Regulation of the human ascorbate transporter SVCT2 exon 1b gene by zinc-finger transcription factors

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Abstract

The sodium-dependent vitamin C transporter (SVCT) 2 is crucial for ascorbate uptake in metabolically active and specialized tissues. The present study focused on the gene regulation of the SVCT2 exon 1b, which is ubiquitously expressed in human and mouse tissues. Although the human SVCT2 exon 1b promoter doesn't contain a classical TATA-box, we found that it does contain a functional initiator (Inr) that binds YY1 and interacts with upstream Sp1/Sp3 elements in the proximal promoter region. These elements in turn play a critical role in regulating YY1-mediated transcription of the exon 1b gene. Formation of YY1/Sp complexes on the promoter is required for its optional function. YY1 with Sp1 or Sp3 synergistically enhanced exon 1b promoter activity as well as the endogenous SVCT2 protein expression. Further, in addition to Sp1/Sp3 both EGR-1 and -2 were detected in the protein complexes that bound the three GC boxes bearing overlapping binding sites for EGR/WT1 and Sp1/3. The EGR family factors, WT1 and MAZ were found to differentially regulate exon 1b promoter activity. These results show that differential occupancy of transcription factors on the GC-rich consensus sequences in SVCT2 exon 1b promoter contributes to the regulation of cell and tissue expression of SVCT2.

Keywords

ascorbic acid; Sp; YY1; SVCT2; EGR

Introduction

Ascorbic acid (the reduced form of vitamin C) maintains metal ions in the reduced form used in many dioxygenase enzyme reactions and scavenges free radicals to protect tissues from oxidative stress. Substantial evidences indicate vitamin C is implied in general health and disease, with special implications for chronic inflammatory disorders [1–3]. Even though most mammals can synthesize the vitamin, all are strictly dependent on the presence of vitamin C transporters, which determine the distribution of this molecule between extra- and intracellular fluids. Ascorbic acid is imported into cells by an active mechanism that is mediated by two sodium-dependent vitamin C transporters (SVCT1 and SVCT2) identified in 1999 [4]. The SVCT1 and SVCT2 are highly homologous with 65% sequence identity in the human and the rat [4] and 60% in mouse [5]. These two transporters nonetheless exert distinct functions, depending on their cell and tissue location. The SVCT1 transcript is

expressed in epithelial tissues [6,7]. The SVCT2 is more widely expressed and its mRNA is detected in many tissues such as brain, lung and muscle [8–10].

Distribution and kinetic parameters suggest that the SVCT2 is crucial for ascorbate uptake in non-epithelial cells. Sotiriou et al. demonstrated that mice lacking the SVCT2 have apparently normal intrauterine development, but they die within minutes of birth and show cortical capillary hemorrhage and failure to inflate the lungs [11]. More recent results show modest, localized oxidative stress in the brain of $SVCT2^{-/-}$ fetuses and that capillary hemorrhage extends to the hindbrain and was associated with cell death [12]. The cause of this hemorrhage was attributed to deficient type IV collagen in basement membranes in the brain [12]. Thus death in these fetuses or newborns is probably due to neurologic dysfunction secondary to hemorrhage, especially during the physical stress of birth.

Despite its importance for maintaining intracellular ascorbate, little is known about the transcriptional regulation of SVCT2. Concerning SVCT2 regulatory regions, Rubin and co-workers [13] identified two distinct promoters (P1 and P2) located immediately upstream of the fisrst two exons (termed exon 1a and exon 1b) and P2 promoter showed greater activity compared to P1 promoter. Moreover, the 5′UTR variants initiated by exon 1b always substantially exceeded that of exon 1a, suggesting that P2 promoter may play a critical role to maintain the mRNA and protein levels of SVCT2. The proximal region of P2 has multiple putative cis-acting elements, including Krueppel-like factor (KLF), zinc finger transcription factor, Sp1, EGR-1, AP2, metal transcription factor (MRE), and Myc-associated zinc finger protein (MAZ) sites [13,14]. Mutation at either of the two putative overlapping KLF/Sp1sites located 5′ of the minimal −100 bp promoter caused a >50% reduction in promoter activity [14].

To better understand how these sites regulate SVCT2 promoter function is the goal of the current study. Using a combination of biochemical and molecular approaches, we show that the ubiquitously expressed YY1 and Sp1/3 transcription factors bind to the proximal exon 1b promoter and both maintain promoter activity and enhance SVCT2 exon 1b expression. Four Sp1/3 sites in the human SVCT2 exon 1b promoter were also identified as overlapping functional binding elements for EGRs, WT1 and MAZ. These transcription factors differentially regulate SVCT2 exon 1b promoter activity *in vitro,* suggesting that competition between Sp1/3 and EGR/WT1/MAZ may be important for controlling the expression of the exon 1b gene.

Materials and methods

Reagents

The antibodies against YY1 (H-414), NF-YA (C-18), Sp1 (PEP 2), Sp3 (D-20), EGR-1 (588), EGR-3 (C-24), EGR-4 (C-14), WT1 (C-19), MAZ (H-50), TFII-I (H-58) and SVCT2 (S-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The EGR-2 (PRB-236P) antibody was purchased from COVANCE (Emeryville, CA). Biotin end-labeled or unlabeled oligonucleotides and other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Human cell lines HeLa (cervical cancer), U2OS (osteosarcoma) and HEK293 (embryonic kidney) were maintained in DMEM with 10% FBS. EA.hy926 cells were a gift from Dr. Cora Edgel. They were derived from fusion of HUVEC with A549 (lung adenocarcinoma epithelial cell line) and were cultured in DMEM that contained 10% FBS and HAT media supplement (Sigma Chemical Co., St. Louis, MO).

Plasmid constructs

The reporter construct of −1940/+108-luc was prepared as previously described [13]. To generate the reporter constructs for exon 1b minimal promoter and the various exon 1b mutants as indicated in Fig. 2B, the promoter regions were prepared by polymerase chain reactions with −1940/+108-luc as the template. The PCR products were digested, inserted into the pGL3-basic vector and verified by nucleotide sequence analysis. The expression vectors for EGR-1, -2, -3, -4, WT1-EGR-1 fusion protein, YY1, dominant-negative mutant YY1S339/S342, the short isoform (pPac/Sp3) and the long isoform (pPac/Sp3FL) of Sp3, WT1 and MAZ were all previously described [15–21]. The EGR-1 mutants (I293F, T288A, T288D, T288C and P289G) were generously provided by Dr. Jeffrey Milbrandt, Washington University School of Medicine, St. Louis, MO. pPac/Sp1 was purchased from Addgene, Inc.

Transient transfection and luciferase assays

Cells were seeded in 24-well plates and grown to ~70% confluence. On the following day, the cells were co-transfected with 0.1 to 0.5 μg of reporter plasmid, 5 ng of *Renilla* plasmid pRL-CMV, 0.1 to 1 μg of plasmids expressing the genes of interest or empty-vector plasmid to compensate for the amount of DNA transfected. Fugene HD reagent (Roche Applied Science, IN) was used for the delivery of plasmids into cells. At 24 h after transfection, cell lysates for measurement of luciferase activities were prepared using Passive Lysis Buffer (Promega, WI) according to the manufacturer's instructions.

Involvement of Sp1 family proteins in regulating SVCT2 exon 1b gene expression

Drosophila melanogaster Schneider SL2 cells were co-transfected with YY1 and Sp1/Sp3 expression vector driven by Drosophila actin promoter, along with −100/+108-luciferase or +1/+108-luciferase. Alternatively, U2OS cells were treated with a GC-rich DNA-binding protein inhibitor mithramycin A (MMA) at 100 nM at the time of transfection of promoter constructs. All transfection experiments were carried out as described above. Finally, U2OS cells were treated with various concentrations of MMA for different times followed by RNA isolation and RT-PCR analysis using SVCT2 isoform-specific primers [22].

Electrophoresis mobility shift assays (EMSA)

The exon 1b promoter, as well as its mutants, was prepared as biotin end-labeled duplexes. Nuclear extract (2 μl) derived from U2OS or HeLa cells was incubated with the biotin end-labeled probes at room temperature for 20 minutes either in 25 mM Hepes pH 7.5, 12.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 5% glycerol, 0.1% NP-40, 70 mM KCl, 10 μM $ZnCl_2$ and 50 ng/μl poly dI-dC for YY1/Sp/EGR in vitro binding reaction, or in 10 mM Tris pH 7.5, 50 mM KCl, 1 mM DTT, 0.2 mM EDTA, 5% glycerol, 2 mM $MgCl_2$, 0.5 mg/ml BSA, and 50 ng/μl poly dI-dC for optimized EGR-2 in vitro binding reaction. For competition or super-shift experiments, the nuclear extracts were treated with excess unlabeled probes or 1 to 4 μg of antibody for 30 minutes at room temperature prior to the addition of the biotin end-labeled probes. The reaction products were then loaded onto 4.5% polyacrylamide gel electrophoresis and electrophoresed at 100 V in 0.5x TBE buffer for 1.5 hours followed by the detection according to the instruction of LightShift Chemiluminescent EMSA (Pierce Biotechnology, IL).

Co-immunoprecipitation

To analyze the interaction of Sp1/3 with YY1 in intact cells, SL2 cells were co-transfected with pPac-YY1 and pPac-Sp1/Sp3 isoforms. The transfected cells were rinsed twice in phosphate-buffered saline and suspended in lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 2

mM phenylmethylsulfonyl fluoride. The expression levels were monitored by immunoblotting of the cell lysate with antibodies specific for YY1, Sp1 and Sp3. For immunoprecipitation, the lysate was incubated with anti-Sp1 or anti-Sp3 antibodies at a final concentration of 2 μg/ml with 25 μl of protein G Plus-agarose (Santa Cruz Biochemicals, CA) for at least 2 h at 4 °C. The precipitates were separated on polyacrylamide gels and then probed with YY1 antibody. Co-immunoprecipitation in U2OS cells was also carried out as described above.

Immunoblotting

Whole-cell lysates were prepared in RIPA buffer supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland), and 2 mM phenylmethylsulfonyl fluoride. Proteins were subjected to polyacrylamide gel electrophoresis and were then electro-transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% non-fat dry milk for 1 h at room temperature and then incubated for 2 h at room temperature with primary antibodies. After three rinses in PBS-Tween-20, the membranes were incubated at room temperature for 2 hours with a 1:10,000 dilution of a horseradish peroxidase-conjugated secondary antibody (Sigma) and detection was carried out with ECL (Amersham, UK).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assay (ChIP) assays were performed using a commercially chromatin immunoprecipitation kit (Cell Signaling Technology, MA), using either anti-YY1, anti-Sp1, anti-Sp3, anti-EGR-1-4, anti-MAZ, or anti-WT1 antibodies. U2OS cells were first cross-linked for 10 min by adding formaldehyde directly to culture medium to a final concentration of 1%. Cross-linked cells were then rinsed twice with cold PBS containing protease inhibitors, scraped, pelleted, suspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0), and incubated for 10 min on ice. The lysates were then digested by *Micrococcal* nuclease. After digestion, the samples were centrifuged and the supernatants diluted 5-fold in ChIP buffer with protease inhibitors. Cross-linked chromatin was incubated overnight at 4°C with 4 μg of the respective antibody, or with normal rabbit IgG in a total volume of 500 μl. Antibody-protein-DNA complexes were isolated by immunoprecipitation with 30 μl of protein G magnetic beads. After extensive washing, the pellets were eluted by freshly prepared elution buffer (1% SDS, 0.1 M NaHCO3). Formaldehyde cross-linking was reversed by a 2-h incubation at 65°C after adding 2 μl of proteinase K and 6 μl of 5 M NaCl. Samples were purified through PCR purification kit columns and used as a template in PCR. ChIP primers: 5-TCG GGG GCG GGG AGG GAG GT-3 and 5-AGC CGC CTG CAA AAT GGC GCC GCG GAG-3 were used to amplify a 105-base pair fragment corresponding to the core exon 1b.

Assay of ascorbate transport

Ascorbate transport was measured in U2OS cells following 48 h of transfection with YY1 or/and Sp1/3. Prior to the assay, transfected U2OS cells were rinsed with cell incubation buffer (15 mM Hepes, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$). The transport assay was carried out by incubation of the cells at 37 °C in 1 ml of the same medium that contained 50 μM L-[1-^{14}C]ascorbic acid, 0.5 mM reduced glutathione and 30 mM 3-*O*-methylglucose. Glutathione was included to prevent the oxidation of ascorbate, and 3-*O*-methylglucose was included to block any uptake of any remaining DHA on glucose transporters. Uptake was stopped at 20 min by rinsing the cells with three times in ice-cold PBS. Cells were dissolved in 1 M NaOH and the incorporated radioactivity was measured by liquid scintillation spectrometry.

RNA isolation and RT-PCR

Total RNA was isolated using TRIZOL reagent (GIBCO, Grand Island, NY), and 2 μg was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Carlsbad, CA). One microliter of this mixture was amplified in a 25-μl reaction using Advantage 2 PCR kit (Clontech, Mountain View, CA). The PCR products were separated by electrophoresis on 1.5% agarose gels and visualized with ethidium bromide. The amplification parameters and primers for SVCT2 and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were described previously [22].

Results

Analysis of the putative exon 1b promoter

As noted in the Introduction, Rubin and co-workers [13] previously showed that the human SVCT2 promoter activity is mediated by two variants (P1/exon 1a and P2/exon 1b variants). The ~2.1 kB genomic region immediately upstream of the hSVCT2 exon 1b sequence (putative P2 promoter, GenBank accession: DQ11868) was found to be TATA-less, CAAT-less and was highly GC-rich. Deletion analysis suggested the possibility that regulatory elements exist within ~100 bp upstream of the transcription start site (TSS) [13]. We therefore performed sequence analysis on this proximal exon 1b promoter, using Gene Regulation at www.gene-regulation.com and MatInspector at www.genomatix.de. This analysis revealed new features of the proximal promoter region. First, most TATA-less promoters utilize the so-called initiator element (Inr; consensus PyA(A/T)PyPy, where Py is a pyrimdine) as the TSS (Strachan and Read, 2000; Levine and Tijan, 2003). The exon 1b TSS (CATTTT) conformed to the consensus Inr sequence and overlapped with a conserved YY1 consensus sequence (CATTTT) (Fig. 1A), suggesting that it might be involved in the regulation of exon 1b function. Second, multiple potentially important transcription factor-binding sites were concentrated in the proximal exon 1b promoter region (Fig. 1A). Within 100 bp upstream of the TSS were four tandem GC boxes (consensus GGGGCGGG) that contained overlapping binding sites for the EGR/WT1 (consensus GCGGGGGCG) and Sp (consensus GGGGCGGG) families of transcription factors, as well as two MAZ binding sites (consensus CCCTCCC). Third, sequence analysis revealed that exon 1b promoter is also contained within a CpG island (Fig. 2A). This region has a G+C content of nearly 90% (0.898) and a CpG/GpC ratio of 0.867 [23]. This exceeds the 0.6 threshold for designation as a CpG island [24]. All of the features are well conserved between the human SVCT2 exon 1b promoter and mouse SVCT2 promoter, especially the Inr, GC box 1 and GC box 2 (Fig. 1A).

Transcriptional activity of the human SVCT2 exon 1b promoter

The −36/+108 fragment demonstrated the same promoter activity as the −1940/+108 fragment, so it probably includes the full promoter (Figure 1B). This was observed in 4 different cell types (HeLa, U2OS, EA.hy926 and HEK293). However, inclusion of 64 additional base pairs of the upstream sequence up to −100 bp increased luciferase activity to 2 to 4-fold as compared to −36/+106 (Fig. 1C), suggesting the presence of negative regulatory elements upstream of the −100/+108 region [13]. Further, the −100/+108 fragment contains all four GC boxes and the Inr site, whereas the −36/+108 fragment, contains only GC box 1, GC box 2 and the Inr site (Fig. 2A). The −13/+108 and −6/+108 fragments bearing only the Inr site showed relatively low activity (Fig. 2C). In contrast, the +1/+108 fragment, which lacks all four GC boxes and Inr site, showed 2~3-fold promoter activity compared with pGL3-basic vector (data not shown).

We next carried out site-specific mutagenesis and deletion analysis in these regions to investigate whether these putative elements might regulate promoter activity of the exon 1b

gene (Fig. 2B). The reporter assay results shown in Fig. 2C demonstrated that promoter activity was decreased when each of these elements was mutated (−36/+108D2, −36/+108M2, −36/+108MY, −36/+108D1 and −36/+108M1) in each of the three cell lines tested. The promoter activity was reduced still further when GC boxes 1 and 2 were mutated simultaneously (−36/+108M1,2). The reporter activity fell to the level of the +1/+108 control when GC boxes along with YY1/Inr were mutated (−36/+108M1,Y, −36/+108M2,Y, and −36/+108M1,2,Y). Similarly, site-specific mutagenesis also clearly showed GC boxes 3 and 4 play a crucial role to maintain the maximal activity of exon 1b promoter (Fig. 2D), consistent with the published data [14]. These results indicated that each of these elements were essential for the activity of exon 1b promoter.

The transcription factor YY1 binds to the exon 1b Inr

The functional analysis suggested that a transcription factor may bind to and mediate the basal transcription machinery. Because of the homology between residues −2 to +6 and a consensus binding sequence for the transcription factor YY1, we tested whether YY1 binds to this site by carrying out EMSA with nuclear extracts from HeLa and U2OS cells. We observed the formation of a slowly migrating complex that is consistent with YY1 binding to the YY1 probe sequence (Fig. 3A). Wild-type YY1 DNA and the YY1 consensus oligonucleotide competed with each other for binding, but the YY1 mutant did not (Fig. 3A). A 33-bp nonspecific competitor oligonucleotide containing Inr2 of the xanthine dehydrogenase/xanthine oxidase promoter (XDH/XO) also failed to affect the binding of YY1 to DNA (Fig. 3A).

To confirm this observation, nuclear extract from HeLa cells was preincubated with either preimmune serum, anti-NF-YA, or anti-YY1 antibody. The antibody against YY1 super-shifted the DNA-protein complex, whereas the pre-immune serum and anti-NF-YA antibody did not (Fig. 3B).

To directly confirm that YY1 exerts a positive effect on the exon 1b promoter, we tested whether the promoter could be activated by YY1 expressed in cells. As shown in Fig. 3C, transfection of cells with a plasmid expressing YY1 resulted in a 2- to 3-fold activation of the exon 1b promoter. In contrast, basal transcription was substantially inhibited by transfection with a plasmid carrying the YY1 dominant-negative mutant YY1S339/S342. This mutant lacks the ability to bind specific YY1-target sequences, but retains capacity of wild type protein to undergo protein–protein interactions [17]. Thus, ectopically expressed YY1 enhances transcription of exon 1b gene.

The YY1 binding site overlapping the Inr is required for the transcription from the exon 1b promoter

Previous studies have shown that mutating the adeno-associated virus (AAV) p5 ($CCA^{+1}TTTT$) or the RNA polymerase II (RNAPII; $CCA^{+1}TTGT$) Inr at +2 affects promoter recognition of various sequence-specific Inr-binding proteins, whereas mutating the thymidine present in the consensus Inr element at +3 affects transcriptional activity [25–27]. To further analyze the possible role of the YY1 binding site overlapping the Inr in the basal transcription machinery of the exon 1b promoter, point mutations at +1 (Inr+1; equivalent to +2 in p5/RNAPII Inr) and +2 (Inr+2; equivalent to +3 in p5/RNAPII Inr) were made. Thus, Inr+1 was changed from a thymidine to a guanosine residue and Inr+2 was changed from a thymidine to a cytosine residue. The ability of YY1 to bind to the Inr+1 and Inr+2 mutant promoters was analyzed by EMSA. YY1 binding to the Inr+1 mutant exon 1b promoter was completely abolished; however, the Inr+2 mutant promoter was able to bind an equivalent amount of YY1 as the wild-type sequence (Fig. 4A). The activity of each of the mutant promoters was also analyzed in cultured cells. Mutation at Inr+2 had no effect on

the promoter activity; however, mutation at the Inr+1 nucleotide reduced transcriptional activity by at least 75% (Fig. 4B). These results collectively demonstrate that the YY1 binding site overlapping the Inr is indispensable for exon 1b promoter activity. The implication of these findings is that the formation of a transcription-competent complex at the exon 1b promoter is enabled by a YY1-dependent mechanism.

Multiple complexes form in the GC-rich regions of the exon 1b promoter upstream of YY1 binding site

Next, we tested whether the GC-rich region immediately upstream of YY1 binding site in the exon 1b promoter binds specific transcription factors with GC box 1, 2, 3 and 4 probes and nuclear extracts from U2OS cells (Fig. 5A). There were at least 5 retarded complexes, that is, complexes 1–5 that bound to the respective GC box probes. Probes containing consensus sequences for Sp1 and EGR-1/2/3/4 were used as controls. Complexes 1, 2 and 4 were formed on the Sp1 probe and complexes 3 and 5 were formed on the EGR probe (C5 band is best shown in Fig. 5C). When a GC box 1 probe was used, at least three complexes (C1, C2 and C4) were observed. Formation of these complexes was prevented by excess unlabeled Sp1 probe, but not by the EGR or XDH/XO probes. Similarly, excess unlabeled Sp1 oligonucleotide completely prevented formation of complexes 1, 2 and 4 from the GC box 2, 3 and 4 probes, but not complexes 3 and 5. On the other hand, excess unlabeled EGR probe prevented formation of complexes 3 and 5 from GC boxes 2, 3 and 4 without affecting the other three complexes. Similar results were found for HeLa cells (data not shown).

To confirm the identity of the specific Sp-family and EGR-1 transcription factors that bind to the GC boxes, we employed antibody super-shift experiments. As shown in Fig. 5B, for each of the GC boxes, Sp1 antibody specifically blocked Sp1-containing complex formation (C1) and Sp3 antibody resulted in loss of complexes 2 and 4. When Sp1 and Sp3 antibodies were used together, both Sp1 and Sp3 complexes (C1, C2 and C4) were specifically targeted. A similar loss of Sp1/3 containing complexes was previously observed in another study in which the same Sp1/3 antibodies were used [28]. As expected from the results in 5A, the GC box 1 probe did not form complexes 3 and 5. When the nuclear extract was incubated with EGR-1- or EGR-2-specific antibodies before addition of the other 3 GC box probes, EGR protein complexes (C3 and C5) were super-shifted to slower migrating species (Fig. 5B and C). Experiments using antibodies to other family members, EGR-3, EGR-4, Wilms' Tumor protein-1 and MAZ, failed to super-shift or to decrease protein binding in any of the complexes (Fig. 5B).

To further strengthen the above observations, mutational analysis was executed (Fig. 5D). When U2OS cell nuclear extracts were combined with the biotin-labeled probes containing a mutated EGR binding element without affecting the binding of Sp1/3, only Sp1/3 containing complexes (C1, C2 and C4) were produced (left panel). In contrast, the mutation of both Sp1 and EGR sites simultaneously eliminated Sp1/3 and EGR binding (right panel).

Taken together, these results indicate that all four GC boxes of the exon 1b promoter bind Sp transcription factors and three GC boxes bearing the overlapping EGR/Sp sites are recognized by EGR transcription factors.

Physiological binding of transcription factors to the exon 1b promoter in U2OS cells

To directly assess the presence or absence of the transcription factors on the proximal promoter of the endogenous exon 1b gene in U2OS cells, we analyzed the occupancy of the GC-rich region in intact cells by chromatin immunoprecipitation. Cross-linked, digested chromatin was immunoprecipitated with antibodies against YY1, Sp1/3, EGR1-4, WT1 or MAZ, and purified DNA was analyzed by PCR with primers spanning the GC-rich region of

the exon 1b gene. The results in Fig. 6 show that the immunoprecipitated DNA from U2OS cells was substantially enriched for YY1 and Sp3 in the 105-bp region spanning the exon 1b proximal promoter, whereas Sp1 was weakly detected. No enrichment for control IgG and the other transcription factors tested was detected in the immunoprecipitated DNA. These results indicate that YY1, Sp3 and Sp1 are present on the GC-rich region of U2OS cells. This suggests that constitutive gene expression from the exon 1b promoter may indeed depend on these transcription factors, especially YY1 and Sp3.

Displacement of Sp1/3 by EGR at the proximal Sp1/3 binding elements of the exon 1b promoter

Our results suggest that the GC-rich elements of the exon 1b promoter are occupied in the cell by Sp1/3 transcription factors rather than by EGR transcription factors. This may occur due to higher Sp1/3 binding affinities or higher Sp expression. To test the latter possibility, transfection of EGR-2 followed by EMSA was used to determine whether EGR can displace Sp1/3 proteins from their consensus sequences. Transfection of U2OS cells with EGR-2 resulted in the appearance of EGR-2 in immunoblot (Fig. 7A) and EMSA (Fig. 7B). In mock-transfected cells, GC-rich elements were predominantly occupied by Sp proteins (Fig. 7C, vector alone). Increased expression of the EGR-2 protein following its transfection abolished (GC boxes 2 and 3) or decreased (GC box 4) Sp1/3 occupancy. This was associated with appearance of a band containing the respective probe that migrated as expected for EGR-2. However, no effect of EGR-2 over-expression was noted on Sp1/3 binding to the GC box 1 probe. The displacement of Sp1/3 by EGR-2 was also detected in U2OS cells that were transfected with EGR-2 construct (Fig. 7D). These data indicate that the increased EGR expression is capable of interacting with some GC-rich elements in exon 1b promoter by displacing Sp1/3.

Mithramycin A rapidly decreases exon 1b mRNA levels and inhibits exon 1b promoter activity

To investigate mechanisms that might control expression of exon 1b mRNA, we treated cells with mithramycin A (MMA), a cell-permeable agent that binds to GC-rich DNA sequences and is frequently used to explore the sequence dependency of DNA-binding factors. Binding of MMA is thought to impede binding of GC-specific transcription factors by steric hindrance [29]. In U2OS cells treated with 100 nM MMA, there was a time-dependent decrease in exon 1b mRNA levels, whereas SVCT2 Exon 1a was not affected (Fig. 8A). After treatment for 24 h, exon 1b mRNA was also decreased in a concentration-dependent manner by MMA (Fig. 8B).

Furthermore, the addition of MMA in U2OS cells led to a significant inhibition in reporter activity driven by the exon 1b promoter (Fig. 8C). Together, these observations lend additional support to the notion that GC-rich sequences upstream of the exon 1b are bound and activated by specific transcription factors.

YY1 and Sp1/3 synergistically activate exon 1b promoter

To evaluate the potential interactions of YY1/Sp proteins with the proximal binding sites in more detail, we performed a series of transfections with the −100/+108 and +1/+108 constructs in Schneider SL2 cells. This is a *Drosophila melanogaster* cell line that lacks YY1 and Sp protein expression and thus can be used to probe interactions between the factors without the confounding effect of endogenous factors [30]. As expected, the −100/+108 construct alone yielded no increased luciferase activity within this cell type, compared with +1/+108 construct (Fig. 8D). Co-transfection with Drosophila-optimized expression plasmids carrying the YY1, Sp1 or Sp3 isoforms induced exon 1b promoter activity in a dose-dependent manner (Fig. 8D). Sp1 has been reported to be able to work

synergistically with YY1 to activate promoters [31]. Therefore, we further investigated whether YY1 and Sp1/3 might synergistically activate exon 1b promoter. To do this, we co-transfected indicated amounts of YY1, Sp1, Sp3 isoform expression constructs together with the promoter reporter constructs into Drosophila SL2 cells. When expressed individually, YY1 or Sp1/3 resulted in up to 10-fold increase in luciferase reporter activity at the plasmid concentration of 0.5 μg. However, when co-transfected with YY1, either Sp1 or Sp3 (Sp3/Sp3FL isoforms) synergistically led to potent activation of exon 1b promoter activity up to 214-fold (Fig. 8E).

We next examined whether YY1 and Sp1/Sp3 also form protein complexes in intact cells using immunoprecipitation. SL2 cells were co-transfected with YY1 and Sp1/3 plasmids. The expression of target proteins was confirmed by immunoblots of whole-cell extracts using the antibodies against YY1, Sp1, or Sp3 (data not shown). The same whole-cell extracts were subjected to immunoprecipitation with either anti-Sp1 or anti-Sp3 antibody followed by immunoblotting with an anti-YY1 antibody (Fig. 9A). We observed that following immunoprecipitation with antibodies to Sp1 or Sp3, YY1 was detected in extracts from cells that had been co-transfected with YY1 and Sp1/Sp3, but not in YY1- or Sp-transfected cells. Moreover, immunoblotting of YY1 after immunoprecipitation with antibodies to Sp1 and Sp3 in U2OS cells also showed that the immunoprecipitates contained YY1 (Fig. 9B). The ability of the anti-Sp1 or anti-Sp3 antibodies to pull down YY1 in cell extracts demonstrates direct protein-protein interactions between YY1 and Sp1/Sp3 in the cells.

To demonstrate likely co-occupancy or complex formation on the exon 1b promoter, we used EMSA to address this question. Using the 56-bp exon 1b promoter probe containing Inr/YY1 site, GC box 1 and GC box 2, we detected two bands (Ca and Cb) that migrated slower than when the GC box 2 probe was used (Fig. 9C). The formation of Ca/Cb bands was specifically blocked by inclusion of excessive amounts of either unlabeled YY1 or consensus Sp1 probe. In contrast, consensus EGR, mutated YY1, mutated Sp1 or XDH/XO competitor did not affect the formation of Ca and Cb bands. Addition of antibody against Sp1 or Sp3 partially super-shifted Ca/Cb bands (Fig. 9D, i) and the combination of the two antibodies completely super-shifted Ca/Cb bands (iii). Addition of antibody against YY1 specifically targeted Ca/Cb bands as well as YY band, and resulted in a clear super-shifted band (ii and iii). In contrast, YY1 antibody did not affect the formation of Sp1/Sp3 complexes on GC box 2 (more evident in Fig. 9D, iv), indicating that YY1 exists in both Ca and Cb. Additionally, super-shifted YY1 band did not interfere with Ca/Cb bands (iv). As a control, TFII-I antibody did not cause any super-shift band or affect the binding intensity. Taken together, these studies demonstrate specific interaction of Sp1/3 and YY1 on the exon 1b promoter, and are consistent with the luciferase reporter activities shown in Fig. 8E, in which both Sp1 and Sp3 synergistically stimulated reporter activity along with YY1.

Our above results imply that YY1 and Sp1/3 are important regulators of endogenous SVCT2 gene expression in addition to activating the SVCT2 exon 1b promoter. To demonstrate that, U2OS cells were transfected with the YY1 and Sp1/3 expression constructs. The expression of SVCT2 exon 1a is absent in the U2OS cells (data not shown), thus total SVCT2 protein level was measured by immunoblotting. As shown in Fig. 9E, when YY1 was co-transfected with Sp1 or Sp3, SVCT2 protein expression levels were elevated markedly. Furthermore, up-regulated SVCT2 protein expression also increased ascorbate uptake (Fig. 9F).

EGR, WT1 and MAZ differentially regulate SVCT2 exon 1b promoter

The significance of the EGR sites with regard to exon 1b gene expression was analyzed by co-transfection experiments and assay of luciferase-dependent promoter activity. Following transient co-transfection into U2OS cells of either the −36/+108 or the −100/+108 promoter

reporter constructs with either EGR-1 or EGR-2, exon 1b promoter activity was decreased in a dose-dependent manner (Fig. 10A, left two panels and inserts). On the other hand, transfection of EGR-3 and EGR-4 resulted in a marked increase in exon 1b promoter activity (Fig. 10A, right two panels). The full-length exon 1b promoter reporter construct −1940/+108 also displayed similar regulatory activity for EGR-4 (Fig. 10A, right insert).

Sequence analysis also revealed that the GC-rich human SVCT2 exon 1b promoter contains three conserved WT1 binding sites overlapping with the EGR sites and two MAZ consensus elements in GC boxes 1 and 4. To examine whether these transcription factors also regulate exon 1b promoter activity, reporter gene assays were performed in U2OS cells. As shown in Fig. 10B, the WT1(-KTS) variant inhibited exon 1b promoter activity in a concentration-dependent manner. In contrast, WT1(+KTS), which has been implicated in mRNA processing [32,33], had little effect on exon 1b promoter activity. Additionally, the c-Myc associated zinc finger protein MAZ also exhibited a strong inhibitory effect on exon 1b promoter activity (Fig. 10B).

Nab proteins bind EGR-1/2 and reduce trans-activation [34,35]. We therefore tested whether EGR-1 function can be reversed by EGR-1 mutants (I293F, T288A, T288C, T288D, P289G and WT1-EGR-1) that are unable to bind to Nab proteins. Unexpectedly, these mutants did not alter the repression of EGR-1-mediated reporter gene expression (Fig. 10C). Consistent with this, the over-expression of Nab1 did not further change the activity of EGR-mediated reporter gene expression (data not shown). Our data support the concept that Egr-1 and -2 negatively regulate SVCT2 exon 1b expression by a Nab-independent mechanism. The above data demonstrate that forced expression of individual EGR family members, WT1(-KTS), and MAZ differentially regulates exon 1b promoter activity.

Discussion

The present study addresses the molecular regulation of SVCT2 exon 1b expression. The results show that: (1) the multifunctional transcription factor YY1 recognizes a conserved YY1 binding site overlapping the Inr site and is required for the transcription from the exon 1b promoter; (2) four Sp1/3 sites in the proximal promoter region are important *cis* elements in the regulation of exon 1b promoter activity; (3) YY1 and Sp1/3 form functional complexes on the exon 1b promoter and synergistically regulates promoter activity; and (4) three tandem EGR/WT1 binding sites overlapping the Sp1/3 binding sites, and two MAZ binding sites overlapping the Sp1/3 binding and/or EGR/WT1 binding sites constitute multiple potential mechanisms for regulating the expression of exon 1b gene to the different stimuli and environments.

YY1 is a multifunctional transcription factor that can exert either positive or negative control on a large number of cellular and viral genes by binding to sites overlapping the transcription start site. In this study, we have identified a transcription start site core (TSSC) region of the exon 1b promoter that encompasses the transcription start site and Initiator and that is required for promoter activity. We found that a consensus binding sequence for the transcription factor YY1 overlaps the Inr within this region and is indispensable for promoter activity. This conclusion is supported by the finding that a point mutation at Inr+1 on the exon 1b promoter, which eliminates the YY1 binding for the Inr-overlapping binding site, significantly suppressed transcriptional activity. These results are contrary to those from AAV p5, DHFR, TdT and RNAPII Inrs in which the same point mutation decreased the affinity of YY1 for its Inr-overlapping binding site without affecting transcription [25,27]. The present results suggest that the YY1 binding site present in the exon 1b promoter regulates the exon 1b promoter, possibly directing the formation of transcription complexes. Consistent with this interpretation are the findings that YY1 interacts with RNAPII and

some general transcription factors, and that YY1 is a component of the human RNAPII holoenzyme complex [36,37].

In TATA-less promoters, the Inr functions analogously to a TATA box and in TATA-containing promoters the Inr can augment the strength of the TATA box. Several proteins have been suggested to recognize the transcription initiation site, including TFIID [38,39], TFII-I [40–43], USF [41,44], E2F [26], specific transcription (TBP)-associated factors (TAF), RNA polymerase II [45,46] and YY1 [47,48]. YY1 possesses the unique property of acting either as an activator or a repressor of transcription depending on the gene context. It is known to associate with several factors, many of which are components of the basal transcription machinery [36,49]. Presumably, it is these interactions that modulate much of the activity of YY1 in the SVCT2. In our analysis of exon 1b promoter in cells, we observed a consistent inhibition in promoter strength upon mutation of the YY1 binding site. This decrease in activity is due to the loss of YY1 binding, and not to a mutation in the putative Inr sequence.

The proximal exon 1b promoter within a CpG island is extraordinarily GC-rich. *In silico* analysis identified multiple potential binding sites for GC-rich binding zinc finger transcription factors upstream of the Inr, including four tandem GC boxes for Sp1/3 that overlapped EGR/WT1/MAZ binding sites. Deletion and site-specific mutagenesis experiments showed that these GC boxes are important for promoter activity. The Sp family (Sp1-Sp4) is known to bind primarily to such GC-rich sequences. Sp1 and Sp3 are ubiquitously expressed. Sp4 is expressed mostly in central nervous and reproductive systems, whereas the expression pattern of Sp2 is largely unknown [50]. Sp1, Sp3 and Sp4 share a high affinity for GC boxes bearing the consensus sequence GGGGCGGGG, whereas Sp2 weakly binds to GT boxes. HeLa and U2OS cells express low levels of Sp1, but they have easily detectable Sp3 (data not shown), suggesting that Sp1 and Sp3 may regulate exon 1b gene expression through binding to GC boxes. Indeed, EMSA experiments showed that all four GC boxes bind Sp1 and Sp3 (short and long isoforms). The ChIP technique confirmed the exclusive binding of Sp1 and Sp3 to the exon 1b promoter in U2OS cells. Using SL2 cells that lack all endogenous YY1 and Sp proteins, we further demonstrated that Sp1 and Sp3 can synergistically activate the exon 1b promoter. Three additional lines of evidence establish that Sp1/3 proteins are required for exon 1b gene expression. First, the GC-rich DNA-binding protein inhibitor, MMA, inhibits exon 1b promoter activity. Second, MMA suppresses endogenous exon 1b mRNA expression in a time- and concentration-dependent manner. Third, Sp1 and Sp3 act synergistically when YY1 is bound to the promoter to enhance transcription in SL2 cells.

Although Sp3 is the only protein in the Sp subfamily that can either positively or negatively modulate the Sp1-dependent gene expression [51], our experiments demonstrated that Sp3 positively regulates the exon 1b promoter activity in SL2 cells. Taken together, these results establish that Sp1 and Sp3 are biologically essential regulators of the SVCT2 exon 1b gene expression along with YY1.

In many genes, Sp1 sites overlap the binding elements of other transcription factors such as EGR-1 [52,53], MAZ [21], and YY1 [54], and competition between Sp1 and these factors for binding site occupancy is thought to be an important mechanism controlling gene expression. Four Sp1/3 sites in the human SVCT2 exon 1b promoter were identified as overlapping functional binding elements for EGR, WT1 and MAZ. EMSA experiments demonstrated that EGR-1 and EGR-2 do indeed form complexes at the three GC boxes bearing EGR binding sites, although their binding is relatively weak. On the other hand, over-expression of EGR-2 in cells in which the GC boxes were apparently occupied by Sp1/3 caused a loss of Sp1/3 bands. These experiments indicated that competition for

binding between Sp1/3 and EGR/WT1/MAZ transcription factors may help to control the expression of the human exon 1b gene. These overlapping *cis* elements, together with their cognate transcription factors, have been implicated in diverse physiological processes, including cell fate determination, proliferation, and differentiation as well as in pathological conditions including tumorigenesis. Conceivably, these elements may participate in the regulation of tissue-specific, differentiated-related, or stage-specific expression of exon 1b.

These findings provide new insight on how SVCT2 exon 1b gene expression may be regulated upon the different stimuli and environments and contribute to the elucidation of SVCT2's role against oxidative stress in the normal cells and in malignancy. The major features in the proximity of the human SVCT2 exon 1b promoter and mouse SVCT2 promoter are well conserved suggesting that basal transcription of these two promoters may be regulated via a similar mechanism. Thus, these results should also serve as a basis for future investigation into the molecular regulation of SVCT2 and some pharmaceuticals in the human and mouse cells/tissues.

Acknowledgments

Abbreviations

|	
SVCT2	Sodium-dependent vitamin C transporter 2
TSS	transcription start site
MMA	mithramycin A
YY1	Yin Yang-1
ChIP	Chromatin immunoprecipitation
EMSA	electrophoretic mobility-shift assay

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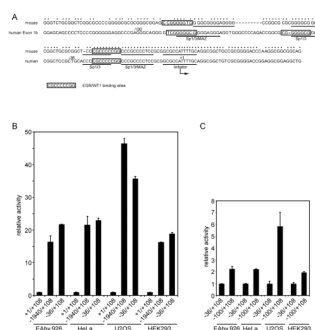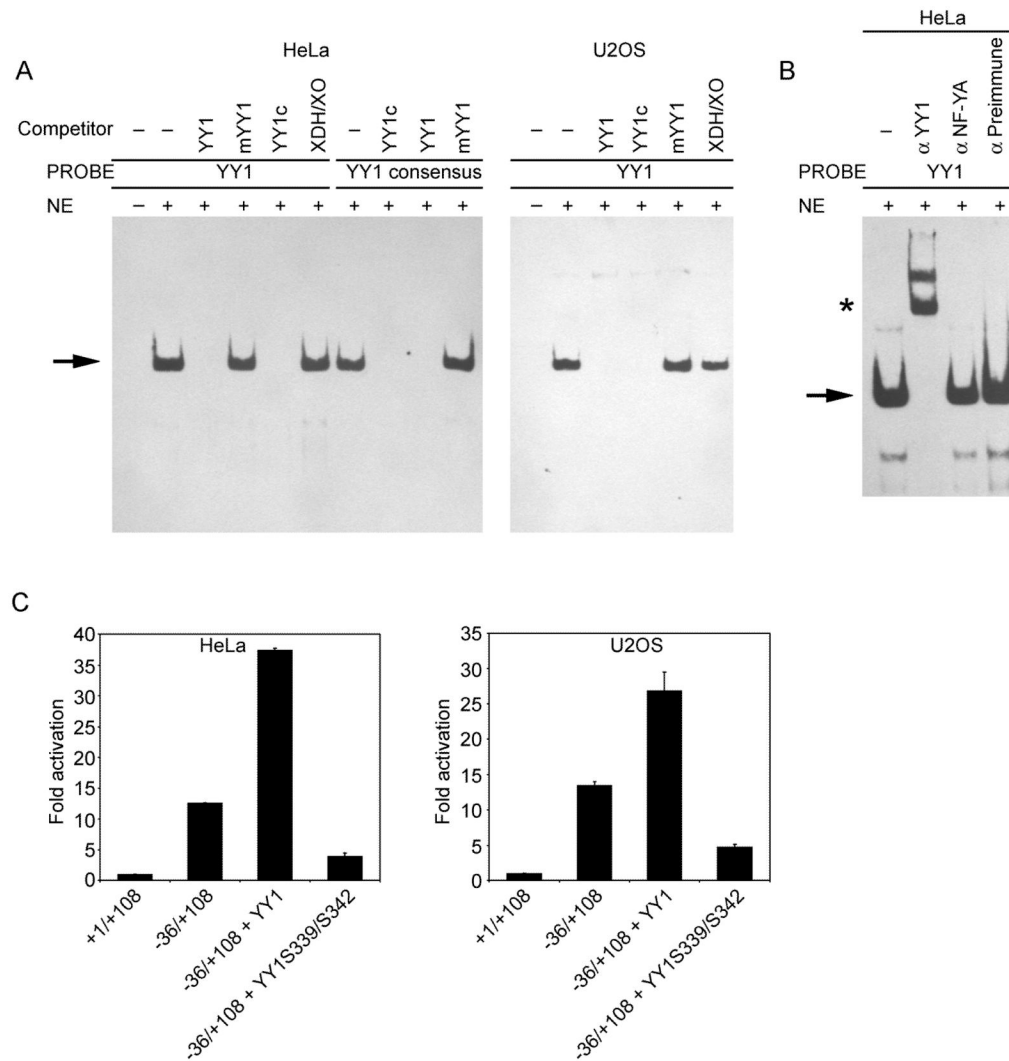
Figure 1.
The proximal human SVCT2 exon 1b promoter contains multiple highly conserved elements required for transcription. (**A**) Nucleotide sequences of the conserved regions of human SVCT2 exon 1b and mouse SVCT2 promoter are aligned. Putative conserved EGR/WT1 binding sites are boxed and other transcription factor binding sites are underlined. The transcription start sites for the human and mouse promoters are indicated by the arrow (As determined in reference [13]). (**B**) and (**C**) Analysis of exon 1b promoter activity. 500 ng of the reporter constructs were transfected into HeLa, U2OS, EA.hy926 or HEK293 cells, and analyzed for luciferase activity 24 h later after transfection. In these experiments, 5 ng of pRL-CMV was added as an internal control and enabled normalization for transfection efficiency. Relative luciferase activity is shown as the data means and the error bars on each column show range of values of duplicate measurements from 1 of 3 similar experiments, based on the activity of +1/+108 (**B**) or −36/+108 (**C**).

Figure 2.
Exon 1b promoter activity depends on the Inr and upstream GC boxes. (**A**) Genomic sequences upstream of the beginning of the mRNA sequence of exon 1b are shown. Putative conserved transcription factor binding sites are marked and potential CpG sites are indicated by an *. (**B**) Luciferase reporters were prepared to test the activity of different lengths of the exon 1b promoter, including the effect of targeted deletions and mutations. The schematic shows the design of the pGL3 reporters to test the relative contribution of different portions of exon 1b promoter. (**C**) and (**D**) The activity of the exon 1b promoter depends on the intact Inr and upstream GC boxes. 500 ng of each of the reporter constructs shown in **B** were transfected into HeLa, U2OS or EA.hy926 cells, and analyzed for luciferase activity. 5 ng of pRL-CMV was added as an internal control and enabled normalization for transfection

efficiency. The data presented are means of duplicate measurements from 1 of 3 similar experiments, based on the activity of +1/+108 (**C**) or −36/+108 (**D**), and the error bars show range of values of duplicate measurements.
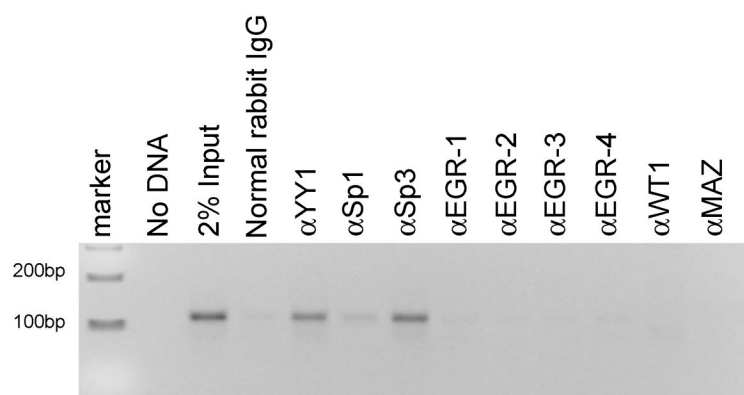
Figure 3.
The exon 1b Inr binds the transcription factor YY1. HeLa or U2OS nuclear extract (NE) was incubated with a labeled probe containing the Inr of the exon 1b promoter and the reaction mixture was electrophoresed on a 4.5% non-denaturing gel to detect the specifically retarded migrating band. The indicated unlabeled probes or antibodies were added prior to labeled probes for competition (**A**) or super-shift (**B**) analysis. The YY1 consensus oligonucleotide (YY1c) served as a positive control. A super-shifted complex is indicated by an *. (**C**) HeLa and U2OS cells were transfected with 250 ng of the exon 1b promoter reporter plasmid along with 250 ng of YY1 or its dominant-negative mutant YY1S339/S342. Luciferase activities were measured 24 hours after transfection and normalized by *Renilla* reporter activities. The data are shown as means and range error bars of duplicate measurements. The experiment was repeated three times with similar results.
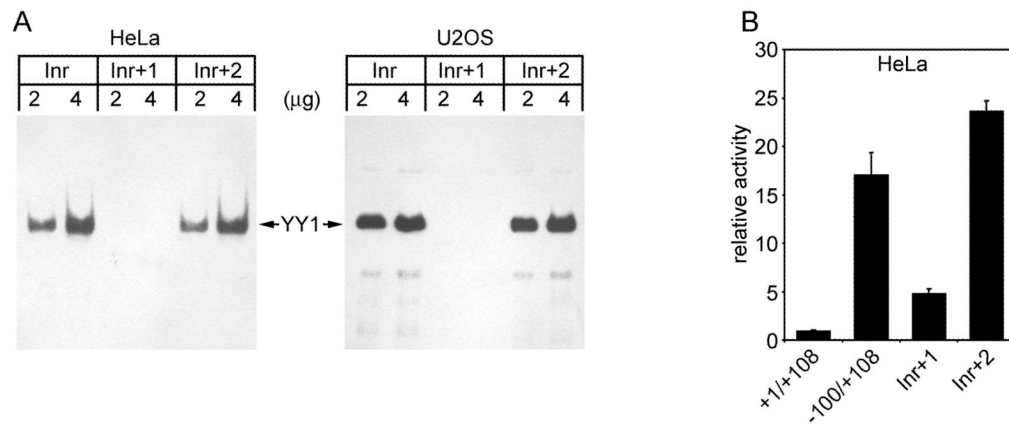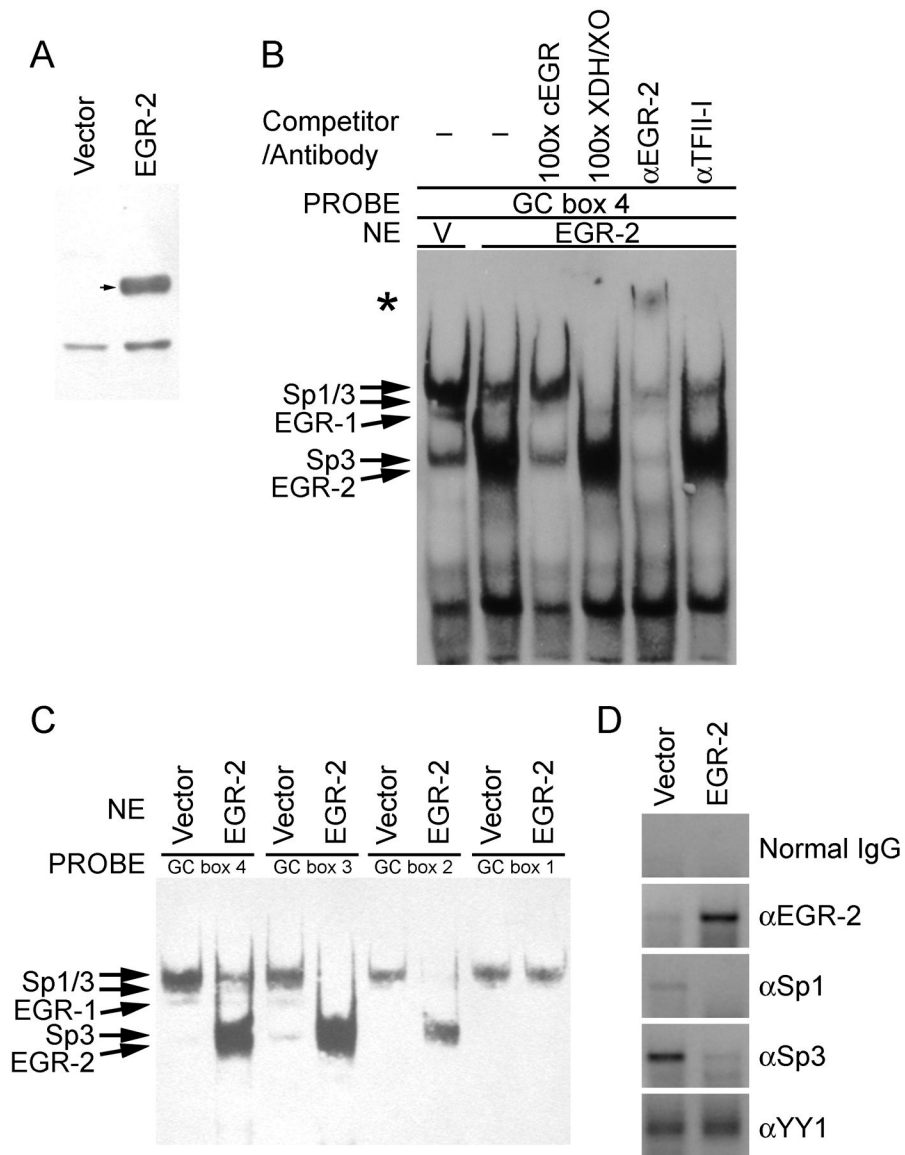
Figure 4.
YY1 binding is required for the transcription from the exon 1b promoter. (**A**) Point
mutations at Inr+1 and Inr+2 were analyzed for their ability to bind to YY1 (2 μg and 4 μg
nuclear extracts from HeLa and U2OS cells) by using EMSA. (**B**) Transient transfection
analysis of the Inr+1 and Inr+2 exon 1b mutant promoters fused to the luciferase gene. 500
ng of the reporter constructs were transfected into HeLa cells, and analyzed for luciferase
activity 24 h later after transfection. 5 ng of pRL-CMV serves as an internal control for
normalization of transfection efficiency. Relative luciferase activity is shown as the means
based on the activity of +1/+108 and the error bars show range of values of duplicate
measurements. Three separate transfection experiments were performed in duplicate.

Figure 5.
EMSA using GC box 1, 2, 3 and 4 probes and nuclear extracts (NE) from U2OS cells. Shown are competition (**A**) and super-shift (**B** and **C**) assays. The unlabeled competing probes and the antibodies used are indicated above the gel pictures. Distinct complexes are indicated on the left. An asterisk indicates super-shifted bands for EGR-1 or EGR-2. (**D**) EMSA was performed with NE from U2OS cells and biotin-labeled DNA probes. The nucleotide sequences for each of the probes are depicted in Table 1. The mutation in the left panel only abolished EGR binding, and the mutation in the right panel disrupted both Sp1/3 and EGR binding.
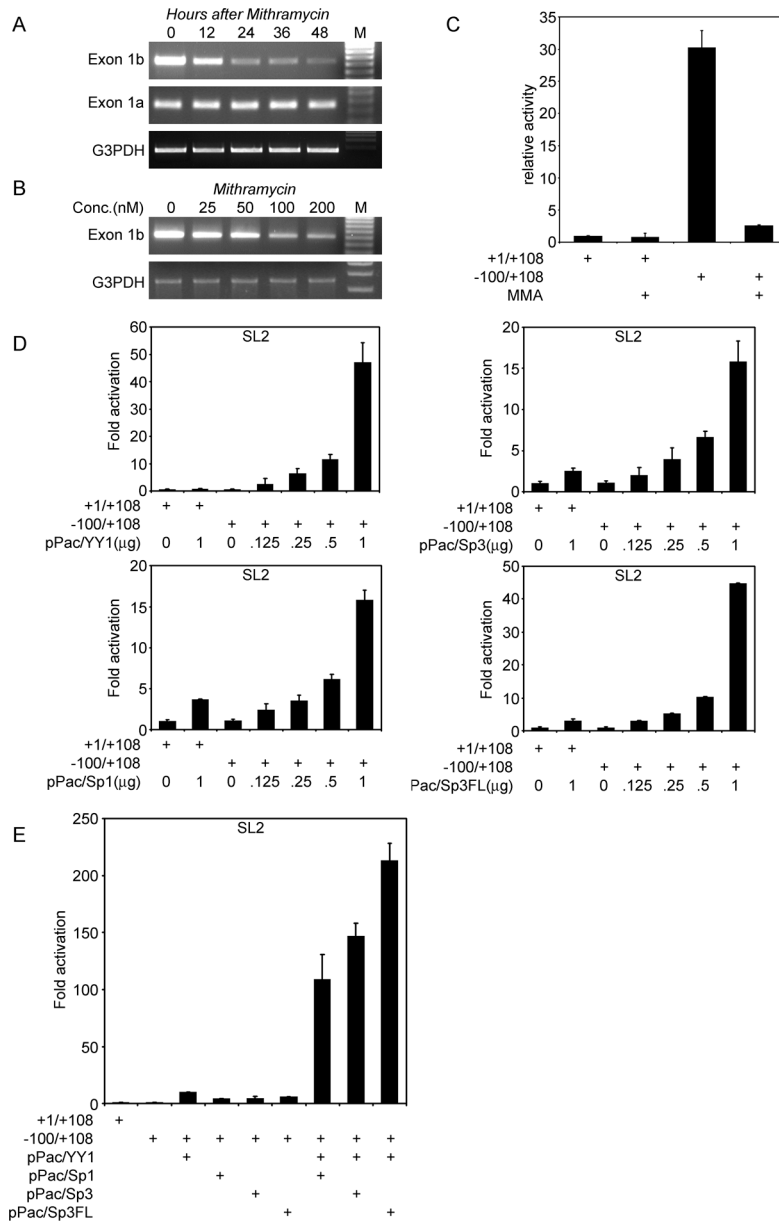
Figure 6.
Analysis of the transcription factor bindings to exon 1a promoter. ChIP assay was performed using U2OS cells. Antibodies for Sp1/3, EGR-1~4, WT1, MAZ or normal rabbit IgG were used to precipitate DNA fragments. These and 2% of the total DNA in the samples were amplified by PCR using primers specific for the human exon 1b promoter (−91 to +14). PCR products were separated on a 2% agarose gel and stained by ethidium bromide.
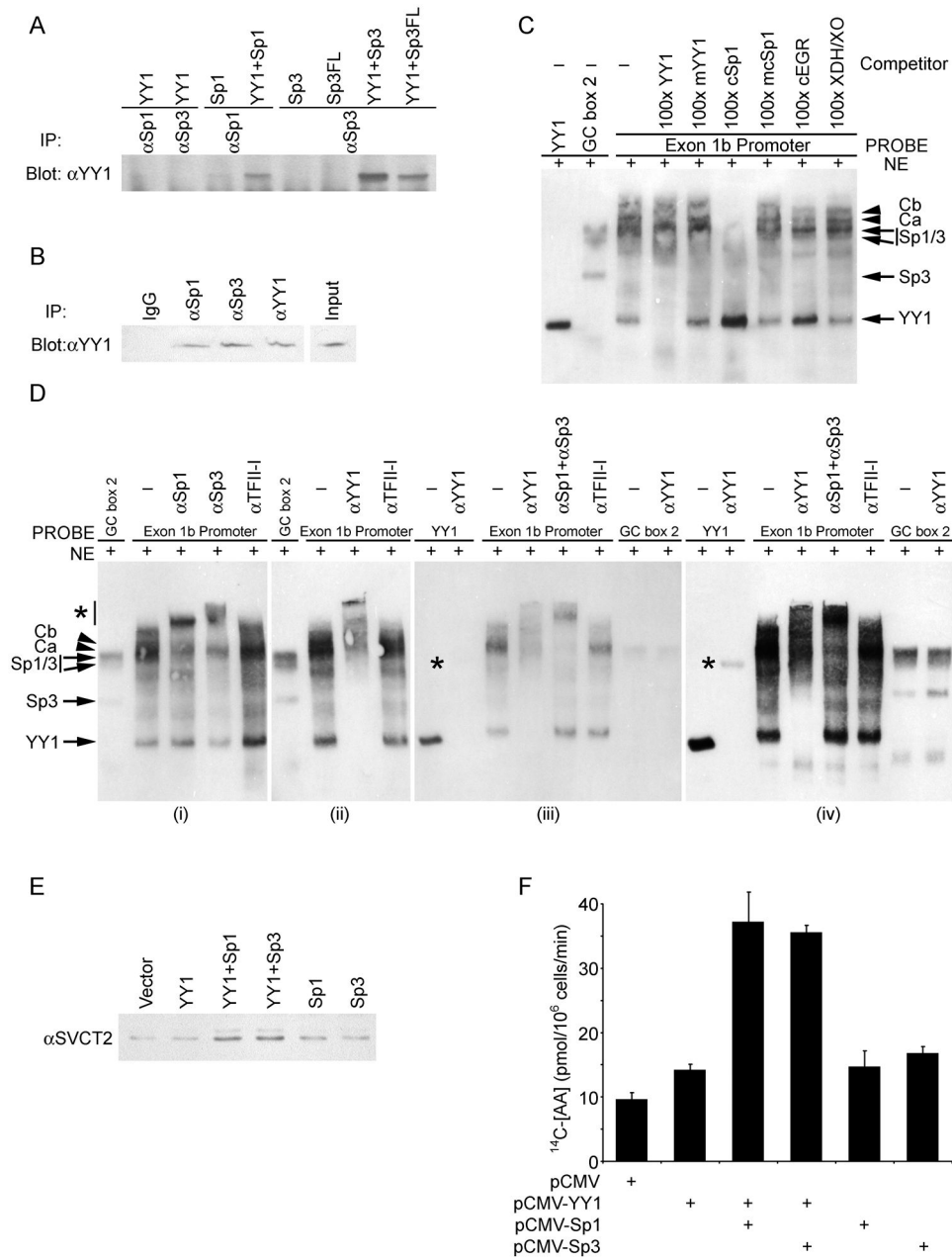
Figure 7.
Over-expression of EGR attenuates the ability of Sp1/3 to interact with the exon 1b promoter. (**A**) U2OS cells were transiently transfected with the empty vector or EGR-2, harvested 24 h later and 10 μg of nuclear protein was immunoblotted with antibody to EGR-2. (**B**) EMSA using GC box 4 probe and nuclear extracts (NE) from U2OS cells transfected with the empty vector (lane 1) or EGR-2 (lanes 2–6). Shown are competition (lanes 3 and 4) and super-shift (5 and 6) assays. Distinct complexes are indicated on the left. * indicates the super-shifted band for EGR-2. V, empty vector. (**C**) Nuclear protein prepared from U2OS cells that has been transiently transfected to express EGR-2 was subjected to EMSA with probes derived from each of the 4 GC boxes. (**D**) ChIP assay for the displacement of Sp1/3 by EGR-2 was performed using U2OS cells. Transfection efficiency was monitored by co-transfected GFP marker.

Figure 8.

YY1 and Sp1/3 synergistically activate exon 1b promoter activity. (**A**) and (**B**) U2OS cells were treated with 100 nM MMA for various time (**A**) or with various concentrations of MMA for 48 h (**B**) before performing semi-quantitative RT-PCR. (**C**) U2OS cells were transfected with exon 1b promoter constructs in the presence or absence of MMA (100 nM). After 24 h, cells were harvested and prepared for luciferase activity assays. (**D**) and (**E**) SL2 cells (5×10^5 cells/well) were transfected with 0.5 μg of exon 1b promoter constructs along with the indicated plasmids (pPac was cotransfected to maintain equal plasmid amount). Cell lysates were made 48 h after transfection for luciferase activity measurement. pRL-dA5C was added as an internal control for each transfection, and each experimental firefly luciferase measurement was normalized to the *Renilla* luciferase value. Relative luciferase activity is shown as the means based on the activity of +1/+108 and the bars on each column

show range of values of duplicate measurements. The experiment was repeated twice with similar results. M, DNA Marker.

Figure 9.
Interaction of Sp and YY1 with the exon 1b promoter. (**A**) SL2 cells were co-transfected
with YY1, SP1, or Sp3 constructs singly or in combination as noted along the top of (**A**).
Cell lysate were immunoprecipitated with antibodies against Sp1 or Sp3, and the resulting
precipitates were immunoblotted with a YY1 antibody. (**B**) Co-immunoprecipitation assays
showing the interactions between Sp1/3 and YY1 in U2OS cells. Cells were lysed and
immunoprecipitated using Sp1, Sp3, YY1 antibodies or normal IgG followed by
immunoblotted with anti-YY1 antibody. (**C**) and (**D**) EMSA using Exon 1b Promoter probe
(−42/+14) and nuclear extracts (NE) from U2OS cells. Note two slowly migrating bands (Ca
and Cb; arrow head) detected behind Sp1/3 bands when Exon 1b Promoter probe (−42/+14)
containing Inr/YY1 site and two GC boxes (1 and 2) was used as the probe with U2OS cell
nuclear extracts. YY1 or GC box 2 probe was used in the absence of competing probe. The

unlabeled competing probes (**C**) and the antibodies (**D**) used are indicated. * indicates the super-shifted bands. Panel (iv) is simply overexposure of the film shown in panel (iii). (**E**) Effects of Sp1/3 and YY1 on endogenous SVCT2 protein level. U2OS cells were plated into 100-mm culture dishes and transfected with the YY1 and/or Sp1/3 expression constructs. Cell lysates were immunoblotted with a SVCT2 antibody. (**F**) Effects of transfected YY1 and Sp1/3 on radiolabeled ascorbate uptake were measured in U2OS cells as described under **Materials and methods**. Data represent means of duplicate measurements from 1 of 2 similar experiments and the range error bars are shown. Transfection efficiency was monitored by co-transfected GFP marker.
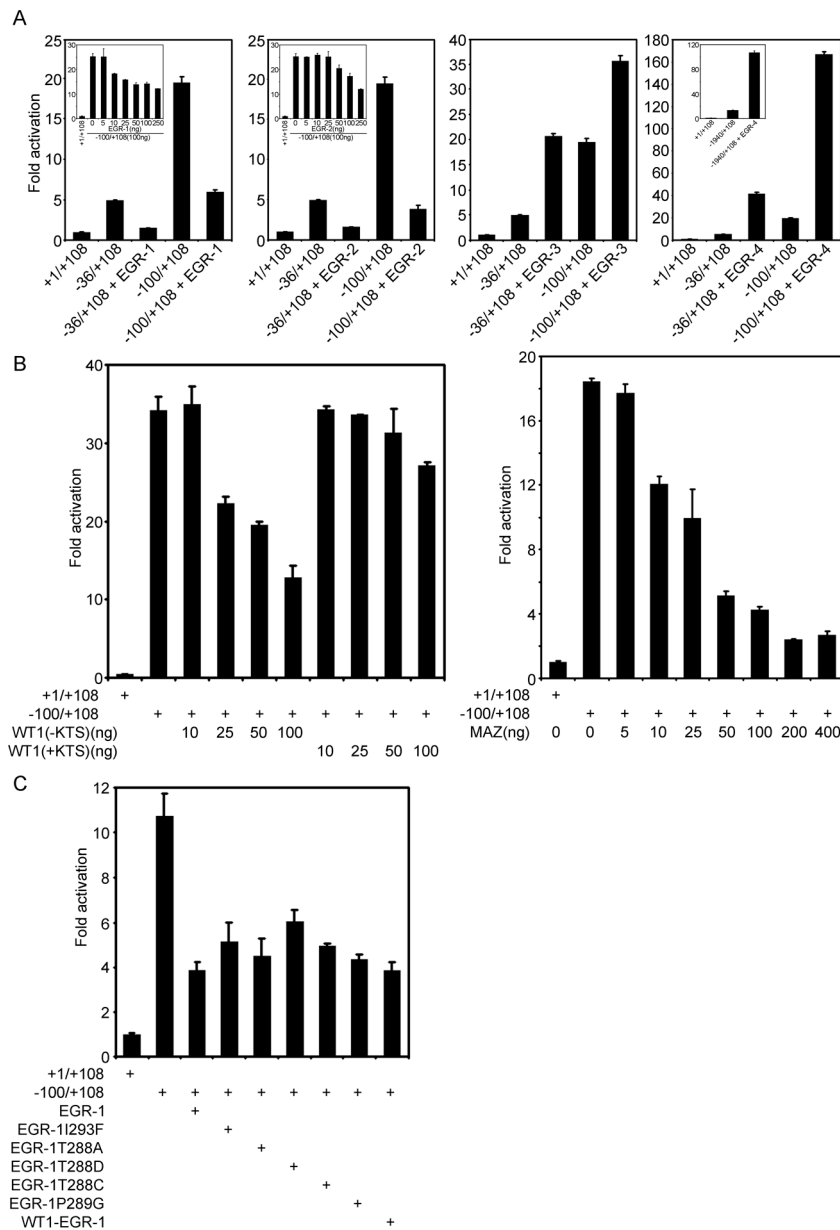
Figure 10.
EGR, WT1 and MAZ differentially regulate SVCT2 exon 1b promoter activity. (**A**) 100 ng of the +1/+108, −36/+108 and −100/+108 promoter constructs were co-transfected into U2OS cells with 400 ng of the indicated expression vectors for different EGR. Description of the insert panel is provided in the text. (**B**) 400 ng of promoter constructs with 100 ng of WT1 or 100 ng of promoter constructs with 400 ng of MAZ were transfected into U2OS cells. (**C**) U2OS cells were co-transfected with 400 ng of Nab binding-ablating EGR-1 mutants along with 100 ng of the −100/+108 promoter construct. When necessary, empty vectors were used for compensation. The promoter activity was analyzed in the luciferase reporter assay. pRL-CMV was added as an internal control and relative luciferase activity is shown as the means of duplicate measurements from 1 of 3 similar experiments and the error bars show range of values.

Table 1

Probes used in the current study

Name	Sequence
YY1/Inr	5-CTCCGCGGCGCCATTTTGCAGGCGGCT-3
YY1 consensus/YY1c	5-CGCTCCGCGGCCATCTTGGCGGCTGGT-3
mYY1	5-CTCCGCGGCGTTGTTTTGCAGGCGGCT-3
XDH/XO	5-CCGGGAGGCGTATCTTTCAAGTTGCAGGGCAGT-3
Exon 1b Promoter	5-CTCCGCTGCACCCCGCCCCCGGCCCGCC CCTCCGCGGCGCCATTTTGCAGGCGGCT-3
Inr+1	5-CTCCGCGGCGCCAGTTTGCAGGCGGCT-3
Inr+2	5-CTCCGCGGCGCCATCTTGCAGGCGGCT-3
cSp1	5-ATTCGATCGGGGCGGGGCGAG-3
mcSp1	5-ATTCGATCGGAACGGGGCGAG-3
cEGR	5-GGATCCAGCGGGGGCGAGCGGGGGCGAACG-3
GC box 1	5-CCCCGGCCCGCCCCTCCGCGGCGCC-3
mGC box 1	5-CCCCGGCCAAACCCTCCGCGGCGCC-3
GC box 2	5-CTCCGCTGCACCCCGCCCCCGGCCCGCC-3
GC box 2mEGR	5-CTCCGCTGCACCCCGCCCTTGGCCCGCC-3
mGC box 2	5-CTCCGCTGCACCCAAACCCCGGCCCGCC-3
GC box 3	5-CAGACCGGCGCGGGGGCGGGCGGCTCCGC-3
GC box 3mEGR	5-CAGACCGGCGAAGGGGCGGGCGGCTCCGC-3
mGC box 3	5-CAGACCGGCGCGGGGAAAGGCGGCTCCGC-3
GC box 4	5-GGGCAGGGGTCGGGGGCGGGGAGGGAGGTG-3
GC box 4mEGR	5-GGGCAGGGGTCAAGGGCGGGGAGGGAGGTG-3
mGC box 4	5-GGGCAGGGGTCGGGGAAAGGGAGGGAGGTG-3