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Transcription of the β-galactoside α2,6-sialyltransferase gene in B lymphocytes is directed by a separate and distinct promoter†

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A single human gene, SIAT1, encodes the β-galactoside α2,6-sialyltransferase from which multiple mRNA isoforms are generated. In rat, expression of the hepatic mRNA isoform (*Form 1*) has been defined with respect to the transcriptional initiation site and promoter region. We show here that a similar hepatic SIAT1 mRNA isoform exists in human. Another human mRNA isoform, a mature B-cell-specific mRNA isoform (*Form 2*), was previously reported. Here, we used 5′-RACE and S1 nuclease protection analysis to define the 5′-untranslated region of *Form 2* human SIAT1 mRNA. We demonstrate conclusively that *Form 2* mRNA is initiated from a point completely distinct from that of *Form 1* mRNA. A number of *cis*-acting regulatory elements residing immediately 5′ of the *Form 2* initiation site includes AP-1, AP-2, NF-κB, NF-IL6, C/EBP, and CREB. A TATAA box is also present 29 bp 5′ of the transcriptional initiation site. CAT reporter gene expression from serially-truncated segments of the 5′-flanking region of the *Form 2* initiation site indicates that the segment between $^{-}$784 and $^{+}$125 was sufficient to promote high level CAT expression in Loukes, a mature B-cell line. The 5′-flanking region to the human *Form 1* initiation site is competent in expression of CAT upon transfection of the fusion construct into HepG2, a human hepatoma cell line. Cellular specificity of expression is apparently retained. Loukes cells expressed CAT efficiently from *Form 2* promoter but only marginally from the *Form 1* promoter. In contrast, CAT expression from *Form 1* promoter is more efficient than from the *Form 2* promoter in HepG2 cells.

Introduction

The β-galactoside α2,6-sialyltransferase (SIAT1 (EC2.4.99.1)) mediates the attachment of sialic acid to galactose in SA(α2,6)Gal(β1/4)GlcNAc-R, an oligosaccharide structure common on cell surface and secreted mammalian glycoproteins and glycolipids. Although SIAT1 and its cognate sialyl linkage are present in most tissues and cells, the level of expression among different cell types is often dramatically different. Functional specialization of the α2,6-sialyl linkage in different tissues has been implicated as well. Among serum glycoproteins where the α2,6-sialyl linkage is the predominant form of sialic acid modification, it contributes to the longevity of these serum components in systemic circulation (Steer and Ashwell, 1980). In liver, the principal biosynthetic site of serum glycoproteins, SIAT1 is expressed to high levels and is transcriptionally regulated by glucocorticoids (Wang *et al.*, 1990b). In mature B-lymphocytes, cell-surface α2,6-sialyl structures serve as recognition ligands to the adhesion molecules CD22β on B-cells and CD45 on T-cells (Stamenkovic *et al.*, 1991; Aruffo *et al.*, 1992). B-Cell maturation is accompanied by a dramatic induction of SIAT1 expression and consequent display of cell-surface α2,6-sialyl linkage (Wang *et al.*, 1993). Aberrant α2,6-sialylation is also closely correlated with the tumorigenicity of colorectal carcinoma (Dall'Olio *et al.*, 1992) as well as susceptibility to killing by NK cells (Bezouska *et al.*, 1994). α2,6-Sialyl modification may also serve a masking function by depleting the Gal(β1/4)GlcNAc-precursor needed for the elaboration of other biologically significant glycan structures (Grollman *et al.*, 1992; Nemansky and Van den Eijnden, 1992; Grollman *et al.*, 1993).

A complex network of regulatory pathways is implicated by the apparent functional heterogeneity and tissue differences in SIAT1 expression. In human, the single SIAT1 gene is located on chromosome 3 (q21–28) (Wang *et al.*, 1993). Multiple SIAT1 mRNA forms, differing only in the 5′-untranslated region, have been described in both human and rat. In rat, a hepatic/small intestine-specific mRNA isoform has been extensively studied (Svensson *et al.*, 1990; Vertino-Bell *et al.*, 1994). In rat liver, SIAT1 expression is modulated by glucocorticoids (Wang *et al.*, 1990b). Recently, a similar hepatic-specific mRNA isoform has been shown to exist in human (Aas-Eng, 1995). Two additional SIAT1 mRNA forms have been documented in human cells. One isoform that appears to be restricted to mature B-cells is thought to be responsible for the elaboration of the sialyl ligand for CD22β and CD45. Another SIAT1 isoform was initially characterized in placenta (Grundmann *et al.*, 1990), but its expression in B-lymphocytes has also been documented (Wang *et al.*, 1993). The apparent lack of sequence similarity in the 5′-untranslated region among the known SIAT1 mRNA isoforms has led to the hypothesis that multiple promoter regions and multiple transcription initiation sites result in the generation of the mRNA isoforms.

Here we provide conclusive evidence that the B-cell-specific SIAT1 mRNA isoform is transcriptionally initiated from a site distinct and separate from the initiation site of the human hepatic SIAT1 mRNA isoform. We characterized the genomic sequences that encompass the distinct and separate promoter regions. Residing immediately upstream of the B-cell-specific SIAT1 transcription site are *cis*-elements with consensus to AP-1, AP-2, NF-κB, NF-IL-6, C/EBP, and CREB. We demonstrate efficient expression of a reporter gene under the control of the respective SIAT-1 promoters. Reporter gene expression in Loukes cells, a B-lymphoblastoid cell line, is optimal when regu-

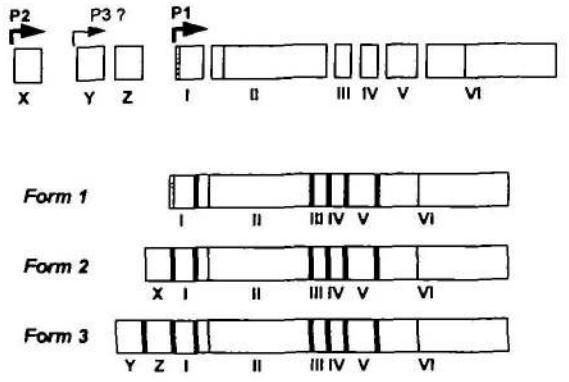

Fig. 1. SIAT1 gene structure. The exon structure of human SIAT1 is diagrammatically represented in the top figure. Shaded boxed regions represent protein coding domains. The three known forms of SIAT1 transcripts are also diagrammed below. Transcription of the three mRNA forms is initiated from initiation regions *P1*, *P2*, and *P3*.

lated by the B-cell SIAT1 promoter. In contrast, reporter expression in HepG2, a cell line of hepatic origin, is optimal when under the control of the putatively hepatic SIAT1 promoter.

Results

Assessment of tissue-specific expression of SIAT1 mRNA isoforms

Figure 1 is a schematic diagram of human SIAT1 gene and the three known forms of SIAT1 mRNA that differ only in the structure of the 5′-untranslated region (Grundmann *et al.*, 1990; Stamenkovic *et al.*, 1990; Aasheim *et al.*, 1993; Aas-Eng *et al.*, 1995). Experimentally, these mRNA isoforms can be distinguished from each other by the presence or absence of sequence contributions from Exon X or Exon Y + Z. SIAT1 is subjected to different programs of expression in different tissues. This is illustrated in Figure 2. Northern blots of mRNA from various human tissues and cells were hybridized with a probe recognizing the coding region that is common to all SIAT1 mRNA isoforms (Figure 2A). Among the tissues examined, SIAT1 mRNA levels are highest in liver. SIAT1 mRNA is also abundant in spleen, thymus, and prostate, with lesser levels in heart, kidney, ovary, small intestine, colon, and peripheral blood leukocytes. Brain, placenta, lung, pancreas, skeletal muscle, and testis express lowest levels of SIAT1 mRNA. The blots were also probed for sequences corresponding to Exon X (Figure 2B) and to Exon Y + Z (Figure 2C) to assess the differential expression of the SIAT1 mRNA isoforms. Using this approach, Form 3 mRNA is detected by presence of Exon Y + Z; Form 2 mRNA is detected by the presence of Exon X; Form 1 mRNA by the absence of Exons Y, Z, and X. It is clear that liver expresses predominantly the *Form 1* mRNA that lacks sequences from either Exons Y, Z, or X. *Form 1* is also the predominant SIAT1 mRNA isoform in HepG2, a human hepatoma cell line. With the exception of liver, tissue differences in the signal for Exon Y + Z closely parallel those observed when the coding region was probed (compare Figure 2C with Figure 2A). This is suggestive that Form 3 comprises a comparatively constant percentage of SIAT1 expression in most tissues. Another SIAT1 isoform,

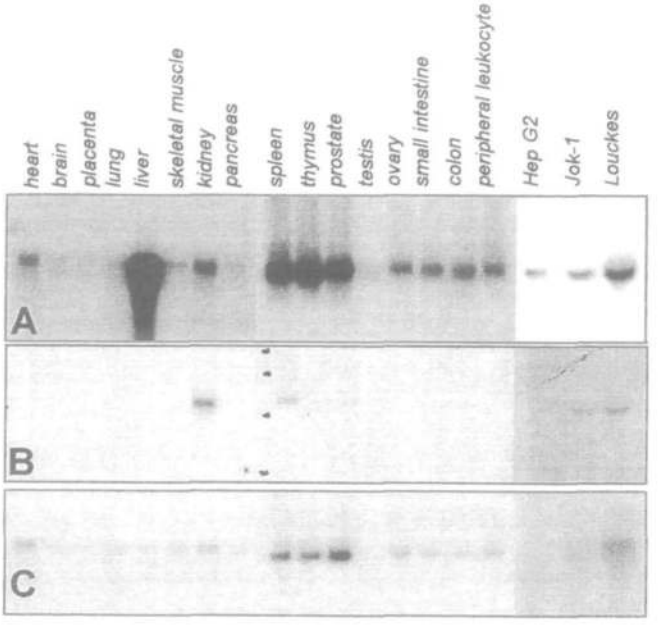

Fig. 2. Tissue specificity of SIAT1 mRNA isoform expression. Blots containing RNA from various human tissues and cells were probed with a human SIAT1 DNA fragment spanning Exons II to VI (**A**), a 68 bp fragment from Exon X (**B**), and a 239 bp fragment from Exons Y and Z (**C**). The same blot was used for all three hybridizations. The initial hybridization was with Exon X, followed with Exons Y + Z, and finally with the probe from Exons II–VI. Between hybridizations, the blots were stripped and assessed for complete removal of signal by film or phosphorimager before rehybridization with a different probe.

Form 2, contains Exon X but not Exon Y + Z (see Figure 1). Our lab has previously documented the expression of *Form 2* SIAT1 mRNA in mature B-lymphoblastoid cells (Wang *et al.*, 1993). As shown in Figure 2B, Louckes and Jok-1 cells, both lymphoblastoid lines representing the mature stage of B-cell differentiation, express *Form 2* SIAT1 mRNA. Among tissues examined, *Form 2* mRNA was detected only in kidney and spleen.

Transcription initiation region of Form 2 SIAT1 mRNA

The synthetic oligonucleotide HST-p5 that is complementary to a region within Exon I was used to prime reverse transcription of Louckes RNA. Subsequent PCR amplification (see Materials and methods) resulted in the amplification of a 331 bp product (Figure 3A) that putatively contained the extreme 5′ end of SIAT1 mRNA expressed in Louckes. Positive hybridization of the blotted PCR product by a probe for Exon X indicates that the 331 bp product is derived from *Form 2* mRNA (data not shown). The PCR product was cloned, and eight clones were chosen for sequence analysis (see Materials and methods). Previously published data on the *Form 2* SIAT1 mRNA contains sequence information only up to nucleotide +27 as shown in Figure 3C (Bast *et al.*, 1992). All eight clones contain an additional 26 bp of 5′ sequences, up to nucleotide $^{+}1$ as depicted in Figure 3C.

Sequence comparison with the previously isolated (Wang *et al.*, 1993) Exon X genomic region confirmed complete colinearity of the additional 5′ cDNA sequence with genomic Exon X. This observation strongly suggests

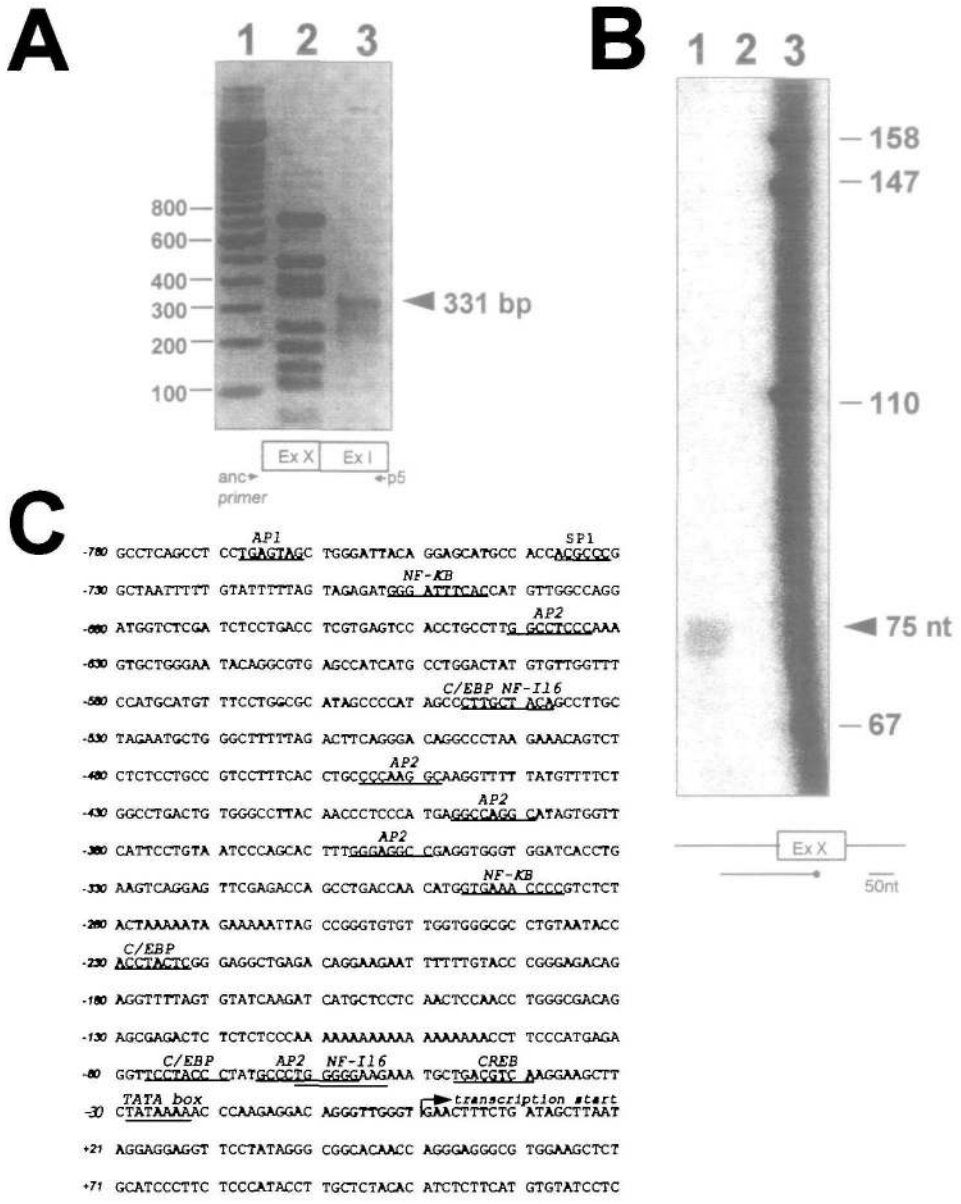

Fig. 3. Transcriptional initiation and 5′ flanking region of *P2*. **A**, mRNA from Louckes cells were subjected to 5′-RACE analysis using oligonucleotides HST-p5 and an anchor sequence as PCR primers. Shown in A is the ethidium bromide stained pattern of the 5′-RACE result. Lane 1 shows the 100 bp molecular weight markers (GIBCO BRL); lane 2 are sized markers generated by Hpa II digestion of BlueScript plasmid (from top to bottom: 711 bp, 489 bp, 404 bp, 364 bp, 242 bp, 190 bp). Lane 3 shows the resultant 331 bp 5′-RACE product from Louckes mRNA Exact length of the 5′-RACE product was derived by sequence analysis. **B**, S1 nuclease analysis of Louckes RNA. A 209 nt, 5′ end-labeled probe that is complementary to Exon X and 5′ flanking sequence was generated. Total RNA from Louckes (lane 1) or *E.coli* RNA (lane 2) was solution-hybridized to the probe, subjected to S1 nuclease digestion and analyzed in a denaturing 8% acrylamide gel. Lane 3 is radioactively labeled Hpa II fragment of BlueScript as molecular size standard. **C**, Nucleotide sequence of 5′-flanking sequence adjacent to Exon X (see Materials and methods). Nucleotide position #1 is the predicted transcription initiation site of *P2*. Potential regulatory elements are identified using SIGNAL SCAN program (Prestridge, 1991), underlined and illustrated above the sequence This sequence has been deposited into GenBank with accession number U27570.

transcriptional initiation of *Form 2* mRNA at position $^{+}1$. To demonstrate conclusively transcription initiation at position $^{+}1$, a single-stranded, end-labeled probe complementary to Exon X and adjacent 5′ region was generated and used for S1 nuclease protection analysis of Louckes mRNA. The result of this analysis is shown in Figure 3B. A major S1 nuclease protected signal of 75 nt is evident in the presence of Louckes RNA (lane 1) but not in a parallel reaction containing only control *E. coli* RNA (lane 2). The predicted size of this nuclease-protected signal is fully consistent with the transcriptional initiation at position $^{+}1$.

Figure 3C also summarizes sequence 5′ of the initiation site that putatively encompasses the transcription promoter region for *Form 2* SIAT1 mRNA. This putative promoter region, *P2*, contains a number of consensus to known *cis*-acting regulatory elements. Among these elements are one AP1 binding site at position $^{-}768$; five AP2 sites at positions $^{-}641$, $^{-}456$, $^{-}397$, $^{-}357$, $^{-}66$; two NF-κB at $^{-}703$ and $^{-}296$; three C/EBP at $^{-}546$, $^{-}230$, and $^{-}77$; two

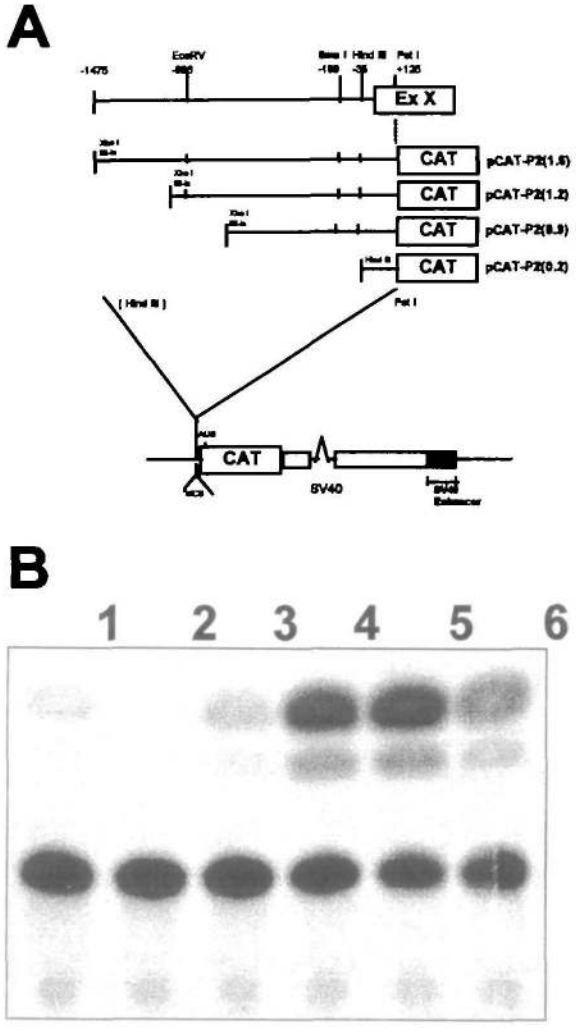

Fig. 4. Expression of *P2* promoter-CAT fusion constructs. **A**, Schematic diagram of serially truncated 5′-flanking regions of *P2* obtained as described in Materials and methods and fused upstream of the CAT reporter sequence in the expression plasmid pCAT-enhancer. **B**, Louckes cells were transiently transfected with pCAT-control, pCAT-enhancer, pCAT-*P2*(0.2), pCAT-*P2*(0.9), pCAT-*P2*(1.2), and pCAT-p2(1.6) (lanes 1–6, respectively). This experiment was performed twice, each in duplicates, with identical results. Shown is the result from one of the two duplicates from one of the two experiments.

NF-IL6 at ⁻546, and ⁻62; one CREB at ⁻47 (Prestridge, 1991). Finally, a consensus TATA box is located between 20 and 30 bases 5′ of the predicted transcriptional start site.

Transcriptional regulatory function of P2 region

Chimeric constructs, containing progressive 5′ truncations of the *P2* promoter region of SIAT1 fused to the CAT structural gene (Figure 4A), were tested by transfection into Louckes, a cell line that normally expresses *Form 2* SIAT1 mRNA. The result of this experiment is shown in Figure 4B. Not unexpectedly, minimal CAT expression was observed in cells transiently transfected with pCAT-enhancer(null), the parental plasmid lacking transcriptional promoter sequences (lane 2). Surprisingly, also minimal CAT expression was observed when pCAT-control, a plasmid containing the SV40 transcriptional regulatory sequences (lane 1) was tested. Four constructs were tested in which progressively shorter segments of the SIAT1 *P2* region were fused upstream to the CAT structural gene in pCAT-enhancer(null). Maximal CAT expression resulted upon transfection of pCAT-*P2*(1.2), containing 1.2 kb of *P2* sequence, and pCAT-*P2*(0.9), containing 0.9 kb of *P2* sequence (Figure 4B, lanes 5 and 4, respectively). Lesser CAT expression resulted upon transfection of pCAT-*P2*(1.6), containing a longer stretch of *P2* sequence (Figure 4B, lane 6), or pCAT-*P2*(0.2), containing only a very short segment of the *P2* region (Figure 4B, lane 3). Together, the data demonstrate the ability of the region immediately flanking the *Form 2* SIAT1 initiation to serve as transcriptional promoters and that the sequence between ⁻784 and ⁺125 is sufficient to promote high level CAT expression in Louckes.

Transcription initiation region of Form 1 SIAT1 mRNA

Synthetic oligonucleotide HST-p27 that is complementary to a region within Exon I was used to prime reverse transcription of HepG2 mRNA. Subsequent PCR amplification (see Materials and methods) resulted in a major 152 bp product and a minor 191 bp product that putatively contain the extreme 5′ ends of SIAT1 mRNA expressed in HepG2 (Figure 5A). The PCR products were cloned. Among the 10 clones chosen for sequence analysis, two clones contained sequence information up to ⁻39, four contained sequence up to ⁺1, the other four up to ⁺4.

Sequence comparison with the previously isolated Exon I genomic region confirms the complete colinearity of the cloned sequence with Exon I, suggesting transcriptional initiation of *Form 1* mRNA from site(s) immediately 5′ of Exon I. To assess the precise transcriptional initiation site, RNase protection analysis of HepG2 mRNA using a probe generated from genomic region 5′ and including Exon I was performed (Figure 5B). In the presence of HepG2 RNA (lane 3), two principal RNase protected regions are visible. The corresponding signals are absent in a parallel reaction containing only control yeast tRNA (lane 2). The sizes of the RNase protected signals (denoted by *) predicts two groups of transcription initiation sites (I and II) that are summarized in Figure 5C. The predicted initiation sites I and II are fully consistent with 5′-RACE products of 191 bp and 152 bp. A consensus region to an 'initiator' sequence (Smale and Baltimore, 1989) resides within 20 bp upstream of each of the initiation sites.

Sequence of this genomic region is shown in Figure 5C. Nucleotide position ⁺1, for convenient purposes, has been labeled at the first transcription start site in group II for *Form 1* SIAT1 mRNA. The promoter region, *P1*, residing upstream of the transcription initiation sites, contains consensus to a number of known *cis*-acting elements. Among these are APRF binding site at position ⁻379, AP2 site at position ⁻249, NF-IL6 at ⁻237, and HNF-1 at ⁻51.

Differential expression of CAT by P1 and P2

Chimeras containing CAT under the control of *P1* and *P2* SIAT1 regulatory regions were tested for differences in efficiency when expressed in Louckes or HepG2 cells.

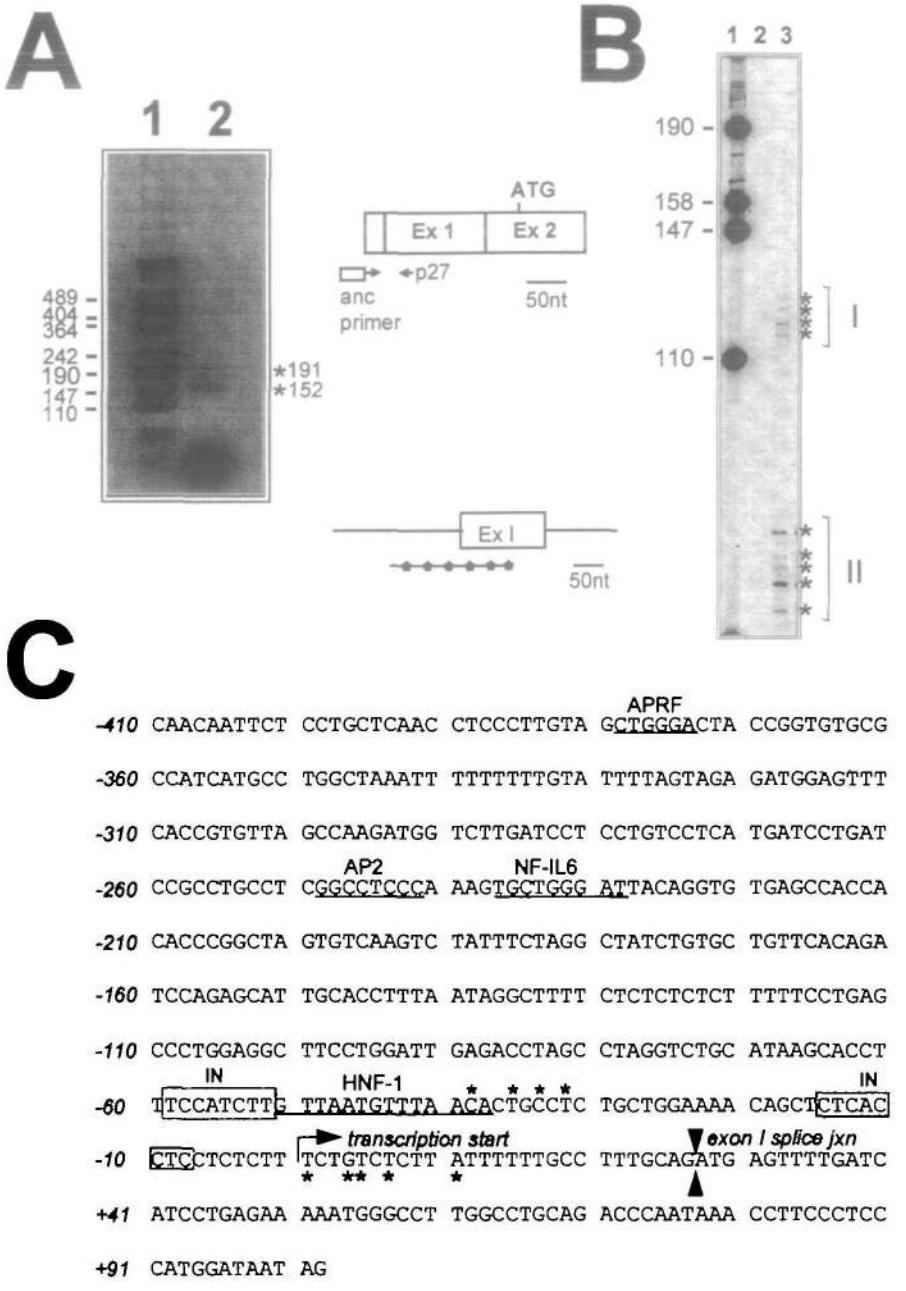

Fig. 5. Transcription initiation and 5′ flanking region of *P1*. **A**, mRNA from HepG2 cells was subjected to 5′-RACE analysis using oligonucleotides HST-p27 and an anchor primer as PCR primers. Shown in A is the ethidium bromide stained pattern of the 5′-RACE result. Lane 1 shows Hpa II digested BlueScript as molecular weight markers. Lane 2 is the product of the 5′-RACE using HepG2 mRNA. A major product at 152 bp and a minor product at 191 bp are visible. Exact length of the PCR products was derived by sequence analysis. **B** summarizes the RNase protection analysis of HepG2 mRNA using an internally labeled single stranded RNA probe complementary to Exon I and 5′-flanking genomic region. End-labeled Hpa II fragments of BlueScript serves as size standards on a denaturing acrylamide gel (lane 1). RNase protected signal in the presence of 10 μg of yeast tRNA as control is shown in lane 2. Two groups (I and II) of RNase protected signals were observed in the presence of 10 μg of HepG2 RNA (lane 3) that correspond with 5′-RACE products of 191 bp and 152 bp (see A). **C** summarizes sequence information for the 5′ genomic region immediately flanking Exon I (see Materials and methods). Starred positions denote initiation points as predicted by RNase protection analysis (**B**). Potential regulatory elements are underlined and illustrated above the sequence. Exon splice junction to generate *P2* and *P3* type mRNAs is also shown. Boxed letters represent initiator sites. This sequence has been deposited into GenBank with accession number U27580.

pCAT-*P2*(0.9), containing 0.9 kb of the *P2* regulatory region (see Fig 4), was compared with CAT-*P1*(0.2), the CAT chimera containing 0.2 kb of the *P1* regulatory region. As shown in Figure 6, CAT is efficiently expressed in Loucke cells from the *P2* regulatory sequences but only marginally from the *P1* sequence. In contrast, reporter expression in HepG2 cells is strong when under the control of the *P1* region and comparatively weaker when controlled by the *P2* region. Chimeras containing *P1* regions longer than that included in pCAT-*P1*(0.2) were also tested. However, CAT expression in HepG2 was severely depressed in these longer *P1* constructs (data not shown)

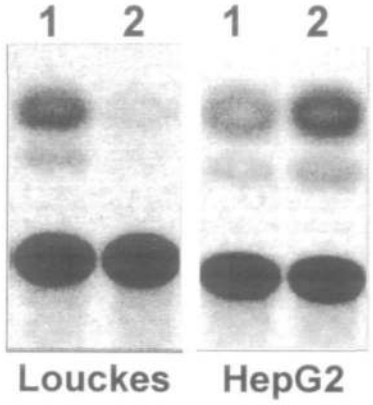

Fig. 6. Transient expression of SIAT1-CAT fusion constructs in HepG2 and Louckes cells. CAT chimeras were transiently introduced into Louckes cells (left panel) or Hep G2 cells (right panel) and assessed for CAT expression as detailed in Materials and methods. In each case, lane 1 is the result of transfection with pCAT-*P2*(0.9); lane 2 is the result of transfection with pCAT-*P1*(0.2). This experiment was performed twice, each time in duplicates. Shown is the result from one of the two duplicates from one of the two experiments. Construction of pCAT-*P1*(0.2) is detailed in Materials and methods

presumably due to the inclusion of negatively acting transcription elements in the additional upstream *P1* regions.

Discussion

Most tissues and cell types express SIAT1 and synthesize the cognate sialyl α2,6 linkage. SIAT1 expression results in a number of mRNA isoforms, most of these differ only in the 5′-untranslated region. The existence of multiple promoter regions has been suggested to account for the complete absence of sequence commonality in the extreme 5′ region of the mRNAs (Grundmann *et al.*, 1990; Stamenkovic *et al.*, 1990; Aasheim *et al.*, 1993; Aas-Eng *et al.*, 1995). In human, three such mRNA forms have been identified (see Figure 1). Two of these, *Form 1* and *Form 2*, have a tightly restricted range of tissue specificity of expression. *Form 1* mRNA is known to be expressed only in hepatic cells (Wang *et al.*, 1990b; Svensson *et al.*, 1992) and in the small intestinal epithelium of pre-weaning animals (Vertino-Bell *et al.*, 1994); *Form 2* mRNA is so far known to be expressed only in mature B cells (Wang *et al.*, 1993). The third form, Form 3, appears to be a constitutively expressed form in most tissues. In this report, we have established that two physically distinct promoter regions regulate the transcription of the *Form 1* and *Form 2* SIAT1 mRNAs. On this basis, it is likely that a third distinct promoter region is responsible for transcription of the third known SIAT1 mRNA form.

Form 1 SIAT1 mRNA has been extensively characterized in the rat (Wang *et al.*, 1989, 1990; Svensson *et al.*, 1992). In the differential Northern blot analysis (Figure 2), we have demonstrated a similar restriction of *Form 1* expression to hepatic cells. Here, we used the parallel approach of RNase protection and 5′-RACE analysis to define the transcription initiation sites of *Form 1* mRNA at the 5′ junction of Exon 1. A heterogenous but closely spaced pattern of transcription starts was noted by both 5′-RACE and RNase protection assays (see Figure 5). A similar undertaking was reported by Aas-Eng *et al.* (Aas-Eng *et al.*, 1995). Our data are in essential agreement except for the notable exception that the heterogenous initiation sites were not reported by Aas-Eng *et al. Form 1* promoter, *P1*, residing immediately 5′ of Exon I contains a number of consensus to *cis*-elements also noted in the rat *P1* region. These include HNF-1 and NF-IL6, a human homolog of murine DBP (Courtois *et al.*, 1988; Ramji *et al.*, 1991). HNF-1 and DBP are liver-enriched factors that are believed to participate in the hepatic specificity of *Form 1* SIAT1 mRNA expression (Svensson *et al.*, 1992). As in the rat promoter, human *P1* lacks the canonical TATA box. A GC box, a binding site for the transcription factor Sp1 that is present in many 'housekeeping' genes lacking TATA boxes, is also notably absent (Reynolds *et al.*, 1984). Nevertheless, *P1* did have two 'initiator' sequence regions (YYCAYYYY) which is required for initiation in some TATA-less genes (Smale and Baltimore, 1989). These regions reside immediately upstream of the two P1 initiation points (see Figure 5C).

Expression of *Form 2* SIAT1 mRNA is largely restricted to mature B lymphocytes (Wang *et al.*, 1993). In Northern blot survey of a number of human tissues (Figure 2), kidney is the only tissue besides spleen in which *Form 2* SIAT1 mRNA was detected. 5′-RACE and S1 nuclease protection analysis established the transcription initiation of *Form 2* mRNA to the 5′ end of Exon X (see Figure 3). *Form 2* promoter, *P2*, resides immediately 5′ of Exon X and contains a canonical TATA box. A number of regions with consensus to transcription factor binding sites are also noted (Prestridge, 1991), including AP1 (Wasylyk *et al.*, 1989), AP2 (Imagawa *et al.*, 1987; Mitchell *et al.*, 1987; Faisst and Meyer, 1992), Sp1 (Janson *et al.*, 1987), NF-κB (Hoyos *et al.*, 1989; Lenardo and Baltimore, 1989), NF-IL6 (Faisst and Meyer, 1992), CREB (Jameson *et al.*, 1988), and C/EBP (Johnson, 1987; Costa *et al.*, 1988). Among these, the presence of two consensus regions for NF-κB binding is most noteworthy. The reported presence of NF-κB binding activity in nuclear extracts of mature B cells but not in pre-B cell lines (Sen and Baltimore, 1986) coincides with SIAT1 induction and the appearance of *Form 2* mRNA during B cell maturation (Wang *et al.*, 1993).

P1 and *P2*, when fused upstream to the CAT reporter gene, efficiently regulate CAT expression in transiently transfected cells. In Louckes cells that normally express *Form 2* mRNA, maximal efficiency in CAT expression was achieved in constructs containing 1.2 kb and 0.9 kb of *P2* sequence. Another construct, containing 1.6 kb of *P2*, expressed CAT comparatively inefficiently (see Figure 4). The reason for this is not clear, although the presence of negative acting elements in the longer *P2* construct is a likely possibility. Cellular specificity is apparently retained in CAT expression under the control of SIAT1 regulatory regions. *P1* regulated constructs are expressed most efficiently in HepG2, a cell line of hepatic origin; *P2* regulated constructs are expressed most efficiently in Louckes, a B-lymphoblastoid cell line.

Central to the elucidation of mechanisms for tissue and developmental specificities of sialic acid moieties on glycoconjugates is the understanding of their cognate sialyltransferases. Although the β-galactoside α2,6-sialyltransferase activity is ubiquitously present, available data implicate cellular differences in regulation of SIAT1 expression. In

hepatocytes, SIAT1 expression is responsive to glucocorticoids (Wang *et al.*, 1990b) but apparently unaffected by the cAMP pathway (Wang *et al.*, 1989). SIAT1 expression in thyrocytes, in contrast, is negatively modulated by TSH via the cAMP pathway (Grollman *et al.*, 1993). Transient high level SIAT1 expression in the intestinal epithelium of new born animals (Vertino-Bell *et al.*, 1994) and the induction of SIAT1 during B-lymphocyte maturation (Wang *et al.*, 1993) further suggest independent regulation of SIAT1 in different tissues. The existence of multiple promoter regions is ideally suited for such differential requirements for SIAT1 expression in different cell types.

The existence of multiple promoters for the expression of a single gene is a relatively common phenomenon among procaryotes. An example is the *ptsH* operon of *E.coli* under the control of two distinct promoters *P0* and *P1*. These promoters endow two separate regulatory mechanisms, one via the CRP-cAMP, the other through glucose, to *pts* expression (Ryu and Garges, 1994). Multiple promoters are unusual, although not without precedent among mammalian genes. A number of different promoters regulate the transcription of the human fibroblast growth factor 1 gene. These promoters respond to different physiologic signals and stimuli that result in the differential production of fibroblast growth factor 1 protein in different cells (Chotani *et al.*, 1995). Multiple promoters and associated alternate transcription initiation sites have also been reported in the rat rolipram-sensitive cAMP phosphodiesterase gene (Monaco *et al.*, 1994) and the inhibin/activin (β-subunit gene (Feng *et al.*, 1995). Among the glycosyltransferases, the (β1,4-galactosyltransferase (Shaper *et al.*, 1988) and the N-acetylglucosaminyltransferase I (Yang *et al.*, 1994) are known to be regulated by multiple promoters. Unlike SIAT1 in which the multiple promoters are physically distinct, galactosyltransferase expression is regulated by separate but overlapping promoter regions (Shaper *et al.*, 1988; Harduin-Lepers *et al.*, 1992; Shaper *et al.*, 1994).

Among some other glycosyltransferase activities, most notably the α1,3-fucosyltransferase activities (Weston *et al.*, 1992) and the α-galactoside α2,3-sialyltransferase activities (Chang *et al.*, 1995), multiple genes encode enzymes with closely related catalytic specificities but different patterns of tissue expression. While differences in their catalytic specificities have been amply documented, whether one enzyme may adequately substitute for a related enzyme in normal physiologic functions remains to be determined. What remains a possibility, nevertheless, is that these multiple genes evolved via evolutionary forces similar to that which gave rise to the multiple promoters of SIAT1. The need for multiple regulatory pathways may be common among glycosyltransferases for which expression is ubiquitous, but the degree of expression may need to be differentially modulated in different cell types. In any event, detailed knowledge of the precise functionality and regulation of the individual glycosyltransferases is essential for the overall understanding of the correct expression of biologically significant glycan epitopes.

Materials and methods

Cell culture and northern analysis

The human lymphoblastoid lines Louckes and Jok-1 and the human hepatoma line HepG2 were obtained and maintained as previously described (Wang *et al.*, 1993). Total cellular RNA was prepared from human cell lines mentioned above using the guanidine isothiocyanate method (Chirgwin *et al.*, 1979). Ten micrograms of each total RNA were separated on formaldehyde agarose gels (Boedtker, 1971), and electrotransferred onto nylon filter (Zetabind, Cuno Inc., Meriden, CT). Human multiple tissue blots of poly A+ RNAs were purchased from Clontech Laboratories (Palo Alto, CA). Approximately 2.0 μg of poly A+ RNA were in each lane on the purchased blots. Hybridization procedure and generation of the radiolabeled probes ($\sim 1 \times 10^6$ c.p.m./ml) specific to human SIAT1 coding region, Exon X, and Exon Y + Z were as described previously (Wang *et al.*, 1993). The blots were exposed to XAR-5 films (Eastman Kodak Co., Rochester, NY) for 1–4 d with an intensifying screen and stripped according to the manufacturer's instruction for rehybridization. Alternatively, the blots were quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Cloning of the 5′ end of the human SIAT1 transcripts

Total cellular RNA was isolated from Louckes cells and HepG2 cells and poly A+ RNA purified by oligo(dT) cellulose chromatography (Aviv and Leder, 1972). First strand cDNAs were synthesized from mRNA derived from 200 μg of total RNA from Louckes cells (5′-AmpliFINDER RACE Kit, Clontech Lab, Palo Alto, CA) using HST-p5 (Wang *et al.*, 1993) and subsequently ligated with AmpliFINDER Anchor (5′-P-CACGAATTCACTATCGATTCTGGAACCTTCAGAGG-3′NH2) in which an EcoRI restriction site has been included. The anchor-ligated cDNAs were then subjected to PCR amplification as described previously (Wang *et al.*, 1993). PCR primers used were anchor primer (5′-CTGGTTCGGCCCACCTCTGAAGGTTCCAGAATCGATAG-3′) and HST-p5. To clone the PCR product, HST-p28 (5′-CTATTATCCATGGGGAGGGAAGCTTTATTG-3′) which contains a mutation to generate a HindIII restriction site was used as a nested primer. PCR product digested with EcoRI and HindIII was cloned into pBS-SK+/EcoRI/HindIII (Stratagene, La Jolla, CA). Clones of interest were screened using the Exon X probe and sequenced using Sequenase (modified T7 polymerase from U. S. Biochemicals, Cleveland, OH) by the dideoxynucleotide chain termination method. For HepG2 cells, first strand cDNAs were synthesized using HST-p27 (5′-CTATTATCCATGGGAGGGAAGGTTT-3′) and ligated with AmpliFINDER Anchor. The anchor-ligated cDNAs were PCR-amplified using HST-p27 and anchor primer. PCR product was then cloned into plasmid pCRII using TA cloning kit (Invitrogen, San Diego, CA).

S1 nuclease protection assay and ribonuclease protection assay

Two plasmids, pBS-PX and pBS-PL which, respectively, contained Exon X and Exon I as well as their respective 5′ flanking genomic sequences, were constructed to generate probes for the assays. To construct pBS-PX, a 234 bp fragment was PCR-amplified from λHG4 (Wang *et al.*, 1993) using HST-P40 (5′-ACTCCAACCTGGTCGACAGAGC-3′) and HST-P41 (5′-TGGGAGAAGGGATCCAGAGCTT-3′), digested with BamHI and SalI. The 209 bp digested fragment was cloned into pBS-KS+/BamHI/SalI. For pBS-PL, a 277 bp fragment was PCR amplified from previously isolated λHG9 (Wang *et al.*, 1993) using HST-P28 and HST-P39 (5′-TGTGCTGTTCACGGATCCAGAGC-3′), and digested with BamHI and HindIII, and cloned into pBS-SK+/BamHI/HindIII. The integrity of both clones was sequence-verified. S1 nuclease assay was performed using S1 Assay Kit (Ambion Inc., Austin, TX). Briefly, pBS-PX was digested with BamHI followed by dephosphorylation using alkaline phosphatase (Boehringer Mannhein, Indianapolis, IN) and subsequent digestion with SalI to generate a 209 bp fragment. Two picomoles of the fragment were 5′-end labeled with γ^{32}P-ATP (3000 Ci/mmol, Amersham, Arlington Heights, IL). Probe (4×10^4 c.p.m.) was added to 50 μg of either *E.coli* RNA or Louckes total RNA and ethanol coprecipitated. The pellet was resuspended in 10 μl of hybridization solution (80% deionized formamide, 100 mM sodium citrate, pH 6.4, 300 mM sodium acetate, pH 6.4, 1 mM EDTA), denatured at 90°C for 3 min, cooled down slowly to 42°C, and then allowed to hybridize at 42°C overnight. Following hybridization, 50 U of S1 nuclease were added and samples were incubated at 25°C for 30 min. After incubation, 40 μl of S1 nuclease inactivation buffer were added, and the samples were ethanol-precipitated. The pellets were resuspended in TE and sequencing loading dye and then analyzed on a denaturing 8% polyacrylamide gel.

For the RNase protection assay, 0.5 μg of BamHI-linearized pBS-PL was used to synthesize RNA probe using T7 RNA polymerase (Promega, Madison, WI) and α^{32}P-CTP (800 Ci/mmol, NEN, Boston, MA). Labeled RNA probe (1×10^5 c.p.m.) was then added to 10 μg of HepG2 total RNA or yeast tRNA and the mixtures precipitated with ethanol. The

Guardian RNase Protection Assay Kit (Clontech Laboratories, Palo Alto, CA) was used for the RNase protection assays.

Construction of plasmids for transient cell transfection and transient transfection analysis

A plasmid, pSK-HGX4Ps containing 4.6 kb flanking sequence of Exon X, was constructed by subcloning of the genomic clone λHG4 (Wang *et al.*, 1993) into Bluescript. Serially 5′-truncated constructs of pSK-HGX4Ps were obtained by Exo III and Mungbean nuclease digestion. Clones were sequence-analyzed to determine the precise degree of 5′ deletion. Inserts were placed upstream of the CAT coding region in pCAT-enhancer (see schematic in Figure 4). An additional construct, pCAT-*P2*(0.2), was obtained by inserting the 160 bp Hind III/Pst I restriction fragment into pCAT-enhancer.

For CAT constructs, another clone, pHG9N2 (Wang *et al.*, 1993), containing 5′ genomic sequence flanking Exon I was used as starting material. A fragment released by Pst I digest that contains the 70 bp Exon I and 1 kb of 5′-flanking sequence was placed upstream of the CAT structural gene to obtain pCAT-*P1*(1.0). The second construct pCAT-*P1*(0.2) contains 70 bp of Exon I and 162 bp of the adjacent flanking region.

Transient transfection of HepG2 cells was performed using the calcium phosphate transfection technique (Ausubel *et al.*, 1989). Equal molarity of plasmid DNA was prepared in 0.5 ml of 0.25 M $CaCl_2$ at ~10 μg/ml. The DNA solution was added to 0.5 ml of 2 × HBS buffer (280 mM NaCl, 10 mM KCl, 3 mM $NaHPO_4$, 12 mM dextrose, 50 mM HEPES, pH 7.5) and allowed to precipitate at room temperature for 20 min. The precipitate was added to cells grown in 100 mm plastic dishes (40% confluent) and incubated for 5 h. Cells were washed two times with phosphate-buffered saline (PBS) and allowed to recover in complete media for 24–48 h before assaying for CAT activity. Transient transfection of Loucke cells was performed using electroporation (GIBCO, BRL). Cells (5×10^6) were resuspended in 0.6 ml of RPMI 1640 + 10% FBS containing equal molarity of plasmid DNA (~25 μg) and electroporated in 0.4 cm cuvette at room temperature under 250 V, low R, and 1180 μF. Cells were cultured in 10 ml of complete medium for 24–48 h before assaying for CAT activity. CAT assay was performed according to Ausubel *et al.* with some modifications. In brief, transfected cells were washed twice with PBS and once with TEN (40 mM Tris-Cl, pH 7.5, 1 mM EDTA, pH 8.0, 150 mM NaCl) and resuspended in 100 μl of 0.25 M Tris-Cl, pH 8.0. Cells were sonicated briefly, incubated at 65°C for 5 min, frozen and thawed once, and clarified by quick centrifugation. Protein concentration in each cellular extract was normalized by the Bradford reagent (Bio-Rad, Richmond, CA). Twenty microliters of cell extracts was used for each measurement for CAT activity. CAT activity was quantitated by thin layer chromatography

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