Functional significance of Sp1, Sp2, and Sp3 transcription factors in regulation of the murine CTP:phosphocholine cytidylyltransferase α promoter

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Abstract **The transcription factor Sp1 has been implicated in regulation of the expression of the murine CTP:phosphocholine cytidylyltransferase α (CTα) gene, *Ctpct* (M. Bakovic, K. Waite, W. Tang, I. Tabas, and D. E. Vance. 1999. *Biochim. Biophys. Acta*. 1438: 147–165). We have utilized transient transfections, mutation analysis, electromobility gel-shifts, and immunoblot analysis to test the hypothesis that expression of the CTα gene is controlled in part by the binding of three *trans*-acting nuclear factors, Sp1, Sp2, and Sp3. Sp1 and Sp3 activate CTα gene transcription through sequence specific binding within three promoter domains. In Sp1-mediated transcription, Sp3 acts as an activator in a dose-dependent manner and vice versa. Sp2 represses Sp1- and Sp3-driven transcription in *Drosophila* SL2 cells, but stimulates transcription in C3H10T1/2 mammalian cells. Our results suggest that the predominant action of Sp proteins is a direct function of local organization of three *cis*-acting elements in the regions A (−31/−9), B (−88/−50), and C (−148/−128). The ability of distal C (−148/−128) and proximal A (−31/−9) regions to activate or repress transcription depends upon the cellular background. The multiple binding elements at position B (−88/−50) confer a positive regulation independent of the cell context. However, the effectiveness of Sp proteins at this site is strongly governed by neighboring sites A and C. The results suggest that the level of expression of the CTα gene will depend on the cell type, the availability of Sp proteins, and the structure and organization of three *cis*-acting elements.**—Bakovic, M., K. A. Waite, and D. E. Vance. **Functional significance of Spl, Sp2, and Sp3 transcription factors in regulation of the murine CTP:phosphocholine cytidylyltransferase α promoter.** ***J. Lipid Res.*** 41: **583–594.**

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CTP:phosphocholine cytidylyltransferase (CT) is a primary regulatory enzyme in phosphatidylcholine biosynthesis (1). Two genes encoding enzymes with CT activity, CTα and CTβ, have been characterized. CTα has been identified in many tissues (1) while CTβ has been only recently identified in human tissues as two 3′-end splicing variants, CTβ1 and CTβ2 (2). CTα and CTβ have essentially identical catalytic properties and both require lipids for maximum activity. The two proteins are dissimilar in their amino-terminal domains, and CTβ1 lacks most of the phosphorylation sites located in the CTα carboxyl-terminal domain. CTα has been detected in the nucleus (3), both nucleus and cytoplasm (4), and associated with Golgi apparatus (5, 6), endoplasmic reticulum (7) and transport vesicles (7). CTβ has currently only been found in the cytosol (2).

Concerning mechanisms that regulate CT gene expression, earlier work (8–10) showed that CTα gene and mRNA turnover are regulated during cell growth and development. While both isoforms are ubiquitously expressed, CTβ mRNA is particularly abundant in placenta and testis (2), suggesting tissue-specific regulation. Furthermore, transcripts of different size were identified for both forms, indicating that alternate splicing mechanisms or promoter usage might occur.

Only the murine CTα gene (*Ctpct*) has been cloned and characterized (11). We isolated the *Ctpct* promoter and characterized some of its binding elements and associated factors (12). The 5′-terminal ~200 bp sequence proximal promoter contains four consensus elements for nuclear factor Ap1, overlapping sites for NFκB, E2F, and Elk-1, one SRE (sterol response element), and three Sp1-related motifs, indicating potentially complex regulation of the *Ctpct* gene. We have established the importance of three regions on the promoter that are responsible for suppression, activation, and basal promoter activity (12). These regions contain *cis*-elements that are transactivated by the Sp1 transcription factor, but the functional relationship among them has not yet been established.

Recently, studies with several other promoters (13–15) have begun to explore the functional contributions of newly identified members of the expanding Sp family of transcription factors (16). In this regard, the cDNAs en-

Abbreviation: CT, CTP:phosphocholine cytidylyltransferase.

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coding Sp2 and Sp3 proteins that are closely related to transcription factor Sp1 have been cloned and characterized (17). Sp1 and Sp3 are structurally and functionally highly related, whereas Sp2 appears to have distinct DNA binding specificity and its functional role is not clearly defined (17). It seems that the function of these nuclear factors is context and cell-type dependent, which underlies the importance of investigating their role in *Ctpct* regulation.

To test the hypothesis that all three Sp transcription factors may regulate *Ctpct* promoter activity, we co-transfected Sp2 and Sp3 expression vectors, in the presence or absence of Sp1 expression vector, with a series of mutated *Ctpct*-reporter genes. Similar to Sp1, Sp3 was a strong activator in *Drosophila* (SL2) cells and mouse embryo fibroblasts (C3H10T1/2). Sp2 was a bifunctional transcriptional regulator showing repression in insect cells and activation in mammalian cells. Our results suggest that the predominant action of Sp transcription factors is a direct function of the abundance of the specific factor and the structure and organization of three *cis*-acting elements in distal, proximal, and minimal promoter region.

MATERIALS AND METHODS

Plasmid construction

Luciferase reporter plasmids containing serial deletions at the 5′-end of the murine *Ctpct* gene, LUC.C5 (−2068/+38), LUC.C7 (−1268/+38), LUC.C8 (−201/+38) and LUC.D1 (−90/+38), LUC.D2 (−130/+38), and LUC.D3 (−52/+38), have been described (12). Single and double mutant variants of LUC.C7 were generated by an overlap extension PCR methodology (18). For single mutants, alterations in the promoter sequence were introduced by incorporating changes into specific promoter primers: ΔA (−31/−9), 5′-TCAGATGTTTCggtacCGTCTCC-3′, ΔB (−75/−51), 5′-CAAGAGGG-aattca-GGAGGCGGGAACTT-3′, ΔC (−148/−128), 5′-ACGCGCCC-gagct-CTCTGGAA-3′, and by using GLPR2 (5′-CTTTATGTTTTTGGCGTCTTCCA-3′) and RVPR3 (5′-CTAGCAAAATAGGCTGTCCC-3′) as vector primers (pGl3, basic, Promega). The mutated promoter fragments were ligated into *Nhe I/Hind III* cut pGl3, basic vector and screened for positive colonies. The screening procedure was simplified by the presence of restriction enzyme sites, as shown in the mutated sequence above: *Kpn I* in LUC.C7(ΔA), *EcoR I* in LUC.C7(ΔB), and *Sac I* in LUC.C7(ΔC). Mutations were confirmed by sequencing (University of Alberta DNA core facility) and two independently derived clones generated from separate overlap extension reactions were used, with no significant differences in activity.

Double mutants were constructed by the same procedure, except that templates used in the first round of PCR were single mutants: LUC.C7(ΔA) for the preparation of LUC.C7(ΔAΔB) and LUC.C7(ΔAΔC); and LUC.C7(ΔC) for the preparation of LUC.C7(ΔBΔC). One insertion mutant, obtained accidentally, LUC.C7(ΔB,ins), was also included in our study. This mutant contained two ΔB sequences, 5′-CAAGAGGG-aattca-GGAGGC GGGACTT-aa-CAAGAGGG-aattca-GGAGGCGGGACTT-3′ instead of the standard sequence 5′-GGGCGGGCGGGAGGCGGA-3′ at the position −71/−58.

Cytomegalovirus promoter-reporter plasmid, CMV.LUC, was generated by introducing a luciferase *Hind III/Xba I* gene fragment from pGl3, basic vector into *Hind III/Xba I* site of pRcCMV vector (Invitrogen). Vectors enabling expression of recombinant Sp proteins were obtained from Dr. R. Tjian (pPacSp1 and pPacO) (19) and Dr. J. Noti (pPacSp2 and pPacSp3) (20). The vector enabling expression of β-galactosidase, pBKΔGal, has been previously described (12).

Cell culture, transfections, and reporter assays

Mouse embryo fibroblasts (C3H10T1/2) and rat hepatoma (McArdle RH-7777) cells were propagated at 37°C in Dulbecco's minimal essential medium supplemented with either 10% fetal calf serum or 10% fetal calf serum +10% horse serum, respectively. *Drosophila* embryo SL2 cells were maintained at 25°C in Schneider medium supplemented with heat-inactivated 10% fetal calf serum. McArdle RH-7777 cells were transiently transfected with a DOTAP liposomal method and analyzed as described previously (12). C3H10T1/2 cells were transfected by the same methodology with slight modifications. The cells were plated at a density of 2×10^6 cells/60-mm dish and transfected the next day with 2.5 μg of specific *Ctpct*-luciferase plasmid with or without a Sp expression plasmid (pPacSp1, pPacSp2, or pPacSp3) or control vector (pPacO). Transfected cells were grown overnight in normal medium and than changed to the medium supplemented with 0.5% fetal calf serum. After arresting at low serum for 2 days, transfected cells were stimulated to grow for 24 h in medium containing 10% serum. Reporter assays were performed as recommended (Promega) and protein was assayed by the BioRad method.

SL2 cells were plated at a density of $2–3 \times 10^6$ cells/60-mm dish and transfected by a standard calcium phosphate co-precipitation method (21). Each plate received 2 μg of *Ctpct*-reporter plasmid and various amounts (0–2 μg) of Sp-expression plasmid or control vector. No β-galactosidase vector was added because the CMV promoter from the vector would compete with the *Ctpct* promoter for the binding of Sp proteins. Luciferase activities were normalized with protein content and no significant variation among dishes was obtained. Thus, two dishes were used for each data point, cells from both dishes were combined during harvest, and cell extracts assayed as recommended (Promega).

Nuclear extracts and electromobility shift assays

Nuclear extracts were prepared as described by Andrews and Faller (22). The following double-stranded DNA probes were used: the probes containing Sp-consensus sites A (−25/−4), 5′-TTTCCCGGGCGTCTCCCCGCA-3′, B(−88/−50), 5′-GAGGTG GCATTGACAAGAGGGCGGGCGGGAGGCGGGACT-3′, and C (−150/−114), 5′-CCACGCGCCCGGCCCCTCTGGAAGCGGAA CTACTCTG-3′, the mutated probes ΔA, ΔB, and ΔC (as described above), the nonspecific oligonucleotide, 5′-CATGTTATGCATAT TCCTGTAAG-3′ (Stat1 consensus), the probe D1, −98/+38, containing both A and B sites and the probes −52/+38 and −201/−170 containing sites A and C, respectively. Probes −52/+38 and −98/+38 were prepared from LUC.D1 and LUC.D3 vectors by restriction digestion and purification. The probe −201/−170 was prepared by PCR by using specific promoter primers (12). The fragments were 3′-end labeled with ^{32}P-labeled dCTP and Klenow polymerase and assayed as previously described (12). For supershift analysis, 1–4 μl of antibody specific for either Sp1, Sp2, or Sp3 (Santa Cruz Biotechnology) was added for 0.5–1 h after incubation of the probe with nuclear proteins. For competition studies, 50- to 200-fold molar excess of unlabeled DNA was incubated with nuclear proteins, 30 min before addition of the labeled probe. The reaction products were separated on 5–6% non-denaturing polyacrylamide gels and analyzed by autoradiogpahy.

Immunoblotting

Nuclear extracts (20–60 μg) were heated at 95°C for 5 min and separated on 12.5% denaturing polyacrylamide gels. The proteins were transferred onto nitrocelulose membranes and in-

cubated overnight with 5% skim milk in TTBS (20 mm Tris-HCl, pH 7.5, 500 mm NaCl, 0.05% Tween-20). The membranes were washed in TTBS, then incubated at 4°C for 4 h with rabbit polyclonal serum raised against either Sp1, Sp2, or Sp3, and after 5–6 washes with TTBS, the membranes were incubated with goat anti-rabbit antiserum (horseradish peroxidase-conjugated, Boehringer Mannheim) at 4°C for 4 h. The membranes were washed 5–6 times with TTBS, developed with enhanced chemiluminescence reagent (Pierce) and exposed to XAR-5 film (Kodak). To reprobe the blots, the membranes were stripped in 100 mm mercaptoethanol, 2% SDS, 62.5 mm Tris-HCl, pH 6.8, at 50°C for 30 min and subjected to the above procedure.

RESULTS

Sp1 and Sp3, but not Sp2, activate *Ctpct* promoter transcription in SL2 cells

The activation properties of Sp1, Sp2, and Sp3 towards the *Ctpct* promoter were studied in SL2 cells as they lack endogenous Sp-like activity (19). Previously, we have established that *Ctpct* promoter deletion mutants can be activated by Sp1 (12). Here, we tested the activation properties of Sp2 and Sp3 and observed that Sp2 alone was unable to initiate transcription (data not shown). Sp3 was very active and its properties relative to Sp1 are shown in **Fig. 1** (inset). A constant amount of the *Ctpct* reporter plasmid LUC.C7 (−1268/+38) was transfected in SL2 cells along with various amounts of the Sp1 or Sp3 expression plasmids, pPacSp1 and pPacSp3. In both situations, a dose-dependent activation of the *Ctpct* promoter was observed (Fig. 1, inset). The activation by Sp1 was saturable while activation by Sp3 required higher amounts of pPacSp3 for a similar level of activation and did not reach saturation.

To test which promoter elements were sufficient to mediate transcriptional activation by Sp3, we performed functional luciferase assays with the full-length promoter and a series of 5′-deletion mutants. As shown in Fig. 1, the highest activation of the *Ctpct* promoter by Sp3 (75- to 100-fold) was achieved only with the longer constructs, LUC.C5, LUC.C7, and LUC.C8. The shorter constructs, LUC.D1, LUC.D2, and LUC.D3, showed little or no activation. Thus, the minimal sequence requirement for Sp3 activation is 201 bp upstream from the transcriptional start site (LUC.C8, −201/+38). This region contains three putative Sp1-binding elements, that we have designated as A (−31/−9), B (−88/−50), and C (−148/−128) (21). The sequence further upstream played no significant role in the Sp3 *trans*-activation. In addition, deletion mutants containing only sites A and B (i.e., LUC.D1 and LUC.D2) had dramatically lower activity; the construct containing only site A, LUC.D3, was completely inactive. Taken together, these results show that the Sp3 nuclear factor acts as a strong transcriptional activator and, like Sp1 (12), can initiate transcription in insect cells primarily through the distal Sp-element at site C.

Sp proteins transiently expressed in insect cells bind to the *Ctpct* promoter

We have previously shown that recombinant Sp1 can specifically interact with promoter regions A, B, and C (12). We now examined the expression and the binding of Sp1, Sp2, and Sp3 proteins in SL2 cells. Specific antibodies detected protein bands for Sp1 at 95 kDa, Sp2 at 35 and 50 kDa, and Sp3 at 90 kDa (**Fig. 2A**). We next performed super-shift experiments (Fig. 2B and Fig. 2C) using the SL2 nuclear proteins. When a longer probe was used (Fig. 2B) faster-migrating Sp1 and Sp2 complexes were always more intense than the slower-migrating complexes (lanes 7 and 9; lanes 13 and 15). In contrast, Sp3 protein formed a strong, slower-migrating complex (lanes 11 and 17). The intensity of the Sp3 complex does not imply, however, that Sp3 was significantly more expressed than Sp1 or Sp2. Sp1 and Sp2 proteins formed multiple complexes and the overall specific radioactivity was distributed among them. A similar level of expression of all three Sp proteins was also observed with shorter radio-

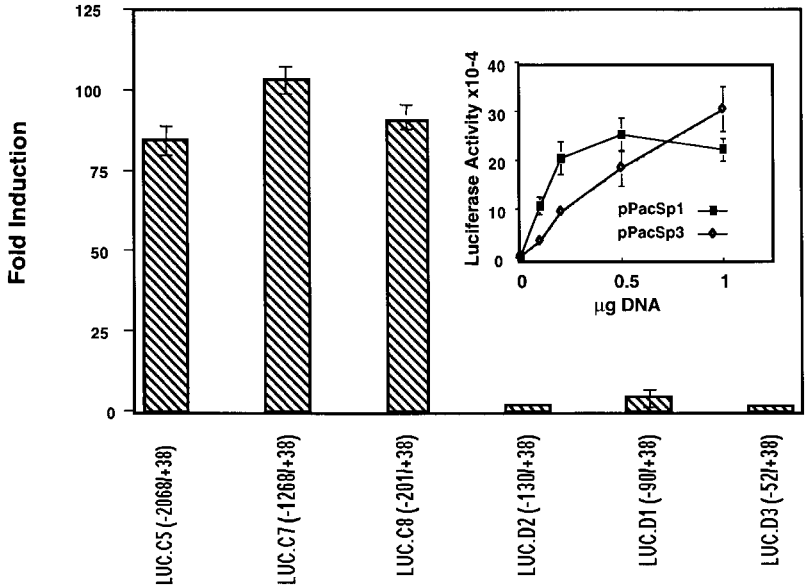

Fig. 1. 5′-Deletion analysis of the mouse *Ctpct* promoter in *Drosophila* SL2 cells. SL2 cells were transfected with 2 µg of the indicated*Ctpct* promoter-reporter constructs and 0.5 µg of pPacSp3. Fold induction of luciferase activity was calculated relative to that in the cells transfected with a reporter plasmid in the presence of control vector pPacO. Inset: Activation of the *Ctpct* promoter by Sp1 and Sp3. SL2 cells were transfected with the indicated amounts of pPacSp1 or pPacSp3 (0–1 µg) and a constant amount (2 µg) of the *Ctpct* promoter-reporter construct LUC.C7 (−1268/+ 38). Luciferase activity is expressed in relative light units/µg of protein and represents the average ± SD from four autonomous transfections. Some error bars could not be seen because of the low values.

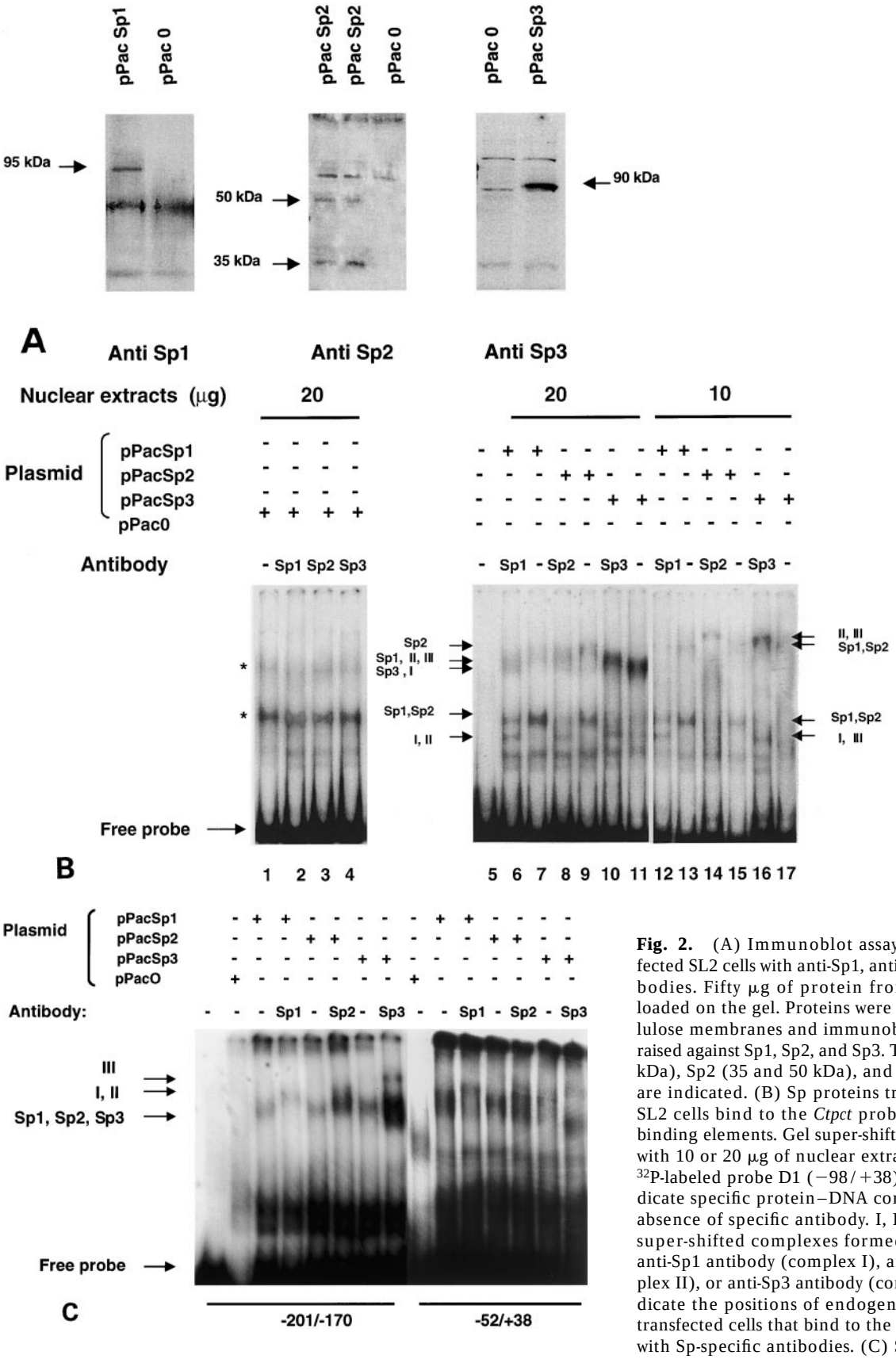

Fig. 2. (A) Immunoblot assays of transiently transfected SL2 cells with anti-Sp1, anti-Sp2, and anti-Sp3 antibodies. Fifty μg of protein from cellular lysates was loaded on the gel. Proteins were transferred to nitrocellulose membranes and immunoblotted with antibodies raised against Sp1, Sp2, and Sp3. The positions of Sp1 (95 kDa), Sp2 (35 and 50 kDa), and Sp3 (90 kDa) proteins are indicated. (B) Sp proteins transiently expressed in SL2 cells bind to the *Ctpct* probe containing multiple binding elements. Gel super-shift assays were performed with 10 or 20 μg of nuclear extracts and 20,000 cpm of ^{32}P-labeled probe D1 (−98/+38). Sp1, Sp2, and Sp3 indicate specific protein–DNA complexes formed in the absence of specific antibody. I, II, and III indicate the super-shifted complexes formed from the binding of anti-Sp1 antibody (complex I), anti-Sp2 antibody (complex II), or anti-Sp3 antibody (complex III). Asterisks indicate the positions of endogenous proteins from untransfected cells that bind to the probe but do not react with Sp-specific antibodies. (C) Sp proteins transiently expressed in SL2 cells bind to the *Ctpct* probe containing single binding elements. Gel supershift assays were performed with 10 μg of protein and 20,000 cpm of ^{32}P-labeled probe −201/−170 (Site C) or −52/+38 (Site A) as indicated. The rest of the figure is the same as in (B).

labeled probes containing single binding site A or C, when single DNA complexes were formed (Fig. 2C).

Synergistic and antagonistic properties of Sp proteins

To investigate how a combination of different Sp proteins can modulate expression of the *Ctpct* promoter, SL2 cells were transfected with a wild-type promoter-reporter construct (2 μg of LUC.C7), a constant amount of the Sp1 expression plasmid (0.2 μg), and various amounts of Sp2 or Sp3 expression plasmids (**Fig. 3A**). Co-transfections with Sp1 and Sp3 showed that Sp3 was a positive regulator whose stimulatory effect was more apparent at higher concentrations. The maximum stimulated activity was 4- to 5-fold above the activity of Sp1 alone (Fig. 3A, left panel). In contrast, Sp2 strongly antagonized Sp1 activity in a dose-dependent manner (Fig. 3A, right panel). Co-transfection of higher amounts of Sp2 led to a profound inhibition of the promoter activity to basal levels.

When Sp3 was expressed at a constant level and the expression of Sp1 was increased (Fig. 3B), Sp1 activated the promoter even at low concentrations: 0.25 μg of Sp1 expression plasmid together with 0.2 μg of Sp3 expression plasmid resulted in ~5-fold promoter activation. This suggests a strong synergism between Sp1 and Sp3. The stimulation did not increase further at higher Sp1 concentrations, in accordance with the tendency of the activation by Sp1 to be saturable at higher concentrations (Fig. 1, inset). Regarding the antagonistic properties of Sp2, there were no fundamental differences between Sp1- and Sp3-mediated transcriptions, but the inhibition of promoter activity was weaker when Sp2 was transfected in combination with Sp3 (Fig. 3B) compared with Sp1 (Fig. 3A).

Endogenous mammalian Sp1 and Sp3 bind to the *Ctpct* promoter

Our earlier gel-shift and DNase protection assays demonstrated that the *Ctpct* promoter interacts specifically with nuclear proteins from C3H10T1/2 and McArdle RH-7777 cells (12). We have now investigated the identity of the bound proteins using antibodies specific for Sp1, Sp2, and Sp3 in gel super-shift assays (**Fig. 4**). The results with untransfected cells (lanes 1 to 5, and 13) and the cells transfected with Sp1 (lanes 7 and 8), Sp2 (lanes 9 and 10), or Sp3 (lanes 11 and 12) expression plasmids are shown. Three Sp-related DNA complexes were detected with C3H10T1/2 nuclear extracts and two complexes with McArdle RH-7777 nuclear extracts. Based upon the super-shifts of bound proteins (Fig. 4A), one complex was identified as the Sp1 gene product (lanes 3 and 8), and two (lane 5) and one (lane 12) complexes as the Sp3-related products. In contrast to insect cells, the antibody against Sp2 caused no specific super-shift with nuclear extracts from untransfected mammalian cells (lane 4). However, a very intense band that migrated with the Sp1 band was observed in the presence of Sp2 antibody when nuclear extracts from Sp2-transfected cells were used (lane 10). Pre-incubation of nuclear extracts from Sp1- and Sp3-transfected cells with the DNA probe caused no significant increase in the Sp1 or Sp3 band intensity relative to that in untransfected cells (lane 7-Sp1 and line 11-Sp3 vs. line 13-control).

Sp2-related proteins could not be detected in McArdle RH-7777 and C3H10T1/2 cells by immunoblotting when using the same antibody used in gel-shift experiments (data not shown). Endogenous Sp1 and Sp3 proteins were, however, detected in multiple forms (Fig. 4B) and even with a high background, at slightly increased levels in over-expressing Sp3 cells (Fig. 4B). The detection of one Sp1/DNA complex (Fig. 4A) relates to the presence of three similar proteins (89, 93, and 105 kDa) that represent different phosphorylation forms of Sp1 as suggested initially (13, 16). The observation of two Sp3-related bands (Fig. 4A) agrees with the presence of multiple Sp3 species of distinct size. A doublet at 70 and 66 kDa was much more prominent than bands at 116 kDa (singlet), 97 kDa (doublet), or 50 kDa (doublet). In accord with previous studies, the bands at 116 and 97 kDa encode an active Sp3 that was enhanced in Sp3-transfected cells (Fig. 4B). The lower migrating species of Sp3 at 66–70 kDa

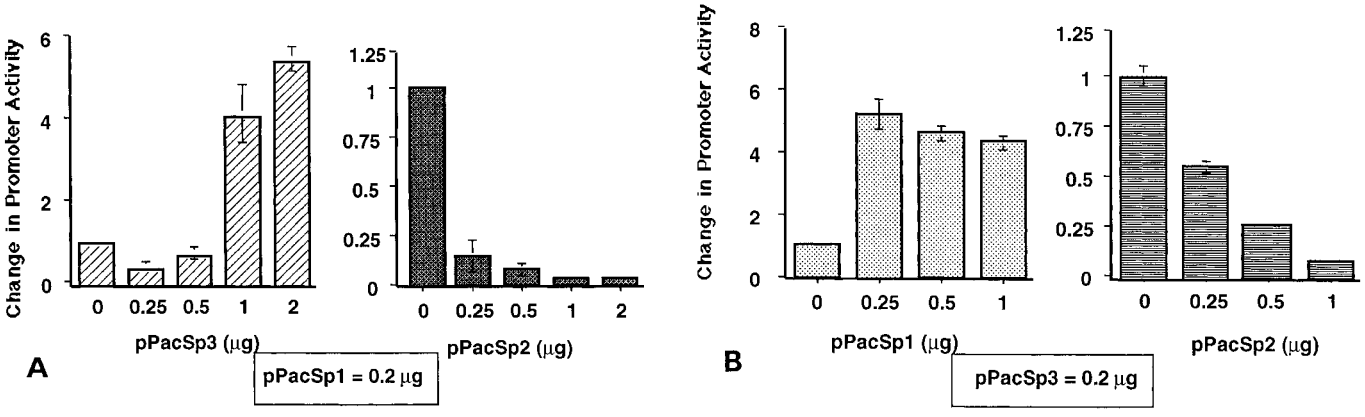

Fig. 3. Sp3 is an activator, and Sp2 a repressor, of the *Ctpct* promoter in SL2 cells. LUC.C7 (−1268/+38) reporter (2 μg) was transfected with (A) 0.2 μg of pPacSp1 and indicated amounts (0–2 μg) of pPacSp2 (right panel) or pPacSp3 (left panel); or (B) 0.2 μg of pPacSp3 and indicated amounts of pPacSp1 (left panel) or pPacSp2 (right panel) vectors. Changes in promoter activity represent the change in luciferase expression relative to the expression driven by pPacSp1 (A) or pPacSp3 (B) alone. The values represent averages of three independent transfections ± SD. Some error bars are too small to be visible.

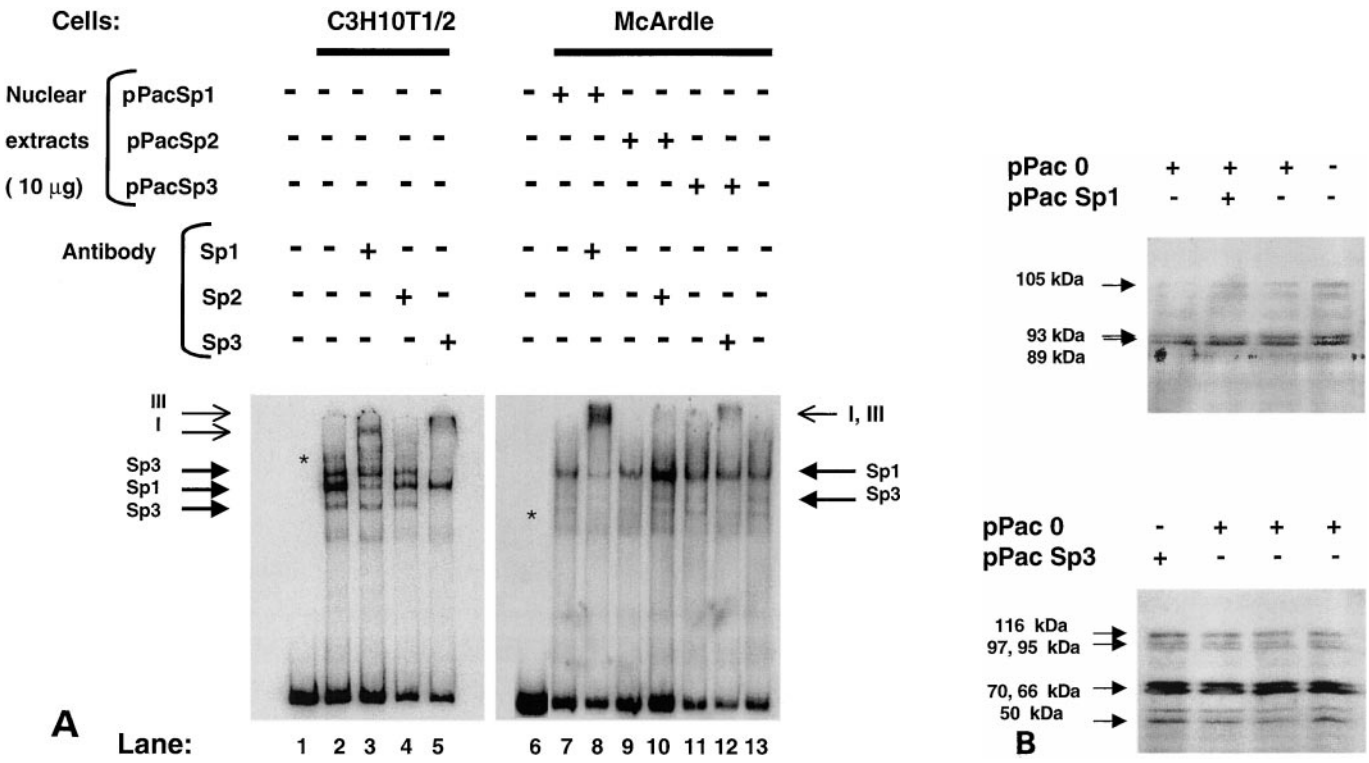

Fig. 4. (A) Gel super-shift analysis of nuclear extracts from C3H10T1/2 and McArdle RH-7777 cells. Nuclear proteins (10 μg) from C3H10T1/2 (lanes 1–5) and McArdle RH-7777 (lanes 6–13) cells were incubated with labeled probe D1 (−98/+38) in the absence or presence of specific antibodies. Specific complexes containing Sp1 and Sp3 are indicated as Sp1 and Sp3. Asterisks depict additional Sp-unrelated complexes formed with McArdle RH-7777 and C3H10T1/2 nuclear extracts. The super-shifted bands for Sp1 are depicted as Complex I and those for Sp3 as Complex III. Lane 13 represent a binding reaction with nuclear proteins from McArdle RH-7777 cells transfected with control vector; lanes 2–5 represent a binding reaction for C3H10T1/2 untransfected cells. (B) Immunoblot detection of multiple Sp1 and Sp3 proteins in McArdle RH-7777 cells. McArdle RH-7777 cells transfected with 5 μg of pPacSp1, pPacSp3, or pPacO were fractionated on 12.5% SDS-polyacrylamide gels, blotted on nitrocellulose, and incubated with anti-Sp1 (upper panel) or anti-Sp3 (lower panel) antibodies. Fifty μg of protein was loaded in each lane on the gels. The arrows indicate the positions of three species of Sp1 (105, 93, and 89 kDa) and six species of Sp3 (116, 97, 95, 70, 66, and 50 kDa).

probably arise from the internal translational initiation within Sp3 mRNA which generates an inhibitory form of Sp3; the 50 kDa species is likely to be the result of proteolytic degradation of Sp3 (23).

Over-expressed Sp2 and Sp3, but not Sp1, stimulate the *Ctpct* promoter in mammalian cells

To test the individual abilities of Sp1, Sp2, and Sp3 to activate or repress *Ctpct* transcription in mammalian cells, we conducted co-transfection experiments in synchronized C3H10T1/2 cells (**Fig. 5**). As controls, we used promoters sensitive to Sp proteins, i.e., SV-40 and CMV promoter/enhancer luciferase constructs. Co-transfection of 1 μg of Sp1 expression plasmid significantly augmented the expression of luciferase gene driven by the SV-40 (7-fold) and CMV promoter/enhancer (4-fold) but had virtually no effect on the luciferase activity driven by the *Ctpct* promoter (LUC.C8, −201/+38). Over-expression of Sp3 enhanced the luciferase expression from both *Ctpct* (4-fold) and viral promoters (6- to 8-fold). Surprisingly, the over-expression of Sp2 also enhanced the transcription driven by all three promoters, *Ctpct* (7-fold), SV40 (4-fold), and CMV (8-fold).

Mutated elements A and B fail to compete for Sp1 and Sp3 binding

To test the hypothesis that the three promoter regions have different roles in regulating transcription and that these roles might be cell-type specific, we examined the functional consequences of mutating the promoter sequence to prevent Sp-protein binding at these sites. The mutations introduced involved changes at positions A, B, and C as indicated: ΔA (5′-TCAGATGTTTC-ggtac-C GTCTCC-3′), ΔB (5′-CAAGAGGG-aattca-GGAGGCGG GAACTT-3′), and ΔC (5′-ACGCGCCC-gagct-CTCTG GAA-3′). The effectiveness of binding of the mutated oligomers was tested in gel-shift competition assays with nuclear extracts from C3H10T1/2 and McArdle RH-7777 cells. As demonstrated in **Fig. 6**, the mutated versions of sites A (ΔA) and C (ΔC) did not effectively compete with the specific non-mutated probe for the binding of Sp1 and Sp3 nuclear proteins. Minor competition with the oligonucleotide for the mutated site B (ΔB) was observed. This site is a cluster of three overlapping Sp-sites (12) and the mutation significantly reduced but did not completely abolish the binding of Sp1 and Sp3.

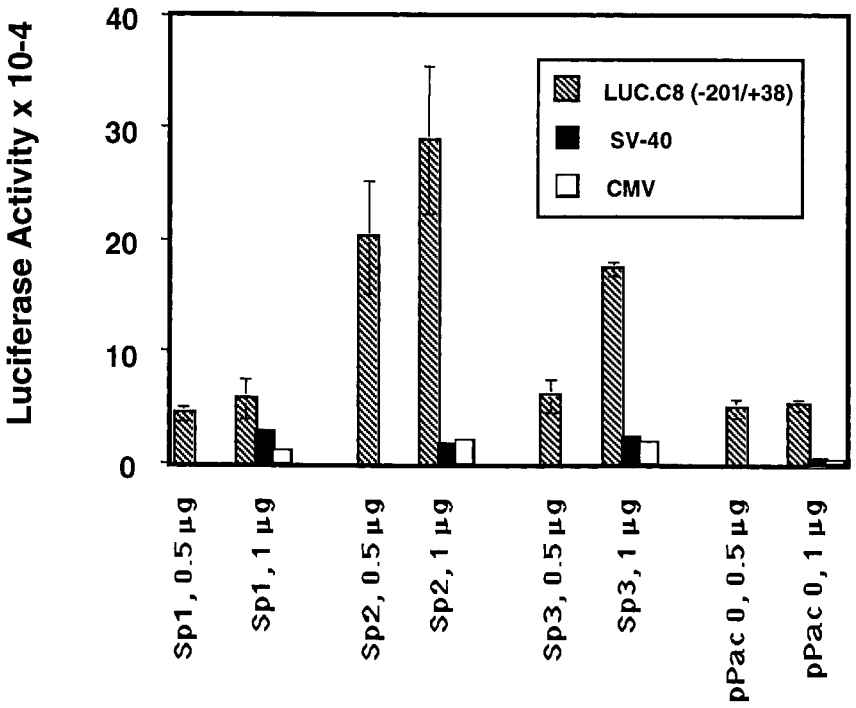

Fig. 5. Overexpressed Sp2 and Sp3 in C3H10T1/2 cells stimulate the *Ctpct* promoter and the viral promoters SV-40 and CMV. The *Ctpct* reporter, LUC.C8 (−201/+38) or viral reporters, CMV.LUC and pGl3-basic (SV-40) were transfected into synchronized C3H10T1/2 cells along with 0.5 and/or 1 μg of the pPacSp1, pPacSp2, or pPacSp3 expression plasmids or the control vector pPacO. Luciferase activity was expressed in relative light units/μg of protein and represents the average ± SD from three independent experiments. Some error bars are too small to be visible.

The *Ctpct* promoter elements B and C negatively cooperate in mammalian cells

Since mutations at sites A, B, and C disabled or significantly reduced Sp protein binding, they were introduced into the LUC.C7 (−1268/+38) construct (**Fig. 7A**) and analyzed in transient expression experiments for their activity (Fig. 7B). In general, the *Ctpct* promoter-reporter mutants had similar trends of expression in McArdle RH-7777 and C3H10T1/2 cells. However, lower expression was observed in C3H10T1/2 cells than in McArdle RH-7777 cells. This may be because the C3H10T1/2 cells were transfected before synchronization and harvested at 3.5, not 2 days after transfection.

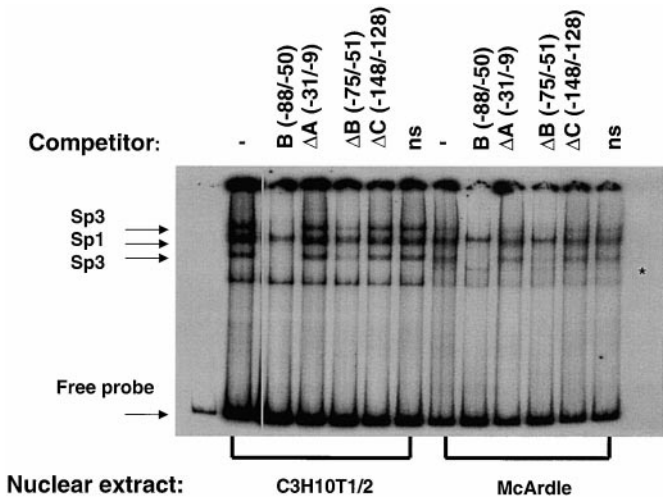

Fig. 6. Competitive gel-mobility shift analyses of the *Ctpct* promoter using wild-type and mutated competitors. The wild-type oligonucleotide competitors are designated as A, B, and C, the mutated competitors as ΔA, ΔB, or ΔC, and an unrelated competitor as ns. The competitors were added in 200-fold molar excess. Specific Sp1 and Sp3 protein–DNA complexes are designated on the left by arrows, and an additional Sp-unrelated complex was indicated on the right by an asterisk.

The order of activity for double mutants with single active site, A (LUC.C7.ΔBΔC), B (LUC.C7.ΔAΔC), or C (LUC.C7.ΔAΔB), was B > C > A, in agreement with previous predictions (12). The activity of single mutants containing two active sites, A and B (LUC.C7.ΔC) or A and C (LUC.C7.ΔB), was an additive function of individual activities of A and B (LUC.C7.ΔBΔC + LUC.C7.ΔAΔC) or A and C (LUC.C7.ΔBΔC + LUC.C7.ΔAΔB). This result indicates that although all three Sp-binding sites could participate in transcription, they have different affinity for the nuclear proteins and no significant synergism exists between the sites A and B or A and C in this process.

To establish the relationship between sites B and C, the activity of a mutant containing both active sites was compared with the activity of the mutants containing either site B or site C (Fig. 7B). Surprisingly, the activity of the mutant containing both sites B and C (LUC.C7.ΔA) was significantly lower than the sum of activities for the mutants containing only site B or site C (LUC.C7.ΔAΔC + LUC.C7. ΔAΔB). This result established the existence of a strong negative cooperativity between sites B and C in mammalian cells.

To examine further the significance of site B, an insertion mutant (LUC.C7.ΔB,ins) was also included in the study (Fig. 7B). This mutant contained two ΔB sequences, 5′-CAAGAGGG-aattca-GGAGGCGGGACTT-aa-CAAGAGGG-aattca-GGAGGCGGGACTT-3′ inserted at the position −71/−58. This particular mutation introduced two identical, but separated, Sp-binding sites. The consensus B sequence is an overlapping cluster of three Sp-sites, 5′-

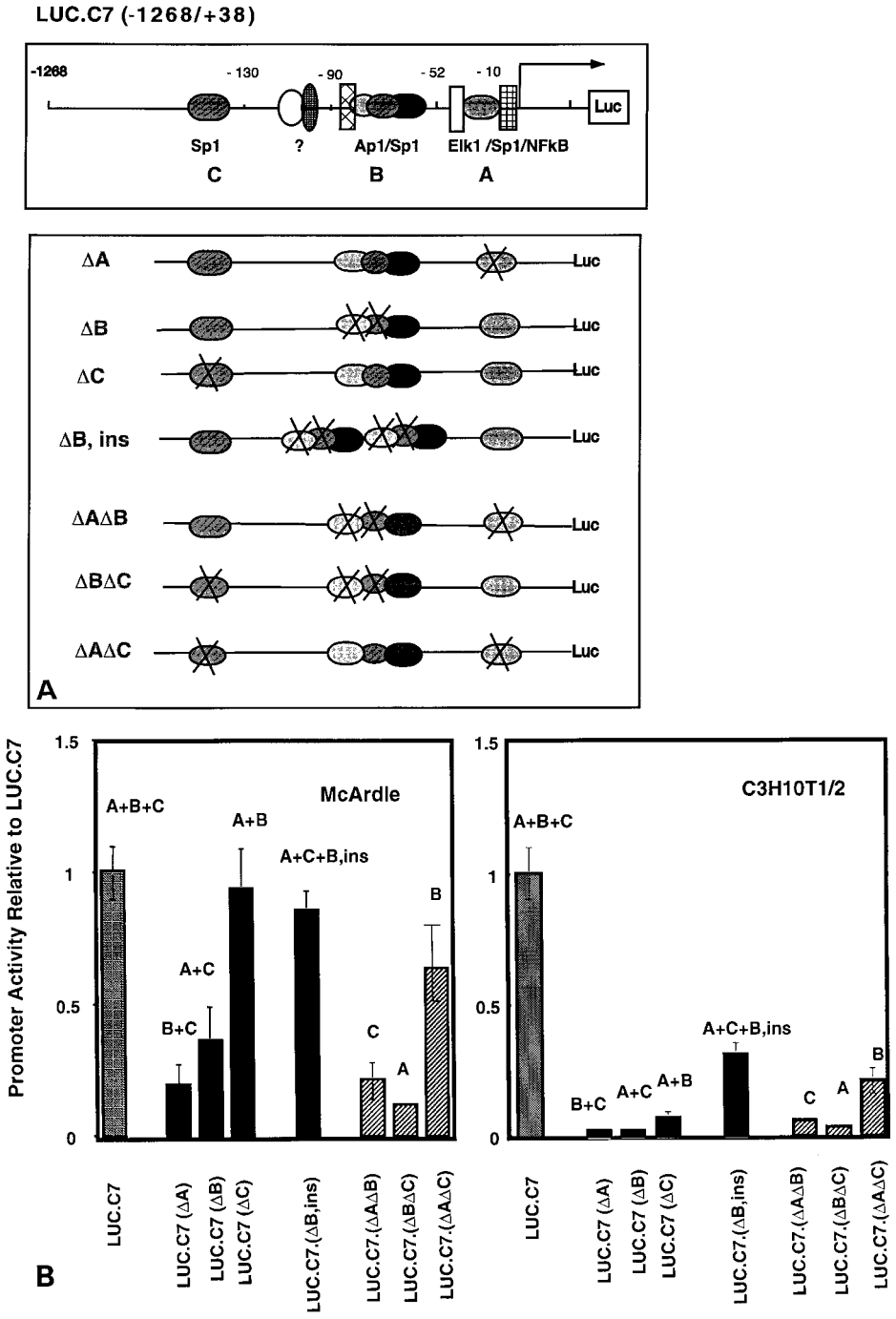

Fig. 7. (A) Positions of mutated sites ΔA, ΔB, ΔC, and ΔB,ins in the *Ctpct* promoter and the tested mutants. Upper panel: structural organization of the *Ctpct* promoter-luciferase reporter construct −1268/+38 bp (LUC.C7) showing the position of three Sp binding sites, A, B, and C, relative to the transcription start site +1 (indicated by an arrow). Putative binding sites for Ap1, Elk1, NFκB, and two unknown transcription factors are indicated. Lower panel: single or double mutations were introduced into the LUC.C7 promoter-reporter construct at positions A (−31/−9), B (−88/−50), and C (−148/−128). All positions contained GC-rich regions that could bind Sp nuclear proteins. A is a "loose" Sp1 site, B is a cluster of three overlapping Sp1 sites, and C is a Sp1 consensus binding site. The GC sequences were replaced with unrelated sequences corresponding to restriction enzyme sites *Kpn I* (ΔA), *Eco RI* (ΔB), and *Sac I* (ΔC). The insertion mutant ΔB,ins contained two ΔB sites. (B) Activation of the *Ctpct* promoter in the absence of one or two functional sites in McArdle RH-7777 and C3H10T1/2 cells. Wild-type (LUC.C7) and mutant constructs, LUC.C7.(ΔA), LUC.C7.(ΔB), LUC.C7.(ΔC), LUC.C7.(ΔB,ins), (LUC.C7.(ΔAΔB), LUC.C7.(ΔBΔC), and LUC.C7.(ΔAΔC), were transfected into C3H10T1/2 and McArdle RH-7777 cells. The unmutated sites are indicated as A, B, C. The results represent the luciferase activity of each reporter construct as the means ± SD of four experiments relative to that of the wild-type construct LUC.C7 (activity 1).

GGGCGGGCGGGAGGCGGGA-3′; the sequence remaining in the ΔB insert is underlined. Thus, the mutation not only changed the number and configuration of the Sp-cluster but also changed the promoter arrangement. Surprisingly, the insertion mutation caused no dramatic changes in the promoter activity in McArdle RH-7777 cells (Fig. 7B) and SL2 (**Fig. 8**) cells.

Altogether, the mutation analysis in mammalian cells (Fig. 7B) shows that the main positive regulation of the *Ctpct* promoter with Sp-related proteins is from the high affinity site located at position B, with minor contribution from site A, and significant antagonistic effect from site C.

Ctpct promoter element C is a positive regulator and element A is a negative regulator in insect cells

To determine the functional significance of the three promoter regions with respect to Sp1- and Sp3-mediated transcription, A, B, or C single and double mutants were co-transfected with Sp1 or Sp3 expression plasmids in SL2 cells (Fig. 8). In agreement with previous results (Fig. 1, ref. 12), Sp1 and Sp3 were similar in initiating transcription from mutated promoter-reporter constructs but the relative activities depended on the number and the position of Sp-binding sites. Single mutations of site B (LUC.C7.ΔB) or site C (LUC.C7.ΔC) resulted in 80–90% reduction in luciferase activity relative to the wild-type construct. In contrast, a 50% increase in luciferase activity was obtained when site A was mutated (LUC.C7.ΔA).

From consideration of the deletion analysis (Fig. 1), one would expect that the double mutations leaving only active site C (LUC.C7.ΔAΔB) could contribute an activity similar to the wild-type promoter and this was observed (Fig. 8). However, in disagreement with deletion analysis, the double mutation showed that site B alone (LUC.C7.ΔAΔC) could also contribute 60–75% of the total activity. These results indicated that interaction with the upstream promoter sequence is important for the action of Sp proteins through site B. Double mutants containing a functional site A (LUC.C7.ΔBΔΔC) possessed only minimal 10–15% activity relative to the wild-type promoter indicating low contribution from this site.

A 50% increase in activity above the wild-type level for the single mutant containing both B and C sites (LUC.C7.ΔA) could be explained with a suppressive role of site A in insect cells. This is confirmed further by other single mutations in Fig. 8. The activity of single mutants maintaining together sites A and B (LUC.C7.ΔC) or A and C (LUC.C7.ΔB) was significantly reduced relative to the activities expected from the individual contributions of these sites (LUC.C7.ΔBΔC + LUC.C7.ΔAΔC, and LUC.C7.ΔBΔC + LUC.C7.ΔAΔB).

Taken together, the mutation analysis in Fig. 8 revealed that the upstream promoter sequence is important for the action of Sp1 and Sp3 proteins at site B, that site A is suppressive, and site C activating. This is different from the data in mammalian cells (Fig. 7B), indicating that the *Ctpct* promoter operates through different mechanisms in insect and mammalian cells.

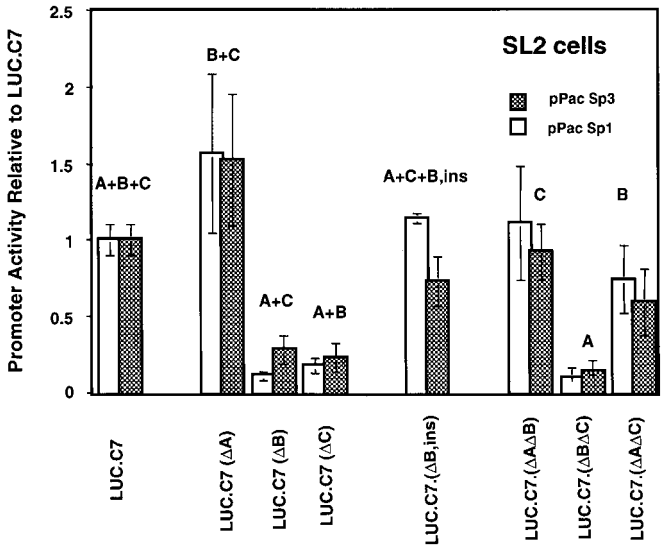

Fig. 8. Activation of the mutated *Ctpct* promoter by exogenous Sp1 and Sp3 in SL2 cells. Wild-type and mutated *Ctpct* promoter-reporter constructs containing the region between −1268/+38 bp were assayed as described in the legend to Fig. 7 and Materials and Methods. SL2 cells were transfected with 2 μg of mutated promoter-reporter plasmids along with 0.2 μg of the pPacSp1 or pPacSp3 expression plasmids or control vector pPacO. The results represent the luciferase activity of each reporter construct as the mean ± SD of 3 to 5 experiments relative to LUC.C7 (activity 1).

Sp2 antagonizes the Sp1- and Sp3-mediated transcription through all three *cis*-acting elements

To explore which promoter regions account for the Sp2-mediated inhibition of the promoter activity in insect cells (Fig. 3), the promoter mutants harboring single active sites A (LUC.C7.ΔBΔC), B (LUC.C7.ΔAΔC), and C (LUC.C7.ΔBΔA) were expressed in SL2 cells along with either Sp1/Sp2 or Sp3/Sp2 expression plasmids (i.e., pPacSp1 + pPacSp2 or pPacSp3 + pPacSp2). The results are summarized in **Table 1** in which "activity" represents the activity of the mutated promoter relative to the wild-type promoter, and "inhibition" is the activity for the particular mutant after addition of Sp2 relative to its activity before the addition of Sp2. When Sp2 was expressed in combination with Sp1 the remaining activity of the mutants containing active sites C or B (LUC.C7.ΔAΔB and LUC.C7.ΔAΔC) was 62 and 66%, respectively. The construct containing site A only (LUC.C7.ΔBΔC) was the least active with only 17% of the activity remaining. When Sp2 was expressed with Sp3, the activities followed a similar trend as with Sp1/Sp2. Taken together these results show that the suppression with Sp2 did not change the general relationship among the mutants relative to the experiments with Sp1 or Sp3-only transcription (compare Table 1 and Fig. 8), as might be expected if Sp2 acted on all three sites, A, B, and C.

The "inhibition" of the wild-type promoter by Sp1/Sp2 and Sp3/Sp2 was 91% and 69% relative to Sp1 and Sp3-only transfections, in agreement with the competition results in Fig. 3. When mutants A, B, or C were co-transfected with Sp1/Sp2, a similar level of inhibition was obtained (88–95%) as for the wild-type promoter (91%). When mutants

TABLE 1. Analysis of Sp2 binding to the *Ctpct* promoter

Transfection[a]	pPacSp1 + pPacSp2		pPacSp3 + pPacSp2	
	Activity[b]	% Inhibition[c]	Activity	% Inhibition
Wild-type *Ctpct* promoter				
LUC.C7	1	91.3 ± 5	1	69 ± 9
Mutants				
LUC.C7(ΔAΔB)	0.62 ± 0.12	95.4 ± 11	0.97 ± 0.3	67.4 ± 11
LUC.C7(ΔBΔC)	0.17 ± 0.01	88.5 ± 19	0.14 ± 0.01	68.6 ± 6
LUC.C7(ΔAΔC)	0.66 ± 0.20	94.0 ± 13	1.1 ± 0.17	42.2 ± 12

[a] SL2 cells were transfected with 2 μg of each *Ctpct* promoter-reporter construct and 0.2 μg of Sp-expressing plasmids. Various amounts of control DNA were included to maintain constant amount of DNA.

[b] The activity represents the mean luciferase activity (relative light units/μg protein ± SD) of the mutants relative to the mean luciferase activity of the wild-type promoter-reporter construct.

[c] Percent inhibition is expressed as the mean percentage inhibition of reporter activity ± SD in the presence of Sp2 relative to its own activity without Sp2.

were co-transfected with Sp3/Sp2, the inhibition was similar to that of the wild-type promoter (70%) for A and C (67–69%), but lower for mutant B (42%). The main conclusion from this analysis is that when competing with Sp1, Sp2 showed no preference for sites A, B, and C. However, when competing with Sp3, Sp2 showed less competition for the high affinity site B relative to the low affinity sites A and C. These results reflect how Sp2 could modulate the promoter activity depending on whether it was in competition with Sp1 or Sp3.

DISCUSSION

Role of Sp family of transcription factors

The recent discovery of the Sp family of transcription factors highlights a previously unknown level of complexity of transcriptional regulation by Sp1. The family includes the Sp1-related proteins, Sp2, Sp3, and Sp4, as well as GC-binding proteins BTEB1 and BTEB2, which have low homology to Sp1 (16). The family members share the DNA binding zinc-finger domain, but differ in domains responsible for interaction with other nuclear factors and co-activators. The functional diversity often seen among the Sp-dependent promoters is still not well understood (13, 14). The arrangements of the binding sites, the functional differences among the family members, the factors' availability, and their interactions with other transcription components, all define conditions for transcriptional regulation (15, 16).

So far, only Sp1, and to a lesser extent Sp3, have been studied extensively. The importance of other members of the Sp-family has yet to be established. In this paper we have analyzed the role of Sp1, Sp2, and Sp3 in the activation of the murine CTα gene. We have also evaluated the functional relationship among the three Sp-binding elements in the *Ctpct* promoter region. We did not investigate Sp4 as it has been reported to be brain specific (16), whereas CTα is expressed in all nucleated cells. We have recently demonstrated that the 5′-flanking region of the murine *Ctpct* promoter contains three *cis*-elements that are critical determinants of CTα transcription (12). The elements are localized in the minimal-promoter region (a low-affinity GC-like motif, site A) in the proximal promoter region (a high-affinity GC cluster, site B), and in the distal promoter region (a medium-affinity GC consensus, site C). Within this study we have established the functional relationship among the Sp-binding elements A, B, and C when they are occupied with Sp1, Sp2, or Sp3 nuclear factors and have examined how the combination of these factors and the cell type can modulate the *Ctpct* promoter activity.

Sp1 and Sp3 function as transcriptional activators of the *Ctpct* promoter

Despite significant structural similarities between Sp1 and Sp3, they can differ dramatically in terms of transcriptional regulatory properties. Sp1 is exclusively a transcriptional activator while Sp3 can function as either an activator or repressor depending on the promoter and the cellular context (16, 24–28). Both the 5′-end deletion analysis (Fig. 1) and the mutational analysis (Figs. 7 and 8) revealed that for *Ctpct* transcription Sp3 is functionally equivalent to Sp1 and acts as an activator. Sp3 acted on the same target sequence as Sp1, but unlike Sp1, was able to stimulate the *Ctpct* promoter when over-expressed in mammalian cells (Fig. 5).

The modulation of the *Ctpct* promoter by Sp3 in insect cells (Fig. 3) is consistent with a mechanism in which Sp3 displaces bound Sp1 at lower concentrations and hinders the access of Sp1 to the promoter at higher concentrations. However, the DNA-displacement mechanism has often been used as an explanation for Sp3 suppression, not stimulation, of Sp1 activity (28). In mammalian cells, we showed that Sp3 was able to increase transcription when Sp1 was at concentrations too high to be displaced by Sp3 (Fig. 5). This stimulatory effect of Sp3 could be explained by a property of Sp3 to enhance transcription by a different type of interaction, protein-protein interactions, on the *Ctpct* promoter (16, 28). Stimulation by Sp3 in mammalian cells, however, could also be possible if the active form of the over-expressed Sp3 protein replaced a transcriptionally inactive form of Sp3. Multiple Sp3-related proteins are present at high abundance in mammalian cells (Fig. 4B) and some of these could potentially serve as inhibitors of *Ctpct* transcription (23).

Sp1, but not Sp3, can form multimeric complexes that can exert transcriptional superactivation by binding to basal transcription factors (16, 28). The transcriptional synergism observed between Sp1 and Sp3 in insect cells could be explained by the presence of similar Sp1 complexes that were able to induce conformational changes by interacting with both, the basal transcription factors and Sp3. This synergistic effect was more apparent at lower concentrations (Fig. 2) when the binding sites were not fully occupied with Sp1 and Sp3. The initial binding of both factors at one site, likely the multiple binding sites B, might be followed by an increased affinity for Sp1 at

other sites, likely site C, which allowed an increased biological response in insect cells.

Sp1 over-expression in mammalian cells, however, did not enhance *Ctpct* promoter activity (Fig. 5). This suggests that binding of Sp1 to the *Ctpct* promoter was saturated. Sp1 showed a similar saturation tendency in insect cells (Fig. 1). Thus, the increased expression of the Sp1 protein may be of little importance in regulating the levels of CTα under basal conditions, in agreement with the proposal (16, 19) that Sp1 is a constitutive transcription factor. However, Sp1-transactivation can be regulatory under certain conditions (29–31). Different factors such as epidermal growth factor (32), insulin-like growth factor I (30), and transforming growth factor β (31) might regulate Sp1 activity. Sp1 also becomes phosphorylated during the process of terminal differentiation, which results in the down-regulation of its DNA-binding ability (33). The possible involvement of Sp1 in mediating Rb function (32), and the ability of Sp1 to regulate the cyclin-dependant kinase inhibitor $p21^{CIP1/WAF1}$ (34), indicate a role in cell-cycle regulation. Hence, Sp1 might play a role in the regulation of the CTα gene during cellular growth, differentiation, and tumor progression.

Sp2 is both an activator and a suppressor of the *Ctpct* promoter

A novel observation in this study is that Sp2 acts as a dual regulator of the *Ctpct* promoter. This nuclear factor has not been studied extensively and only scant evidence is provided about its biological function. Sp2 was isolated from a cDNA library as a GT binding protein, with much weaker binding than Sp3 (17). To date, Sp2 has not been shown to act as either an activator or repressor of transcription (16) and its potential role in transcription activation was unclear. Here, we demonstrated for the first time that Sp2 is potentially an active transcription factor that may play an important role in CTα gene expression. Sp2 did not activate transcription from the *Ctpct* promoter in insect cells, but acted as a strong repressor that could completely block the ability of either Sp1 or Sp3 to drive *Ctpct* transcription (Fig. 3). This is consistent with a DNA-displacement mechanism. The observation that Sp2 completely blocked transcription at high concentrations suggests that Sp1 and Sp3 can be fully displaced by Sp2. The functional contribution of different promoter regions to Sp2 repression was experimentally addressed by using *Ctpct* promoter mutants containing single binding sites, A, B, or C. No functional distinction among the three sites was found (Fig. 3, Table 1), providing further evidence that Sp2 binds to the same regions as Sp1 and Sp3.

The stimulatory function of Sp2 in mammalian cells could not be explained by a simple displacement mechanism, but rather by a complex type of higher order interactions. Sp2 might directly associate with co-activator proteins and thus facilitate the recruitment of other components of the transcriptional machinery. Alternatively, Sp2 complexes might activate the *Ctpct* promoter indirectly, by acting on the transcription of a significant nuclear factor. Similar types of interactions with other transcription factors and co-activators are well established for Sp1 and other nuclear proteins (35–38) but no studies with Sp2 have been reported. Thus, the regulatory mechanism for Sp2 activation remains poorly defined and future studies will be required to understand the role of Sp2 in the regulation of *Ctpct* transcription. Interestingly, the cell-type distribution of Sp2 appears to be more limited than that of Sp1 and Sp3 (17) which would suggest that Sp2 may potentially play a role in the CTα expression in specific tissues.

Positive and negative cooperativity among different promoter regions

Mutation analysis showed that three *Ctpct* promoter regions functionally cooperate to exert full promoter activity in insect cells. Sp1 and Sp3 binding to the *Ctpct* promoter can be both synergistic and antagonistic, depending on the number of available binding sites and their positions (Fig. 8). We have identified promoter elements B and C as the main activators and element A as the main suppressor of the Sp-mediated promoter activity in insect cells.

Different cooperativity among elements A, B, and C was observed in mammalian cells (Fig. 7B). The multiple-binding element B retained the same function as in insect cells and acted as an activator of transcription. The low affinity element A was a weak activator, and element C was a strong suppressor, of the promoter activity. These results suggest that elements A and C might act as dual-function response elements that could modulate *Ctpct* transcription better than element B.

The opposite behavior in insect and mammalian cells also suggests that other auxiliary factors may be important in regulation of the expression of the CTα gene. In insect cells, elements C and B can be activated by Sp1 and Sp3 and suppressed by Sp2. In mammalian cells this interaction is altered, likely by the coordinate action of the neighboring sequence and by the simultaneous interactions with other nuclear factors. Similarly, a mutation at the low affinity site A dramatically increases the promoter activity in insect cells but has low effect in mammalian cells. This site is adjacent to the transcription initiation site and the differences obtained could reflect differences in the basal transcription machinery between the insect and mammalian cells.

CONCLUSION

This study provides evidence that transcription factors Sp1, Sp2, and Sp3 can play distinct roles in transactivation of the murine *Ctpct* promoter. Sp1 can act synergistically with Sp3, and Sp2 can strongly inhibit the action of Sp1 and Sp3 in insect cells. When overexpressed in mammalian cells, Sp3 but not Sp1 can stimulate *Ctpct* transcription. Furthermore, Sp2 can be a positive regulator in mammalian systems, where different types of coactivators and basal transcription factors are present. Thus, differences in the abundance of Sp1, Sp2, and Sp3 and their interactions with other nuclear proteins could be critical for regulating the expression of CTα during cell growth and development, carcinogenesis or in other physiological contexts.

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