Characterization of P1 promoter activity of the β-galactoside α2,6-sialyltransferase I gene (*siat 1*) in cervical and hepatic cancer cell lines

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The level of β-galactoside α2,6-sialyltransferase I (ST6Gal I) mRNA, encoded by the gene *siat1,* is increased in malignant tissues. Expression is regulated by different promoters – P1, P2 and P3 – generating three mRNA isoforms H, X and YZ. In cervical cancer tissue the mRNA isoform H, which results from P1 promoter activity, is increased. To study the regulation of P1 promoter, different constructs from P1 promoter were evaluated by luciferase assays in cervical and hepatic cell lines. Deletion of a fragment of 1048 bp (−89 to +24 bp) increased 5- and 3-fold the promoter activity in C33A and HepG2 cell lines, respectively. The minimal region with promoter activity was a 37 bp fragment in C33A cells. The activity of this region does not require the presence of an initiator sequence. In HepG2 cells the minimal promoter activity was detected in the 66 bp fragment. Sp1 (−32) mutation increased the promoter activity only in HepG2 cells. HNF1 mutation decreased promoter activity in HepG2 cell line but not in C33A cells. We identified a large region that plays a negative regulation role. The regulation of promoter activity is cell type specific. Our study provides new insights into the complex transcriptional regulation of *siat1* gene.

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1. Introduction

Malignant transformation is frequently accompanied by altered glycosylation of cell surface glycoproteins and glycolipids (Dall'Olio 1996; Meany and Chan 2011). Cancer cells contain mainly sialylated structures. Sialic acid is transferred to glycoconjugates by sialyltransferases and the expression level of cell surface sialic acids are mainly related to mRNA level of the sialyltransferase genes (Harduin-Lepers *et al.* 2001). Previous studies have shown that the level of β-galactoside α2,6-sialyltransferase I (ST6Gal I) mRNA is increased in premalignant and malignant cervical tissues compared with normal cervical tissue (Wang *et al.* 2002; López-Morales *et al.* 2009). In cervical cancer, the enhanced ST6Gal I mRNA level is associated with poor differentiation, deep stromal involvement and lymph vascular invasion (Wang *et al.* 2002). The ST6Gal I is encoded by the gene *siat1*. The s*iat 1* gene spans at least 145,000 bp of genomic DNA and comprises 9 exons. Translation initiation codon is in exon II; the upstream exons I, X, Y and Z are 5′-untranslated (UT) regions that contribute to the 5′-UT heterogeneity (Lo and Lau 1996a). Expression is regulated through the use of different promoter regions: P1, P2 and P3. Three mRNA isoforms generated by these promoters have been identified (Wang *et al.* 1990; Svensson *et al*. 1992): (1) a short form isolated from liver called form 1 or H that lacks exons Y, Z

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and X but contains a short sequence adjacent to exon I, (2) a form isolated from human B cells denominated form 2 or X because it contains exon X, and (3) a large transcript denominated form 3 or YZ that contains exons Y and Z (Stamenkovic *et al.* 1990; Aasheim *et al.* 1993; Wang *et al.* 1993; Aaas-Eng *et al.* 1995). These isoforms have been detected in cervical tissue. Isoform YZ was detected at similar levels in normal and cancerous tissues. X isoform was detected in both normal and cancer tissue, but the detection was higher in malignant tissue. Form H was also increased in malignant tissue (Wang *et al.* 2003). It has been demonstrated that H mRNA isoform has a tendency to also accumulate in colon cancer (Dall'Olio *et al.* 2000). Transcriptional regulation of form H mRNA depends on P1 promoter activity, suggesting that promoter-specific transcription factors may be related to neoplastic transformation.

Promoter P1 has been previously characterized in colon and hepatic cell lines (Xu *et al.* 2003). The nt −156 to −1 region is important for transcriptional activity. This region contains a HNF1 recognition element. Mutation of the HNF1 site reduces the promoter activity, suggesting that the HNF1 site is involved in the transcription of mRNA isoform 1 in colon and hepatic cancer cells (Xu *et al.* 2003). The 5′-flanking region of P1 promoter contains putative transcriptional factor binding sites such as AP1, NF-IL6 and HNF1. This promoter does not have TATA and CCAAT boxes. These promoters are characterized for the presence of transcriptional initiator (Inr) sites that function as TATA boxes. In addition, Sp1 can direct accurate transcription initiation of TATA-less promoters. Two initiator sequence regions have been identified in the P1 promoter. These sequences are required for transcription initiation in some TATA-less gene promoters (Lo and Lau 1996b).

In this study we identified the minimal promoter region in cervical C33A and hepatic HepG2 cancer cell lines. We also determined that the HNF1 site is important for the promoter activity in HepG2 cells but not for the C33A cervical cell line. Using in silico analysis, we identified a third initiator sequence that can participate in the transcriptional activity of P1 promoter in HepG2 cells. Meanwhile, in C33A cells, promoter activity is maintained without the three initiator sequences.

2. Methods

2.1 *Cell cultures*

Human hepatocellular carcinoma (HepG2) and cervical carcinoma (C33A) cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C with 5% CO_2 atmosphere.

2.2 *Plasmid construction*

For the preparation of reporter constructs, the pGL3-basic vector containing the *siat1*P1 promoter (1137 bp) (kindly donated by Dr A Taniguchi, National Institute for Materials Science) was digested with *Kpn*I and *Hin*dIII restriction enzymes. The digestion products were electrophoresed in agarose gel and the 1137 bp band was purified and cloned into the *Kpn*I and *Hin*dIII sites of the pGL4.12 (*luc2CP*) (Promega, Madison, WI), a promoter-less and enhancer-less vector. In order to determine the various minimal promoter lengths of DNA fragments upstream of the initiating transcription site, the *siat1* gene was amplified by PCR, digested and inserted into luciferase reporter vector pGL4.12. To eliminate a 5′ fragment of 1014 bp, the *siat1* promoter was digested with *Bgl*I. The product was treated with nuclease S and ligated to obtain the construction pGL-123P1. Other series of constructions were obtained amplifying the promoter fragment by PCR using PCR Master Mix (Promega) along with 0.4 µM of each primer in a final volume of 50 µL. Oligonucleotides were pGL-89P1 5′-GCCTAGGTCT GCATA***GGTACC***TTTCCATC-3′, pGL-66P15′-CTTGTTAATG***GGTACC***ACTGCCTCTGC-3′ and pGL-37P1 5′-GGAAAACAGCT***GGTACC***TCCTCTCTTTC-3′; reverse primer was the same for all constructions (5′-CCAACAGTACCGGAWTGCC***AAGCTT***-3′). The underlined nucleotides represent the restriction sites for *Kpn*I (forward) and *Hin*dIII (reverse) that were incorporated into the primers. All PCR experiments were conducted in the PTC-200 (MJ Research, Watertown, MA).The identity of the products was verified by sequence analysis. Site-directed mutagenesis was created using each promoter plasmid construct as template DNA. Synthetic primers (26–32 bases in length) containing mutation sites in the middle of their sequence were used. To create these mutations, reverse PCR reactions were performed using, for the HNF1 mutation, the forward primer 5′-CTTTCCATCTT GTTAAGCTTTAACACTGCCTC-3′ and reverse primer 5′- GAGGCAGTGTTAAAGCTTAACAAGATGGAAAG-3′; for Sp1 (-32 bp) site the forward primer 5′-GTTTAACAC***GAGCTC***TGCTGGAAAAC-3′ and reverse primer 5′-GTTTTCCAGCA***GAGCTC***GTGTTAAAC-3′, for Inr(-9) site 5′-CTCTCTTTCTGTCTCGAGTT TTTTGCCTTTG-3′ and 5′-CAAAGGCAAAAAACTCG AGACAGAAAGAGAG-3′, and for Inr(+5) site 5′-GGAAAACAGCTCTCGGATCCTCTCTTTC-3′ and 5′-GAAAGAGAGGATCCGAGAGCTGTTTTCC-3′, using 40 ng of template DNA, 0.3 µM of each primer and 2 U/µL Pfu DNA polymerase (Fermentas, Burlington, ON) with initial denaturation of 2 min at 95°C followed by 35 cycles of 1 min at 95°C, 30 s at 50–56°C and 9 min at 72°C. PCR products were treated with DpnI (Promega) at 37°C for 1 h in order to eliminate the original non-mutated plasmid.

2.3 *Transient transfection and luciferase assay*

For transfection assays, HepG2 and C33A were cultured in 24-well plates. C33A cells were co-transfected when cells reached 60% confluence using the calcium phosphate precipitation method with 800 ng of the constructions and 20 ng of an internal reference plasmid (pGL4.72 *hRlucCP,* Promega). HepG2 were transfected by lipofection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were lysed 24 h after transfection, and luciferase activity was determined using the commercially available Dual Luciferase Reporter Assay System (Promega) and a luminometer Glomax 20/20 (Promega). Reporter activity in each lysate was normalized to the co-transfected *Renilla* luciferase activity. All transfections were performed in triplicate in at least three different assays. Statistical significance was determined using Student's *t*-test; $p<0.05$ was considered statistically significant.

3. Results

3.1 *In silico analysis of P1 promoter*

We performed an in silico analysis of P1 promoter region to identify putative transcription factor binding sites that may be important in the regulation of gene expression. We used different promoter analysis software: TESS (Transcriptor Element Search), Sofberry (nsite) and Patch. We identified putative response elements such as YY1, GATA, c-myc, c-Ets-1, Sp1, TFII-I, LBP1, CHOP and one transcriptional initiation sequence at position +5 bp. This sequence has

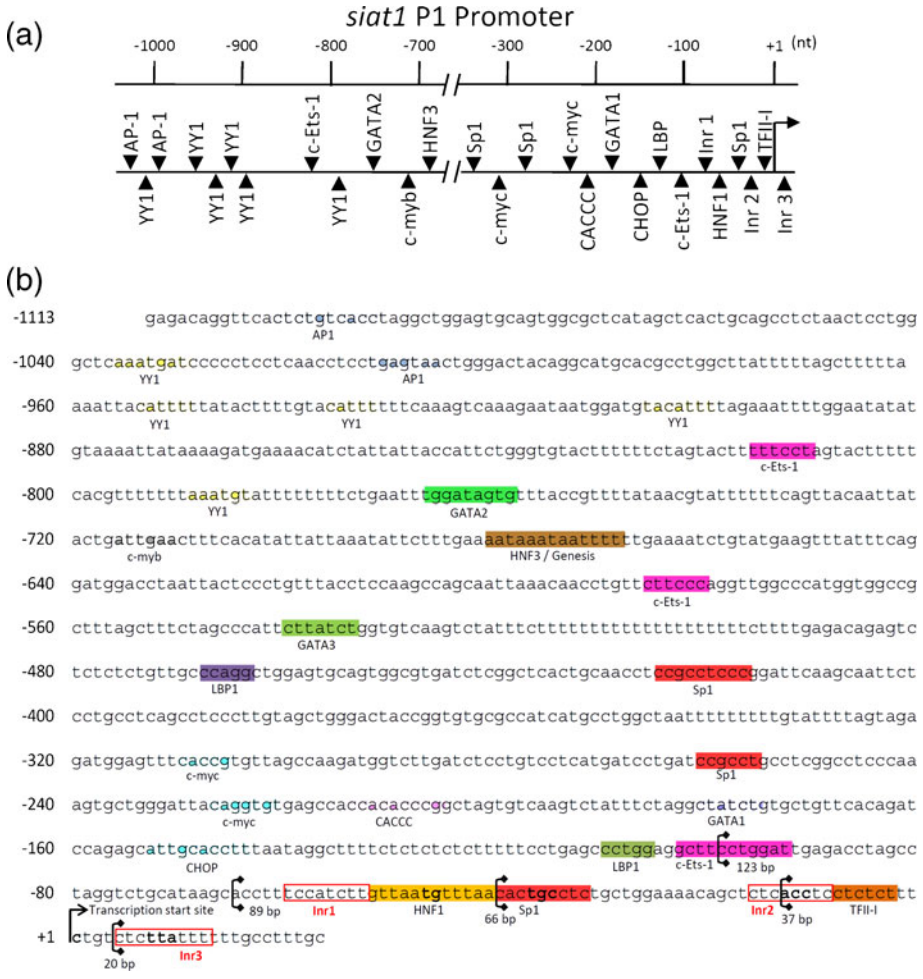

Figure 1. (**a**) Schematic representation of P1 promoter is shown. (**b**) Nucleotide sequence of *siat1* P1 promoter region. Potential regulatory elements are denoted by coloured boxes. Initiator sequences are shown by open boxes. Transcriptional start site is marked with an arrow. Nucleotides are numbered with the transcription initiation site designated as +1. The different promoter regions included in the constructs are also shown.

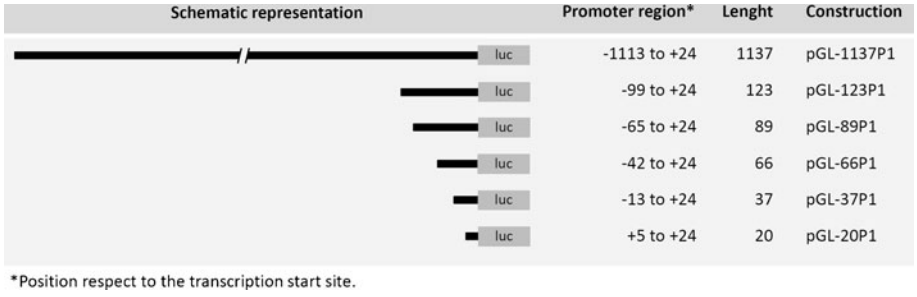

Figure 2. 5′-Deletion mutants of *siat1* P1 promoter and the construct name.

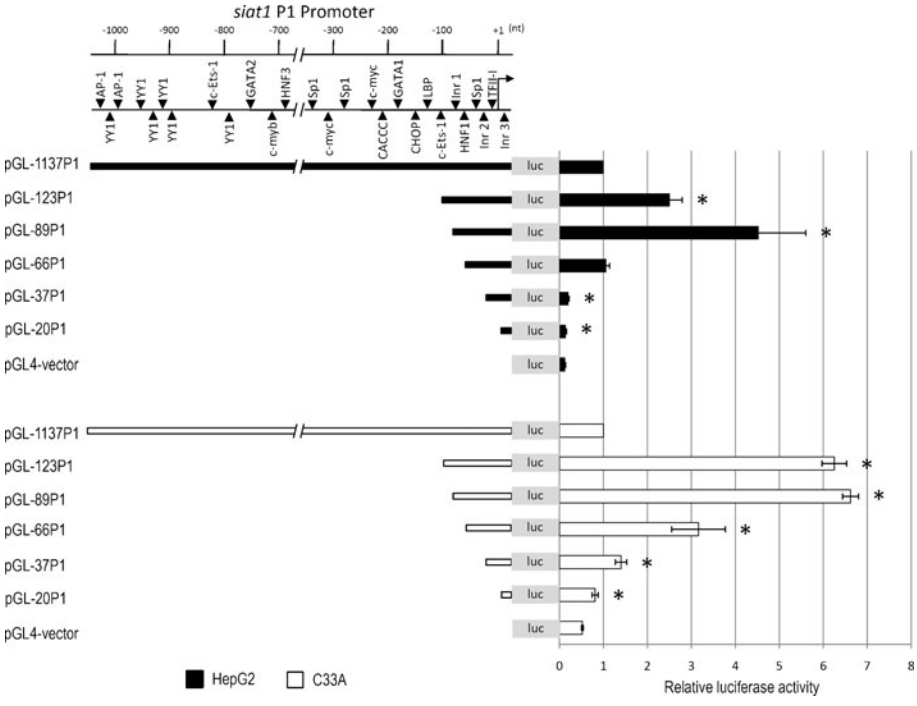

Figure 3. P1 Promoter activity of 5′-deletion constructs. Schematic representation of P1 promoter and the 5′-deletion constructs are shown. C33A and HepG2 cells were transfected with various luciferase reporter constructs containing 5′-deletion mutations of *siat1* P1 promoter. Twenty-four hours after transfection, cells were lysed and luciferase activity was measured. Results were normalized with the *Renilla* luciferase activity. Data are expressed as mean ± SD (n=3). Statistical analysis of luciferase activity was carried out between the complete promoter and the different constructs. *Statistical differences between the complete promoter activity and the different constructs.

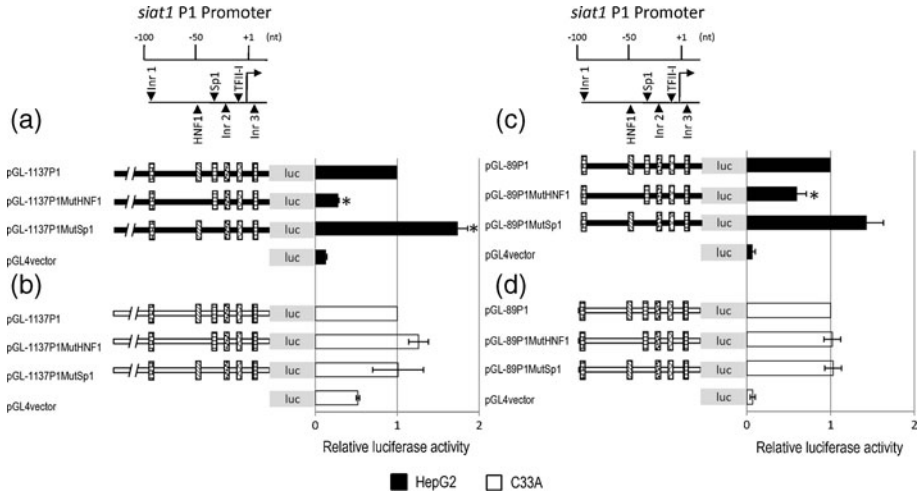

Figure 4. Role of HNF1 and Sp1 (−32 bp) binding sequences in promoter activity in C33A and HepG2 cells. Sp1 (−32) and HNF1 binding sequences as well the three initiator sequences are shown with boxes. C33A and HepG2 cells were transfected with the indicated reporter construct. Twenty-four hours after transfection, cells were lysed and luciferase activity was measured. Results were normalized with the *Renilla* luciferase activity. Data are expressed as mean ± SD (n=3). Statistical analysis of luciferase activity was carried out between the complete promoter and the different constructs. *Statistical differences between the complete promoter activity and the different constructs.

100% sequence homology to consensus YYANWYY. Two transcriptional initiation sequences at positions −53 and −9 bp have previously been reported (Lo and Lau 1996b) (figure 1).

3.2 *Deletion analysis of P1 promoter*

We prepared luciferase gene constructs carrying 5′-deleted P1 promoter pGL-1137P1, pGL-123P1, pGL-89P1, pGL-66P1, pGL-37P1, pGL-20P1 (figure 2) and transfected into C33A and HepG2 cell lines. Deletion of a fragment of 1048 bp (pGL-89P1) increased >4-fold the luciferase activity in HepG2 cell line and >6-fold in C33A cell line. The highest luciferase activity was detected for the pGL-89P1 construct in both cell lines, but pGL-123P1 had a similar activity in C33A cell line. The minimal promoter activity was detected in the promoter fragment of 37 bp in C33A cell line. The activity detected in this construct was similar to the activity of the complete promoter; however, in the HepG2 cell line no activity was detected for this construct. We examined the promoter activity of a pGL-20P1 construct but no activity was detected (figure 3).

3.3 *Effect of point mutation on Sp1 and HNF1 elements*

In TATA-less promoters, Inr sequences and Sp1 direct accurate transcription initiation. To determine the contribution of Sp1 (−32 bp) in the transcriptional activity of P1 promoter, a point mutation in this site was evaluated in the complete promoter and in the 89 bp construct (figure 4). Point mutation at the HNF1 site was also evaluated. It has been reported that this site is important for the P1 promoter activity in HT-29 and HepG2 cell lines (Xu *et al.* 2003). The effect of the mutation in the HNF1 element in the HepG2 cell line diminished the luciferase activity by ~0.7-fold compared with the control complete promoter; Sp1 mutation increased luciferase activity ~0.7-fold (figure 4a). In the C33A cell line, HNF1 and Sp1 mutations in the complete promoter do not modify the luciferase activity (figure 4b). The mutation of HNF1 in the pGL-89P1 construct diminished luciferase activity by ~0.4-fold in the HepG2 cell line (figure 4c), but no significant effect was detected in the Sp1 mutation. In the C33A cell line, the HNF1 and Sp1 mutations had no effect on the pGL-89P1 construct (figure 4d).

3.4 *Role of initiator sequence regions in the promoter activity*

Two initiation sequences have been previously reported for the P1 promoter in the positions −53 and −9 bp (Lo and Lau 1996b), and we identified a third initiation sequence at +5 bp position. In order to determine the role of these sites, we deleted or mutated them and determined the luciferase activity (figure 5). Mutation of the Inr(2) in the pGL-1137P1 construct increased the promoter activity >3-fold, but in the

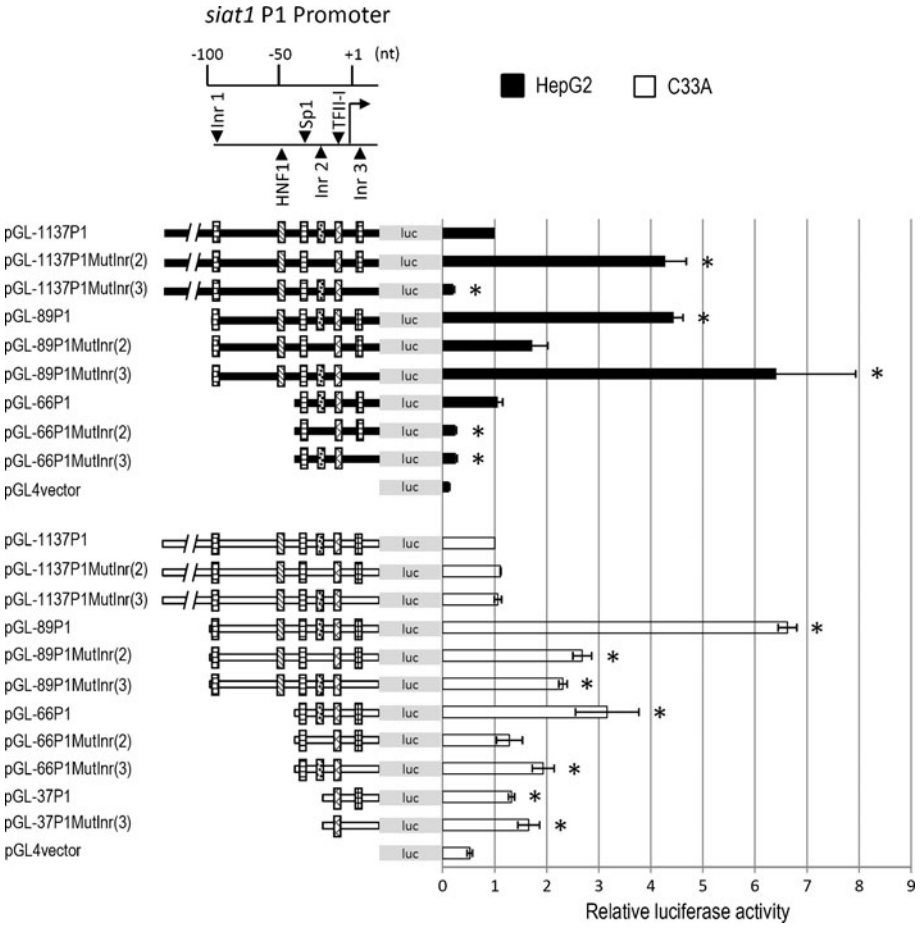

Figure 5. Effect of mutation on the initiator sequences on siat1 P1 promoter activity in C33A and HepG2 cells.Schematic representation of P1 promoter, pGL-89P1, pGL-66P1 and pGL-37P1 constructs; the three initiator sequences, the Sp1 (−32) and HNF1 binding sequences are shown with boxes. Constructs were transfected in C33A and HepG2 cells. Twenty-four hours after transfection, cells were lysed and luciferase activity was measured. Results were normalized with the *Renilla* luciferase activity. Data are expressed as mean ± SD (n=3). Statistical analysis of luciferase activity was carried out between the complete promoter and the different constructs. *Statistical differences between the complete promoter activity and the different constructs.

pGL-89P1 and pGL-66P1 this mutation diminished the promoter activity with respect to the pGL-89P1 and pGL-66P1 controls in the HepG2 cell line. In the pGL-66P1 construct, the Inr(1) was eliminated and the luciferase activity of this construction diminished >3-fold with respect to pGL-89P1 in the HepG2 cell line. When we analysed the mutation of the Inr(3) in the complete promoter and in the 66 bp fragment, the luciferase activity diminished; however, for the 89 bp fragment the activity of the mutated promoter increased (figure 5). When we evaluated these constructions in the C33A cell line, the effects were different. In the pGL4-1137P1, the mutation in the Inr(2) and Inr(3) sites had no effect with respect to the control promoter. Therefore, in the pGL-89P1 and pGL-66P1, the mutation in these initiator sequences diminished the luciferase activity (figure 5). The luciferase activity of pGL-66P1 diminished ~3-fold with respect to the pGL-89P1 that contains the Inr(1). The pGL-37P1 construction only conserved the Inr(3) sequence and mutation in this sequence had no effect on the promoter activity (figure 5).

4. Discussion

In the present study we evaluated the activity of the complete promoter and of the 5′-deleted P1 constructs. The minimal region with promoter activity was the 37 bp fragment in the C33A cell line, but no activity was detected for this construct in theHepG2 cell line. It may be the result of differences in the expression pattern of transcription factors that regulate this promoter between these cell lines. When we determined the promoter activity of a 20- bp construct, no activity was detected.

The minimal promoter activity detected in the pGL-37P1 construct was similar to the activity of the complete promoter.

A previous report identified the nt −156 to −1 region as important for transcriptional activity in HepG2 and HT-29 cell lines, and the activity of this promoter fragment was higher than the activity of the complete promoter (Xu *et al.* 2003). Our results obtained with the construction of 123 bp in the HepG2 cell line are similar to this previous report. The higher activity was detected in the pGL-89P1 construct (−65 to +24 bp). These results suggest that the deleted region contains negative regulatory elements that maintain a low promoter activity. The sequence analysis of this region showed several YY1 recognition elements that could function as negative regulatory elements of the transcription (−1030 bp, −949 bp, −931 bp, −909 bp, −898 bp and −783 bp). YY1 transcription factor is capable of both positive and negative regulation of transcription (Shea-Eaton *et al.* 2001; Deng *et al.* 2010). It is also necessary for the recruitment of HDAC (Rezai-Zadeh *et al.* 2003; Luke *et al.* 1996), and it is capable of inducing conformational changes in DNA by preventing the binding of other transcription factors (Furlong *et al.* 1996). The YY1 transcription factor has been isolated from HeLa cells (cervical adenocarcinoma) (Peters *et al.* 1993) and high concentrations of YY1 protein have been detected in cervical cancer (Shrivastaval and Calamel 1994). We also identified a LBP-1 element at position −110 bp that may be involved in negative transcriptional regulation, preventing TFIID interaction with DNA (Kato *et al.* 1991). Whether or not these transcription binding sites are functional remains to be determined.

HNF1 and Sp1 have been mutated to examine the contribution of these sequence elements in the transcriptional activity of the P1 promoter. The HNF1 mutation had no effect on the cervical cancer cell line; however, in the hepatic cell line, this mutation reduced the luciferase activity as demonstrated in a previous study (Xu *et al.* 2003). HNF1 is abundantly expressed in hepatic tissue (Svensson *et al* 1992). In silico analysis detected three Sp1 sequence elements at positions −416 bp, −255 bp and −32 bp. The mutated Sp1 was at the −32 bp position. This mutation increased the promoter activity in the HepG2 cell line, but no effect was detected on the C33A cell line. The telomerase gene promoter has no TATA box and contains several GC boxes that can be recognized by the transcription factor Sp1 as in the P1 promoter. Mutation in these sequence elements showed different effects (positive or negative) depending on their position (Kyo *et al.* 2000). It has been shown that HDAC1 can mediate repression through the Sp1 sequence elements (Lee *et al.* 2005). The increased activity detected when Sp1 was mutated may be the result of this function.

Two initiator sequence regions have been identified in the P1 promoter; these sequences are required for transcription initiation in some TATA-less genes (Lo and Lau 1996b). In the present study a third initiator sequence at position +5 was identified in the in silico analysis. In order to determine the role of these initiator sequences in the promoter activity, we deleted or mutated these sequences. The Inr(1) deleted in the pGL-66P1 diminished >3-fold the promoter activity compared with the pGL-89P1 in both cell lines, suggesting an important role of this sequence in the P1 promoter activity. In some TATA-less genes, the initiator sequences are required for the transcription (Smale and Baltimore 1989). Mutation in the Inr(2) sequence increased the complete promoter activity in HepG2 cell line 3-fold. These mutations in the full promoter did not alter the promoter activity in the cervical carcinoma cell line. In the pGL-89P1 and pGL-66P1, the mutation in the Inr(2) sequences diminished the promoter activity in cervical and hepatic cell lines, suggesting the importance of the other sequence elements eliminated in these constructs. Transcriptional regulation is complex and the interaction of different transcription factors defines the promoter activity and the effect detected in HepG2 but not in C33A cell lines and confirms the tissue- and cell-specific regulation of this gene. The Inr(2) sequence overlaps with an c-Ets-1 element. The transcription factor c-Ets-1 can function as a promoter repressor when interacting with certain proteins such as EAPIII and DAXX/EAPI. This repression has been reported for *bcl2* y *mmp1* genes. The c-Ets-1 transcription factor is highly expressed in hepatic cancer (Dittmer 2003). The c-Ets-1 may be highly expressed in the HepG2 cell line, but not in the C33A cell line, and the mutation at this binding site may explain why the promoter activity is increased.

Mutation in the Inr(3) sequence drastically diminished the complete promoter activity in the HepG2 cell line, but no effect has been detected for the cervical cancer cell line. The Inr(3) mutation increased the promoter activity in the pGL-89P1MutInr(3) but not in the pGL-66P1MutInr(3) where we observed a minor activity in the hepatic cell line. In the C33A cell line, the Inr(3) mutation decreased the activity in both constructs. The Inr(3) overlapped with a c-Myc element that may be participating in the transcriptional regulation. c-Myc transcription factor can function as a repressor and recruit HDAC that avoids the interaction of transcription factors with DNA (Gardner *et al.* 2002).

The Inr(3) mutation in the pGL-37P1MutInr(3) construct had no effect on the promoter activity. This construct does not have any of the Inr sequences. For the cervical cancer cell line, these sequences are dispensable for the transcription activity because neither the complete promoter nor the minimal promoter modified their luciferase activity.

The promoter must have another important sequence for maintaining the promoter activity. In silico analysis showed a TFII-I binding site. This transcription factor is a crucial protein for the activity of TATA-less promoters that contain initiator sequences (Manzano-Winkler *et al.* 1996). However, additional experiments are required to determine

whether this binding site is critical to the *siat1* P1 promoter on the cervical cancer cell line.

The results obtained confirm that *siat1* gene expression is differentially modulated, depending not only on the promoter regions but also on the cell types. Elucidation of the regulatory mechanisms governing *siat1* expression will contribute to the understanding of its role in carcinogenesis and also in other human diseases. Our study provides new insights into the complex transcriptional regulation of *siat1* gene.

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