels as compared to mRNA levels in liver tissues from untreated animals (Fig. 5).

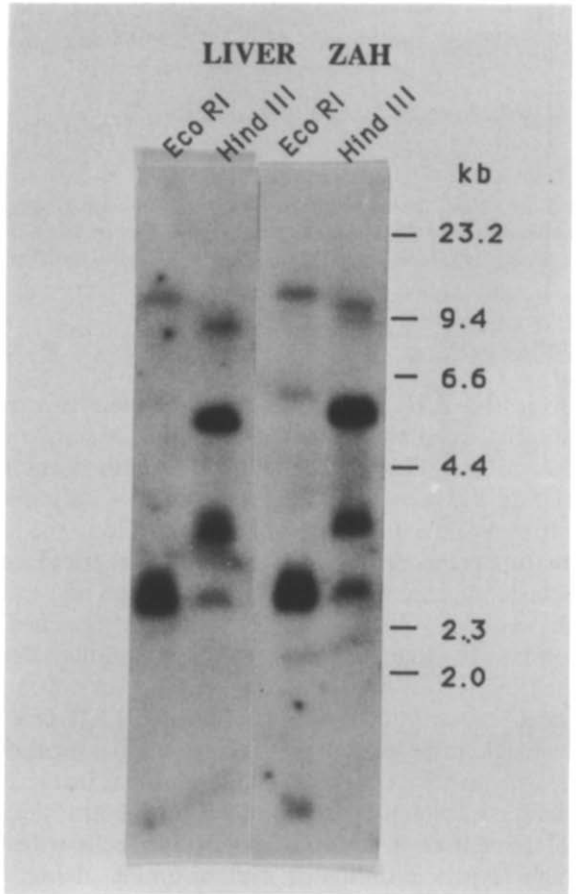

Fig. 2. Southern blot of *Eco*RI- and *Hin*dIII-digested liver and ZAH DNA. 10 μg of digested DNA was loaded on each lane and probed with the pST1 sialyltransferase partial cDNA clone.

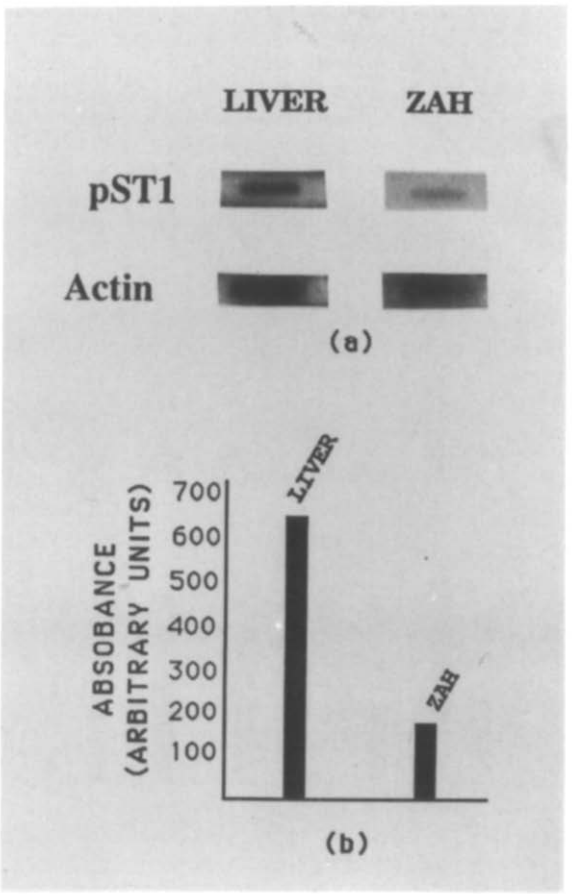

Fig. 3. (a) Nuclear run-on transcription assay showing the level of transcription of sialyltransferase gene in isolated liver and ZAH nuclei. (b) Densitometric scanning of run-on transcript level of sialyltransferase shown in Fig. 1a.

4. DISCUSSION

We have studied the role of alterations in cell surface glycosylation in cellular transformation using ZAH as a model system. We have used this cell line to study the mechanism of regulation of expression of β-galactoside α2,6-sialyltransferase in comparison with that in normal liver cells.

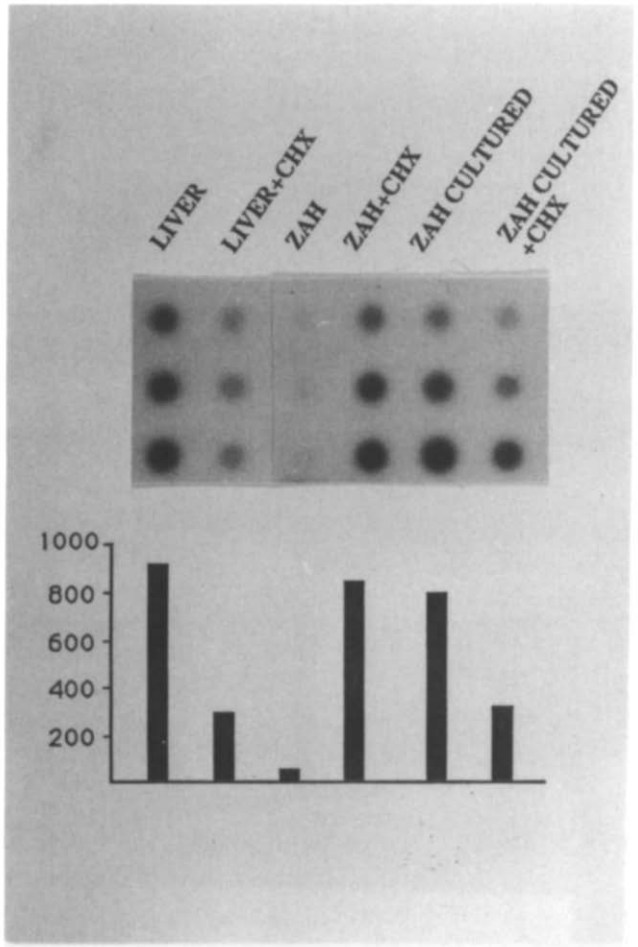

Fig. 4. Dot blot showing the effect of cycloheximide on sialyltransferase RNA in normal liver and ZAH cells in vivo and on ZAH cells in culture (for details see section 2). The bottom panel shows a densitometric scan of the blot. Note changes in basal RNA levels when ZAH cells are put in culture. CHX, cycloheximide.

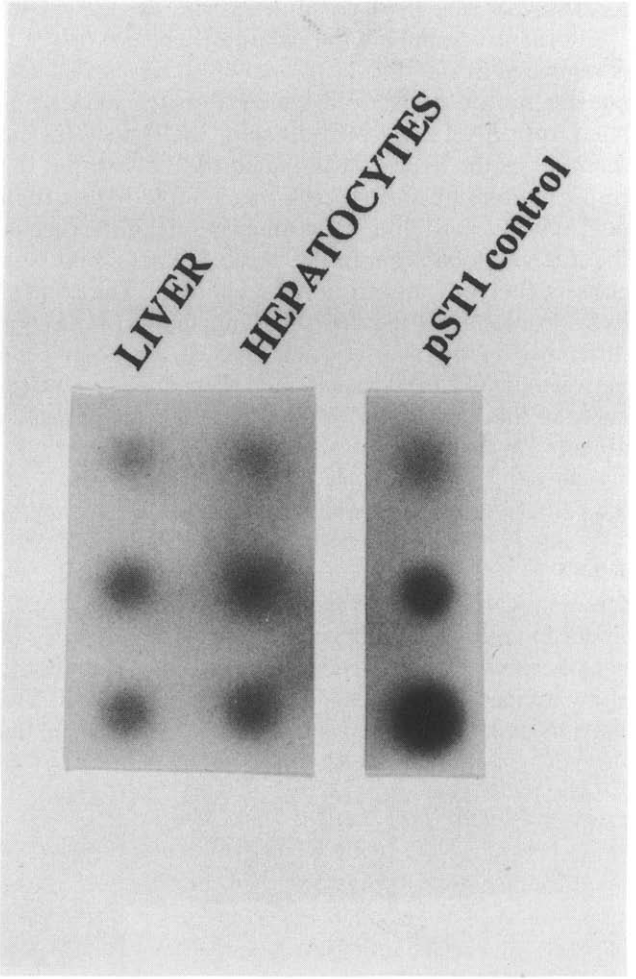

Fig. 5. Dot blot showing that there is no change in sialyltransferase RNA levels when liver cells are cultured as primary hepatocytes. The lane on the right shows hybridization with 10, 20 and 40 pg of control pST1 plasmid.

Quantitative determination of the levels of the sialyltransferase activity showed no detectable amount of the enzyme in the ZAH cell line. Northern blot hybridization using mRNA from liver and the ZAH cells showed that down-regulation of the sialyltransferase activity in the tumor cells is due to a reduction at the mRNA level. It was also observed that the transcript size of ZAH cells (4.7 kb) is slightly larger than that in normal liver cells (4.3 kb) and corresponds to that reported for tissues other than liver, such as brain, heart, lung and spleen [17,29,30]. It has been proposed that these multiple transcripts are generated in a tissue-specific manner from the same gene by alternative splicing [29,30]. This could also hold true for ZAH, since there is no difference in the restriction pattern of the DNA between ZAH and normal liver cells as determined by Southern blots.

Nuclear run-on transcription assays showed a difference in the level of transcripts generated from the nuclei of liver and ZAH cells indicating that there is a transcriptional regulation of the expression of sialyltransferase mRNA. However, the difference in the transcription rate between the two cell types was only about 3.5-fold, while a difference of about 15-fold in the level of mature transcript was observed in Northern blots. It has been suggested that when the changes in steady-state levels of a particular mRNA are not matched by a comparable change in de novo transcription as determined by nuclear run-on assays, it gives an indication of post-transcriptional gene regulation [31,32]. Our results would indicate that post-transcriptional regulation of sialyltransferase mRNA is an additional factor controlling the level of expression of α2,6-sialyltransferase.

Treatment of ZAH and normal liver cells with the protein synthesis inhibitor cycloheximide altered the levels of sialyltransferase mRNA, suggesting that the maintenance of the steady-state levels of the mRNA in liver and ZAH cells requires continued protein synthe-

sis. Interestingly, upon treatment with cycloheximide there was an increase in the level of sialyltransferase mRNA in ZAH cells and a decrease in the case of liver, indicating that the regulation of expression of the sialyltransferase mRNA occurs in a tissue-specific manner. Our results are in contrast with the observations on the levels of this sialyltransferase in H35 hepatoma cells upon treatment with protein synthesis inhibitors [21]. In the H35 hepatoma system, protein synthesis inhibitors have no effect on mRNA levels. It is likely that in the H35 hepatoma, sialyltransferase mRNA is regulated through different mechanism(s). An increase in the level of message upon cycloheximide treatment in ZAH cells could results if any protein factor(s) with a short half-life were involved in either increasing the mRNA stability or negatively regulating the transcription. Direct determination of half-lives of mRNA in ZAH and liver cells is not possible either by the pulse-chase method (which is suitably only for abundantly expressed mRNAs [31,32]) or by using actinomycin D [33].

Interestingly, when ZAH cells were cultured, there was an increase in the level of sialyltransferase mRNA. Changes in the level of the sialyltransferase mRNA in ZAH cells upon short-term culture complements our observation that there are large alterations in the cell-surface sialic acids in ZAH cells upon culturing (manuscript in preparation). When ZAH cells in culture were subjected to cycloheximide treatment, the effect observed was similar to that observed for liver cells in the sense that there was a decrease in the level of transcripts. In contrast, in cultured primary hepatocytes there was no change in the level of the sialyltransferase mRNA level on treatment with cycloheximide as has been demonstrated earlier [21]. It is likely that the milieu in which the cells are present could have an influence on the regulation of expression of the sialyltransferase gene in a tissue-specific manner. This is possible since it has been proposed that transcripts of different lengths (4.3 kb and 4.7 kb) are generated in a tissue-specific manner from different promoters [17,30], and hence regulated by different transcription factors.

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