Characterization of the Human Transcobalamin II Promoter

A PROXIMAL GC/GT BOX IS A DOMINANT NEGATIVE ELEMENT*

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* This work was supported by National Institutes of Health Grants 29 to 163 that contained multiple transcription initiation sites; (c) not dependent on other potential elements, such as a distally localized CCAAT box, a CF1, a HIP1 binding motif and a MED-1 element; (d) modulated weakly by a positive-acting GC box (–568/GAGGCGGTGC) and strongly by a proximal GC/GT overlapping box (–179 CCCCGCCCCCACCACC). Gel shift and immuno supershift analyses demonstrated that both the positive-acting GC box and the negative-acting GC/GT box were recognized by Sp1 and Sp3. Co-transfection studies using Sp1 and/or Sp3 expression plasmids revealed that while Sp1 stimulated, Sp3 repressed Sp1-mediated transactivation of TC II transcription. The proximal GC/GT box also acted as a negative element in human chronic myelogenous leukemia K-562 and HeLa cells. These results suggest that tissue/cell specific expression of the TC II gene may be controlled by the relative ratios of Sp1 and Sp3 that bind to the GC/GT box and the weak promoter activity of TC II is due to the transcriptional repression caused by the binding of Sp3 to the proximal GC/GT box.

Human transcobalamin II (TC II) is a secretory non-glycoprotein of molecular mass of 43 kDa (1) that functions in the plasma transport of cobalamin (vitamin B12) to all cells. TC II gene is expressed in many types of cells and tissues but at different levels (2). Increased levels of plasma TC II have been reported in patients with a variety of diseases (3), indicating that the TC II gene may have a basal expression in many cells, but could be induced under some pathological conditions. Lack of expression or expression of defective forms of TC II leads to the development of intracellular cobalamin deficiency (4). Recent studies (2, 5, 6) from our laboratory have shown that in the most common form of TC II deficiency, lack of immunoreactive plasma TC II is due to lack of TC II protein synthesis, which in turn is due to extremely low levels of TC II mRNA (2). Although in many patients studied the great reduction of TC II mRNA is due to nonsense mutations (5, 6), or to DNA deletions (6), available evidence suggests that TC II deficiency can also result from promoter defects (5).

Despite these studies nothing is known about how the TC II gene expression is regulated. We have recently isolated the human TC II gene and its 5′-flanking region up to about 1 kilobase (7). Primer extension analysis of the mRNA from human kidney revealed multiple transcription initiation sites at the region between nucleotide –128 and –77. The 5′-flanking sequence of the gene is GC-rich, lacks a consensus TATA box, but contains a distally localized CCAAT element (–422/–418). It also contains potential binding sites for a number of transcription factors such as Sp1, CF1, HIP1, Ets-1, and a MED-1 element that have been implicated in the transcription of some TATA-less genes (8–12). In order to begin to understand the transcriptional regulation of this important nutrient transporter we have analyzed a number of plasmid constructs with a series of promoter deletions to identify the cis-elements that are important in control of TC II promoter activity. The results of the present study show that TC II gene transcription is not affected by the housekeeping elements HIP1, CF1, or MED-1. However, its transcriptional activity is dependent on sequences surrounding the transcription initiation sites and is regulated positively by a distal GC box (–568/–559), and negatively by a proximal GC/GT overlapping box (–179/–165). The GC/GT box appears to have a dominant negative effect in control of the weak promoter activity of the TC II gene.

MATERIALS AND METHODS

Construction of Promoter-CAT Reporter Plasmids—The promoterless plasmid, pCAT-Basic (Promega, Madison, WI), was used for the preparation of the CAT (chloramphenicol acetyltransferase) reporter constructs. Various deletions of the TC II-promoter segments were generated by polymerase chain reaction and inserted into the pCAT-Basic (pCAT-B) vector by blunt-end ligation at a site upstream from the CAT gene. A total of 11 promoter segments were amplified and the sequences of each pair of primers used for polymerase chain reaction are shown in Table I. The DNA sequence of each of the promoter segments was confirmed by sequencing prior to transfection.

Site-directed Mutagenesis—The site-directed mutagenesis of GC or GC/GT box in the promoter segment were generated using the Quick-Change Site-directed mutagenesis method (Stratagene, La Jolla, CA). For each mutagenesis a pair of overlapping oligonucleotides with desired mutations was commercially synthesized and the sequences are listed in Table II. The mutations generated were confirmed by DNA sequencing.

Cell Culture and Transient Transfection—The human colon epithelial cell line Caco-2 and HeLa cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. The cells were plated at a density of 1.5 × 10⁶ cells/100-mm dish the day before transfection and transfected by the calcium phosphate precipitation method (13). The transfection was performed using 15 µg of promoter-CAT fusion plasmid and 5 µg of an internal control plasmid pSV-β-galactosidase (Promega) for monitoring the transfection efficiency. In addition, promoterless vector pCAT-Basic and promoter-containing plasmid pCAT-Promoter (Promega) were also transfected in each ex-

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Deletion and mutagenesis of the 5′-flanking region of the human transcobalamin II (TC II) transfected in human intestinal epithelial Caco-2 cells have revealed that TC II promoter activity is: (a) very weak; (b) restricted to a core region (–29 to –163) that contained multiple transcription initiation sites; (c) not dependent on other potential elements, such as a distally localized CCAAT box, a CF1, a HIP1 binding motif and a MED-1 element; (d) modulated weakly by a positive-acting GC box (–568-GAGGCGGTGC) and strongly by a proximal GC/GT overlapping box (–179 CCCCGCCCCCACCACC). Gel shift and immuno supershift analyses demonstrated that both the positive-acting GC box and the negative-acting GC/GT box were recognized by Sp1 and Sp3. Co-transfection studies using Sp1 and/or Sp3 expression plasmids revealed that while Sp1 stimulated, Sp3 repressed Sp1-mediated transactivation of TC II transcription. The proximal GC/GT box also acted as a negative element in human chronic myelogenous leukemia K-562 and HeLa cells. These results suggest that tissue/cell specific expression of the TC II gene may be controlled by the relative ratios of Sp1 and Sp3 that bind to the GC/GT box and the weak promoter activity of TC II is due to the transcriptional repression caused by the binding of Sp3 to the proximal GC/GT box.
The sequences of all wild-type oligonucleotides are taken from the 5'-flanking region of human TCII gene. Mismatched nucleotide for consensus sequence of GC box (22) are double underlined. Mutated nucleotides for SDM are underlined. The GC (179/170) and GT (174/165) overlapping boxes in oligo-GI and a GC box (199/190) with two mismatched nucleotides in oligo-GII are in antisense orientation. The sequence of the GT box is identical to the GC box except that C or A at position 5 is replaced by T.

**TABLE II**

DNA sequence of oligonucleotides used in EMSA or site-directed mutagenesis (SDM)

The sequences of all wild-type oligonucleotides are taken from the 5'-flanking region of human TCII gene. Mismatched nucleotide for consensus sequence of GC box (22) are double underlined. Mutated nucleotides for SDM are underlined. The GC (179/170) and GT (174/165) overlapping boxes in oligo-GI and a GC box (199/190) with two mismatched nucleotides in oligo-GII are in antisense orientation. The sequence of the GT box is identical to the GC box except that C or A at position 5 is replaced by T.

**TABLE II**

PCR primers used in generating TCII promoter segments

| Promoter segment | Forward primer | Reverse primer |
|------------------|----------------|----------------|
| p1(-1014/+37)    | 5'-ACCTCTTGGTTCAAGGATCTCTC | 5'-GGACCCCAAGGAAAGGAAAG |
| p2(-746/+37)     | 5'-CTCAGAGGGCTCCAGGCTCTTCG | +37 to +15 |
| p3(-511/+37)     | 5'-TCCAGCTCCGGTTCCACTCTTG | +37 to +15 |
| p4(-453/+37)     | 5'-GGTAAAGGATCGGGTAGGCCAC | +37 to +15 |
| p5(-330/+37)     | 5'-GGGCTCTTTAAGCAGGAACGTG | +37 to +15 |
| p6(-265/+37)     | 5'-TCCCTTTGTTCCCTACCTCCAGG | +37 to +15 |
| p7(-163/+37)     | 5'-CTGCAAGCTACTGCGTGCATGC | +158 to +165 |

**Preparation of Nuclear Extracts**—Nuclear extracts were prepared from Caco-2 cells essentially as described by Dignam et al. (18). All buffers contained protease inhibitors including phenylmethylsulfonyl fluoride (1 mM), leupeptin (2 µg/ml), antipain (10 µM), and benzamidine (1 mM). Protein concentration of the nuclear extract was determined by the Bio-Rad protein assay method using bovine serum albumin as a standard (19).

**Electrophoretic Mobility Shift Assay**—The promoter segment p6.3 (-265/-158) was cleaved from the plasmid p6.3 (-265/-158)-B by digestion with HindIII and XhoI and labeled with "32P"dCTP using the Klenow fragment of DNA polymerase. Double-stranded oligonucleotide CI and GI (Table II) were labeled at the 5'-termini with [γ-32p]ATP using T4 polynucleotide kinase. Labeled probe (~2 × 10^5 cpm) was incubated for 15 min at 22 °C with 2.2 µg of nuclear extract in 10 µl of reaction buffer (10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, and 50 µg/ml poly(dI-dC) poly(dI-dC)). For competition experiments the nuclear extract was incubated with indicated concentrations of double-stranded oligonucleotide competitors (Table I) at 22 °C for 5 min prior to incubation with the probe. For immunosupershift assay, the nuclear extract was preincubated with 1.5 µg of affinity purified rabbit polyclonal antibody against Sp1 or Sp3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 22 °C for 30 min prior to incubation with the probe. The reaction mixture was then subjected to 4% polyacrylamide gel electrophoresis in 0.5 × TBE buffer (44.5 mM Tris-HCl, pH 8.0, 44.5 mM borate acid, and 1 mM EDTA) at 100 V. The protein-DNA complexes were visualized by autoradiography.
Transcobalamin II Gene Regulation

RESULTS

Human TC II Promoter Activity Is Regulated by Both Positive and Negative Elements—To identify regions that are important for the promoter activity, a series of promoter segments were cloned into a promoterless CAT reporter vector, pCAT-B, and the fusion promoter-CAT constructs were transiently transfected into human intestinal epithelial Caco-2 cells known to express endogenous TC II (20, 21).

Initially, seven plasmid constructs with sequential 5'-deletions and a 3'-fixed end (+37) were analyzed and the results are shown in Fig. 1. The longest promoter segments p1 (1039 bp) and p2 (783 bp) revealed maximal promoter activities, but their levels were only 2.4-fold of the baseline activity. Further deletion from −746 to −511 (p3), which contains a putative GC and E-box, resulted in negligible promoter activity, indicating the presence of positive elements in the region −746 to −511. Deletion from −511 to −453 (p4) caused a slight increase of the promoter activity (1.8-fold of the baseline value), suggesting the presence of a weak negative element in this region. Additional deletions from −453 to −330 (p5) containing a CCAAT element and from −330 to −265 (p6) containing a CF1 binding motif did not significantly change the promoter activity. However, deletion from −265 to −163 (p7) which contains two overlapping Sp1 consensus motifs, GC and GT box, caused an increase of the promoter activity almost to the same level as that of the longest promoter segment.

The 5'-deletion studies suggested that the TC II promoter activity is weak in Caco-2 cells. However, it is not known whether the weak promoter activities detected using these promoter segments are due to the absence of some important positive elements such as MED-1 (12) located downstream from the 3'-end point (+37) of these constructs or to the presence of strong negative elements at the 3'-region. In order to test these possibilities, promoter segments with 3'-extension to position +185 (p2.1 and p6.1), which included a perfect match for a MED-1 element (+159/+160), or with 3'-deletions to position −29 (p6.2) and −158 (p6.3) were fused with the CAT reporter vector. Fig. 2 shows the constructs of these promoter segments and their relative CAT activities. The inclusion of the region +37 to +185 to promoter segments p2 and p6 did not increase, but reduced the promoter activities to the baseline. This result indicated that MED-1 is not functional in the transcription of the TC II gene. However, removal of region from +37 to −29 increased the promoter activity from 1.6 (p6) to 2.9 (p6.2), suggesting the presence of negative elements in the region −29 to +37. Further deletions from region −29 to −158 (p6.3), which contains multiple transcription start sites and a potential Ets-1 binding site, eliminated the promoter activity completely.

These studies revealed that the TC II promoter activity is weak, its core promoter is limited to the region between −163 and −29, and its activity is regulated by both positive and negative control elements. Since the positive (−746 to −511) and one (−265 to −163) of the negative regions contained a putative GC box (−568 to −559) and a GC/GT box (−179 to −165), respectively, the nuclear factors binding to these two regions were characterized by EMSA.

Sp1 and Sp3 Interact with the Positive (−746 to −511) and Negative (−265 to −163) Regulatory Regions of the Promoter—The positive region (−746 to −511) of the TC II promoter contains a putative GC box (−568/−559) GAGGCGGTGGC, with one mismatch of nucleotide T instead of G or A at position 8 of the consensus sequence of the GC box (22). To test the possible interaction of the putative GC box with Sp1, a double-stranded oligonucleotide containing this region (oligonucleotide CI, see Table II) was synthesized, labeled, and subjected to EMSA and immunosupershift analysis (Fig. 3). Incubation with the labeled oligonucleotide CI with Caco-2 nuclear extracts revealed two major distinct protein-DNA complexes, I and II (lane 2). Both the complexes were abolished when the nuclear extract was preincubated with unlabeled wild-type oligonucleotide, CI (lane 3), but not the mutant oligonucleotide, CI-M (lane 4) in which the GC box was mutated (see sequence in Table II). The competition results suggested that the nuclear proteins interacted specifically with the GC box. Preincubation of the nuclear extracts with antibody to Sp1 resulted in more than 50% shift of the complex I without affecting the mobility of complex II (lane 5), indicating that Sp1 is one of the proteins forming complex I.

It is known that the GC box can interact with several human transcription factors other than Sp1. These include Sp3, Sp4, and BTEB2 (25–25). However, among these Sp1-related proteins, only Sp3 is expressed ubiquitously (24). Thus, we examined whether Sp3 contributed to the formation of protein-DNA complex I and/or the rest of the complex II. When the nuclear extracts were preincubated with antibody to Sp3, electrophoresis revealed elimination of complex II and a partial elimination of complex I (lane 6). These observations indicated that complex I was formed as a result of interactions with both Sp1 and Sp3 whereas complex II was formed due to interaction with Sp3 alone and that the positive regulatory region (−746 to −511) interacted with both Sp1 and Sp3.

5'-Deletion analysis of the promoter identified a negative regulatory region from −265 to −163 (Fig. 1). This region
contains two potential Sp1 binding motifs, one is an inverted GC and GT overlapping box (\(2^{179}-2^{165}\)), CCCCCGCCCCACCCC, and the other is an inverted GC box with two mismatches at position 2 and 10 (underlined) of the consensus sequence of the GC box (\(2^{199}-2^{190}\)), TCCCCGCCAC. To identify the functional elements binding to Sp1 or to any other nuclear factors, a DNA fragment (\(2^{265}\) to \(2^{158}\)) spanning the entire negative region was labeled and analyzed by EMSA (Fig. 4A). Three predominant complexes (I–III) were observed when the nuclear extracts were incubated with the labeled probe (lanes 2–3). All three complexes were eliminated (lanes 4–6) by competition with an unlabeled double-stranded oligonucleotide GI (Table II) which contained the overlapping GC/GT box, but not affected when competition was carried out with 50–1000-fold excess (lanes 7–9) of oligonucleotide GII (Table II) which contained the potential GC box with two mismatches. This result indicated that in the negative regulatory region (\(2^{265}\) to \(2^{163}\)), almost all of the nuclear factor binding was due to interactions with the sequence GI. Since the overlapping GC/GT box at position \(-179\) to \(-134\) is not only a potential binding motif for Sp1 but also for AP2 (CCC(A/C)N(G/C)(G/C)) (26), additional competition studies were performed by using oligonucleotides containing consensus sequences for Sp1 and AP2 (see Table II). As shown in Fig. 4A, the three complexes were competed by Sp1 oligonucleotide (lane 10), but not by AP2 oligonucleotide (lane 11).

In order to confirm that the binding of nuclear proteins to the negative regulatory region (\(-265\) to \(-163\)) occurred at the sequence GI (\(-187\) to \(-152\)), oligonucleotide GI was \(^{32}\)P-labeled and subjected to EMSA (Fig. 4B). Consistent with the result from Fig. 4A, three predominant complexes (I–III) were revealed by using the short DNA sequence as a probe (lane 2). All three complexes were competed away with unlabeled double-stranded oligonucleotide CI which contained the putative GC box (Table II) and not with the mutant oligonucleotide CI-M (lane 3) or GI-M (lane 6), in which the GC box or the GC/GT box was mutated (Table II). This result demonstrated specific interactions of the nuclear proteins with the GC or the GT box. Antibody to Sp1 supershifted the complex I almost completely (lane 7) while antibody to Sp3 inhibited the formation of complex II and III (lane 8), indicating that the negative region (\(-265\) to \(-163\)) interacts with both Sp1 and Sp3. Taken together, the results shown in Figs. 3 and 4, A and B, have demonstrated that both the GC box in the positive regulatory region and the GC/GT box in the negative regulatory region interact with Sp1 and Sp3. The functional significance of these two regulatory elements was further investigated by transfection of the plasmid constructs containing mutations in the GC or the GC/GT box.

### FIG. 2. 3' -Extension and deletion analysis of the human TC II gene promoter region. TC II promoter segments with 3'-extension (p2.1-B and p6.1-B) or deletions (p6.2-B and p6.3-B) were generated by polymerase chain reaction and ligated into promoterless vector, pCAT-B. The fusion constructs were transfected into Caco-2 cells. The relative CAT activities shown are mean ± S.D. from four separate transfection experiments. kb, kilobase.

### FIG. 3. Nuclear proteins binding at the positive region containing a putative GC box (−568/−559). The \(^{32}\)P-labeled double-stranded oligonucleotide CI containing the putative GC box (Table II) was incubated with (lanes 2–6) or without (lane 1) nuclear extracts from Caco-2 cells and analyzed by EMSA. The nuclear extracts were preincubated with binding buffer alone (lane 2) or with unlabeled double-stranded oligonucleotide competitors CI (lane 3) or CI-M (lane 4) or with antibodies to Sp1 (lane 5) or Sp3 (lane 6).
antibodies to Sp1 (lane 7) or Sp3 (lane 8) region (stranded oligonucleotide competitors as indicated (lanes 3–6) preincubated with binding buffer alone (lanes 1–3) or without (lane 2) Caco-2 nuclear extracts and analyzed by EMSA. The nuclear extracts were preincubated with binding buffer alone (lanes 1–5) or unlabeled double-stranded oligonucleotide competitors as indicated (lanes 4–11). B, the 32P-labeled oligonucleotide GI (–157 to –152) was incubated with (lanes 2–8) or without (lane 1) Caco-2 cell nuclear extracts and analyzed by EMSA. The nuclear extracts were preincubated with binding buffer alone (lane 2), with unlabeled double-stranded oligonucleotide competitors as indicated (lanes 3–6), or with antibodies to Sp1 (lane 7) or Sp3 (lane 8).

The repressive effect of the proximal GC/GT box on the transcriptional regulation of the TC II gene was also tested in two other cell lines, human chronic myelogenous leukemia K-562 and HeLa cells (Fig. 5). Northern blot analysis using mRNA from K-562, Caco-2, and HeLa cells have shown similar levels of endogenous TC II mRNA in Caco-2 and K-562 cells and its undetectable levels in HeLa cells. Consistent with this observation, transfection using both the long (p2-B) and the short (p6.2-B) promoter fragments resulted in similar levels of the promoter activity in K-562 and Caco-2 cells. In contrast, the long (p2-B) promoter fragment transfected in HeLa cells did not reveal any promoter activity. When the GC/GT box was mutated, the CAT activity with the long (p2/CTM-B) and the short (p6.2/CTM-B) promoter fragments increased 307 and 657%, respectively, in K-562 cells. Very interestingly, in HeLa cells, mutations of the GC/GT box in the long promoter fragment (p2/CTM-B) caused a 432% induction of the CAT activity over the baseline activity detected using its wild-type (p2-B) promoter counterpart. Taken together, these transfections studies have clearly shown that the proximal GC/GT box functions as negative element in multiple cells.

Sp1 Activates and Sp3 Represses Sp1-mediated Transactivation of the TC II Promoter Activity—It is well known that Sp1 is a transcriptional activator (27) and Sp3 is a bifunctional transcriptional regulator that can both repress (14, 28, 29) and activate (30–32) transcription, depending upon the cell and promoter type. Our EMSA experiments (Figs. 3 and 4) demonstrated that Sp1 and Sp3 interact specifically with both the positive GC and the negative GC/GT boxes. The functional roles of Sp1 and Sp3 on the TC II promoter was therefore tested by overexpression of Sp1 and Sp3 in Caco-2 cells.

Four promoter-CAT reporter plasmids with either wild-type sequence (p2-B and p6.2-B) or mutated GC/GT box (p2/CTM-B and p6.2/CTM-B) were co-transfected with expression plasmids for human Sp1 and/or Sp3 and their resultant CAT activities are shown in Fig. 6. With the longer promoter segment, overexpression of Sp1 stimulated the transcription of the wild-type construct (p2-B) by about 420% and the GC/GT mutant construct (p2/CTM-B) by 233%. In contrast, overexpression of Sp3 alone did not increase but slightly suppressed the promoter activity. Co-transfection of Sp3 along with Sp1 suppressed the Sp1-mediated transcriptional activation of both the wild-type or mutant constructs. With shorter promoter segments in which the distal GC box was deleted, overexpression of Sp1 caused a 260% activation of the wild-type construct (p6.2-B), and no activation of the GC/GT mutant construct (p6.2/CTM-B). Like with the longer construct, overexpression of Sp3 alone

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slightly inhibited the transcriptional activities of both the wild-type and the mutant forms of the shorter constructs. This slight inhibition was mostly due to nonspecific inhibition of the pRC/CMV vector in which Sp3 cDNA was harbored (data not shown). Co-transfection of Sp3 along with Sp1 inhibited the Sp1-mediated transactivation of the wild-type construct (p6.2-B), and had no effect on the GC/GT mutant construct (p6.2/CTM-B). Taken together, the co-transfection studies have shown that Sp1 stimulates while Sp3 suppresses Sp1-mediated transactivation of the TC II promoter activity.

**DISCUSSION**

The current studies have provided some interesting insights into the basal and regulated expression of TC II promoter activity. Our results have demonstrated that TC II-promoter activity is weak and its core promoter sequence (−29 to −163) lacks a consensus TATA box or an Initiator (Inr) element, but contains multiple transcription initiation sites (MTIS) and a putative Ets binding motif (−117-CAGGAAGC). In addition, several putative cis-elements identified in the TC II promoter region, such as a HIP1 initiator element (−37-ATTC N 27 GCCA), MED-1 (−155-GCTCCC), and CCAAT box were not required for the TC II promoter activity.

In general, for basal transcription, a core promoter requires a TATA box and/or an Inr element to direct transcription by RNA polymerase II (33). Several types of Inr elements have been described, including TdT-Inr, PBGD-Inr, DHFR-Inr, ribosome protein-Inr, and adeno-associated virus P5-Inr (reviewed in Ref. 34). The sequences around MTIS of the TC II promoter do not fit any of the reported Inr elements, except a putative DHFR-Inr or HIP1 binding motif that is present at the 3′-end of the MTIS of the TC II promoter. However, deletion of the HIP1-binding site actually increased the promoter activity from 1.6 (Fig. 1, p6-B) to 2.9 (Fig. 2, p6.2-B), indicating that

**FIG. 5. Effects of mutations in the distal GC box (−559/−568) and/or proximal GC/GT box (−165/−179) on the TC II promoter activity.** Three promoter-CAT fusion constructs with wild-type sequences (p2-B, p6-B, and p6.2-B) or mutated GC/GT box (p2/CTM-B and p6.2/CTM-B) were co-transfected into Caco-2, HeLa, or K-562 cells. The diagram of each construct is shown and the mutations in either GC or GC/GT box regions indicated by X. The CAT activity corresponding to each wild-type construct minus the basal CAT activity is taken as 100% and has been used as a reference to normalize CAT activity of each mutant construct. The values reported are mean ± S.D. of three independent transfections with each construct. ND, not determined.

**FIG. 6. Effects of co-transfection of Sp1 and Sp3 on the TC II promoter activity.** Two promoter-CAT fusion constructs with either wild-type sequences (p2-B and p6.2-B) or mutated GC/GT box (p2/CTM-B and p6.2/CTM-B) were co-transfected with expression plasmid for Sp1 and/or Sp3 into Caco-2 cells. The relative CAT activity is expressed as a percent of activity of the constructs without co-transfection with Sp1 and/or Sp3. The values represent mean ± S.D. of three independent transfections.
HIP1 initiator element is not functional in TC II basal transcription.

TATA-less promoters are known to transcribe from either a single or multiple sites. While most of the Inr elements identified so far in the transcription from a single site, the mechanisms involved in the selection and activation of MTIS in a TATA-less promoter are not fully understood. Two hypothesis have been proposed for the positioning of the preinitiation complex at multiple sites in a TATA-less promoter. The first hypothesis is that the absence of a strong positioning element, like the TATA or Inr element, is responsible for the utilization of MTIS (35). Recently, a second hypothesis has been proposed by Ince and Scotto (12). According to this hypothesis, the selection and activation of MTIS in TATA-less promoter is regulated by a downstream element MED-1 (GCTCC/G/C). Interestingly, we have found a six-nucleotide sequence, GCTCCC, located at position 232 bp downstream of the 5'-end of the MTIS of the TC II promoter. However, our results (Fig. 2, p2.1-B and p6.1-B) indicated that the MED-1 element identified in TC II is not functional in transcription. It should be noted that although MED-1 element has been identified in five TATA-less promoters, its positive role in transcription has been demonstrated only in P-glycoprotein promoter (12). Thus, our results indicate that the utilization of MTIS in TC II transcription may be due to the lack of a strong positional element.

The only cis-element identified in the core promoter of TC II is a putative Ets binding motif. Recently, Ets binding element has been reported to be important in the selection and activation of MTIS in several TATA-less and Inr-less promoters, including thymidylate synthase promoter (36), cytochrome c oxidase subunit IV promoter (37), and β2-integrin CD18 promoter (11). It is suggested (11, 36) that the Ets binding motif is an important common element in directly recruiting the basal transcription machinery in some TATA-less promoters. The actual role of the putative Ets-binding element in the modulation of TC II core promoter is not known and additional studies are needed to address this issue.

An interesting observation of the current studies is that the GC or GT box could have either a positive or a negative effect on TC II transcription in the same cell, depending upon the position or the context of the element. Our mutagenesis studies (Fig. 5) have revealed that the distal GC box (−568/-559) acts as a positive element while the proximal GC/GT overlapping box (−179/-175) functions as a negative element. Mutagenesis of both sites demonstrated that the distal GC box is a weak positive element and the proximal GC/GT box is a strong negative element, since the repressive activity of the proximal GC/GT box was dominant over the activation by the distal GC box (Fig. 5, p2/GCM.CTM-B). Our in vitro binding study has demonstrated that both Sp1 and Sp3 are capable of binding to the positive-acting GC box (Fig. 3), as well as to the negative-acting GC/GT box (Fig. 4B). Furthermore, overexpression of Sp1 and Sp3 (Fig. 6) demonstrated that Sp1 activated transcription when it bound to either the distal GC box (p2.CTM-B) or the proximal GC/GT box (p6.2-B). In contrast, Sp3 did not activate transcription, but repressed Sp1-mediated activation through binding to either the distal GC box or the proximal GC/GT box. Based on these observations, we speculate that in the cells tested, the distal GC box is mainly bound by Sp1 which functions as an activator whereas the proximal GC/GT box is occupied by Sp3 which acts as a repressor. The finding that overexpression of Sp3 alone did not repress the activity of promoter containing GC/GT box is most likely due to the presence of saturated amounts of endogenous Sp3 bound to this site in vivo.

Several studies (14, 28, 29) have demonstrated that Sp3 acts as a repressor of Sp1-mediated transcriptional activation. It has been suggested (14) that the inhibitory effect of Sp3 is due to its competition for the Sp1-binding site. However, later studies (38, 39) have demonstrated that Sp3 contains a repressor domain that functions independently from the DNA-binding domain. The repressor domain of Sp3 has been shown to inhibit both the multiple activator-mediated transcription as well as the basal transcription (28, 38). It is therefore speculated (28, 38) that Sp3-mediated repression may be due to a direct action on the general transcriptional machinery. Our observation (Fig. 5) that the great enhanced promoter activity resulting from mutation of the GC/GT box supports the speculation that Sp3 can act not only as a passive but also as an active repressor. If the mechanism of Sp3-mediated transcriptional repression is solely by physically blocking the Sp1 binding to the GC/GT box, one should expect that the mutation of the GC/GT box should not result in a strong stimulation of the promoter activity since mutated GC/GT box cannot interact with Sp1 (Fig. 4B).

Transcriptional activation as well as repression are both important mechanisms of transcriptional regulation. Usually, GC box acts as a positive element in both TATA-containing and TATA-less promoters. In TATA-less promoters, the GC box near the initiation sites (36–70 bp upstream) often plays an important activation role in both transcription initiation and efficiency (40–42). Therefore, the finding that the proximal GC/GT box (42 bp upstream of the 5'-end of the MTIS) in the TC II promoter functions as a negative element is somewhat surprising. It is possible that the Sp3 repressor bound at the proximal GC/GT box, which is close to the MTIS of the TC II promoter, impairs the assembly of the preinitiation complex by exposing the repression domain to the component of the general transcription machinery. The presence of a negative-acting GC/GT box in the TC II promoter may partly explain its undetectable transcriptional activity in HeLa cells and very weak activity in Caco-2 and K-562 cells.

There is now a growing list of promoters in which the GC or GT box has been shown to act as a negative element. These include the rat D2 dopamine promoter (43), the rat αA adrenergic promoter (44), the long terminal receptor of the human T cell leukemia virus type I promoter (45), the rat fatty acid synthase promoter (46), and the rat smooth muscle myosin heavy chain promoter (47). The finding that the GC or GT box involved in the negative regulation of gene expression implies that the GC or GT box may play a more complicated role in transcriptional regulation. It is possible that a GC or GT box with potential dual function can act as a molecular switch to control transcription both positively and negatively, depending on the competitive binding by activators and repressors and possible antagonistic or synergistic interactions between the factors. This mechanism may provide a means to control the differential expression of TC II gene noted in tissues/cells. This possibility could be tested in future studies by in vitro transcription assay using nuclear extracts isolated from human kidney (high expression) or colon (low expression) and the TC II promoter template with the wild-type or the mutant sequences.

The transcriptional activity of the TC II promoter in the colon could be further examined by using Sp3-depleted nuclear extracts that are preincubated with antibody against Sp3. In summary, the present studies have demonstrated that TC II, an important nutrient transporter, has a very weak promoter that does not utilize TATA or Inr for its basal transcription. Its promoter activity is regulated positively by a distal GC box, and negatively by a proximal GC/GT box.

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