Published in final edited form as:
J Nutr Biochem. 2011 April ; 22(4): 344–350. doi:10.1016/j.jnutbio.2010.03.001.

Promoter Analysis of the Human Ascorbic Acid Transporters SVCT1 & 2: Mechanisms of Adaptive Regulation in Liver Epithelial Cells

Jack C. Reidling[1] and **Stanley A. Rubin**[2]

[1] VA Medical Center, Long Beach, CA 90822, [1] University of California College of Medicine, Irvine, CA 92697

[2] VA Greater Los Angeles Medical Center, and the Department of Medicine, UCLA School of Medicine, Los Angeles, CA 90073

Abstract

Ascorbic acid, the active form of vitamin C, is a vital antioxidant in the human liver, yet the molecular mechanisms involved in the regulation of ascorbic acid transporters (hSVCT1 and hSVCT2) in liver cells are poorly understood. Therefore, we characterized the minimal promoter regions of hSVCT1 & 2 in cultured human liver epithelial cells (HepG2) and examined the effects of ascorbic acid deprivation and supplementation on activity and regulation of the transport systems. Identified minimal promoters required for basal activity were found to include multiple cis-regulatory elements, whereas mutational analysis demonstrated that HNF-1 sites in the *hSVCT1* promoter and KLF/Sp1 sites in the *hSVCT2* promoter were essential for activities. When cultured in ascorbic acid deficient or supplemented media, HepG2 cells demonstrated significant ($P < 0.01$) and specific reciprocal changes in [^{14}C]-Ascorbic acid uptake, and in hSVCT1 mRNA and protein levels as well as *hSVCT1* promoter activity. However, no significant changes in hSVCT2 expression or promoter activity were observed during ascorbic acid deficient or supplemented conditions. We mapped the ascorbic acid responsive region in the *hSVCT1* promoter and determined that HNF-1 sites are important for the adaptive regulation response. The results of these studies further characterize the *hSVCT1* and *2* promoters, establish that ascorbic acid uptake by human liver epithelial cells is adaptively regulated, and show that transcriptional mechanisms via HNF-1 in the *hSVCT1* promoter may, in part, be involved in this regulation.

Keywords

Ascorbic acid; Nutrient transport; Promoter regulation; SVCT

INTRODUCTION

Although most mammals synthesize vitamin C via the glucuronic acid pathway in the liver, humans must acquire the water-soluble micronutrient from dietary sources. The active form of the vitamin is ascorbic acid (reduced), an effective antioxidant acting as a free radical

Address correspondence to: Jack C. Reidling, VA Medical Center-151, 5901 East 7th St., Long Beach, CA 90822, Tel: (562) 826-2212, Fax: (562) 826-5675, jreidlin@uci.edu.

scavenger, and an essential cofactor in numerous enzymatic reactions (1). Once ascorbic acid has acted as a reducing agent it is converted to the oxidized form dehydro-ascorbic acid and then recycled back to the active form by enzymes and glutathione (2). Dietary deficiency of vitamin C leads to scurvy. Other clinical abnormalities reported during insufficient consumption include delayed wound healing, bone and connective tissue disorders and vasomotor instability (1). In addition, some reports have implied that optimizing vitamin C body homeostasis can offer a protective effect against gallbladder diseases, nonalcoholic liver diseases, cardiovascular disease, cancers and cataract formation (3–6).

In contrast to the well-established characteristics of vitamin C as an antioxidant there is a paucity of knowledge regarding the molecular mechanisms involved in maintaining and regulating vitamin C body homeostasis, we (among others) have recently suggested that the liver is both an important target for the antioxidant effects of vitamin C and plays a role in body vitamin homeostasis (7), adding to a small body of work regarding ascorbic acid uptake mechanisms in the liver (8–10). Our work in liver epithelial cells (7), and studies in other cell/tissue models (reviewed in 11), shows that ascorbic acid transport is Na^+-dependent, temperature-dependent and occurs via a specialized carrier-mediated mechanism. Two isoforms transport ascorbic acid, the human sodium-dependent vitamin C transporters-1 and -2 (hSVCT1 and hSVCT2), the products of the *SLC23A1* and *SLC23A2* genes, respectively. Each protein isoform participates in Na^+-dependent accrual of ascorbic acid in a variety of expression systems (7,12–16). The topology of the two transporters includes predicted 12 transmembrane spanning regions, cytoplasmic COOH- and NH_2-terminal domains, and similar functionality. Although both transporters display a high affinity for ascorbic acid (the K_m values range from 65–237μM for hSVCT1 and 8–62μM for hSVCT2, depending on the cell system utilized) hSVCT1 exhibits a higher V_{max}, suggesting the possibility that hSVCT2 is a high affinity/low capacity transporter, while hSVCT1 is a high capacity/low affinity carrier (11). Studies suggest hSVCT1 expression is confined to epithelia involved in bulk transport, such as the kidney and intestine, and hSVCT2 expression is more widespread occurring in neurons, the endocrine system, bone and other tissue (15–17). Our simultaneous comparison of the expression levels in the liver shows that hSVCT2 is expressed at slightly higher levels than hSVCT1 (7).

In this investigation we provide the first detailed analysis of the *hSVCT* promoter regions activity and regulation in cultured human liver cells. Using a combination of deletion analysis and mutagenesis, the minimal promoter regions were mapped and specific cis-regulatory elements located in those regions were identified. Next, we examined the effects of ascorbic acid deprivation and supplementation on the activity of the transport systems and determined if transcriptional mechanisms were involved in any observed regulatory events. We compared functional ascorbic acid uptake, expression of hSVCT1 and 2 protein and message levels, and promoter activities during substrate deprivation and supplementation. Finally we mapped the minimal promoter responsive region and identified the cis-regulatory elements involved.

METHODS AND MATERIALS

Materials

[^{14}C]-Ascorbic acid (13mCi/mmol) was purchased from Amersham (Arlington Heights, IL). Custom-made [^{3}H]-thiamin (specific activity >30Ci/mmol; radiochemical purity >98.0%) was obtained from American Radiolabeled Chemical (St. Louis, MO). All chemicals and reagents used in this study were of analytical/molecular biology grade and were purchased from commercial sources.

Cell culture and uptake studies

The human-derived hepatic epithelial HepG2 cells (passage 20; ATCC, Manassas, VA; these cells were derived from a 15 year old male Caucasian) were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), glutamine (0.29g/L), sodium bicarbonate (2.2g/L), penicillin (100,000U/L), and streptomycin (10mg/L) in 75cm^2 plastic flasks at 37°C in a 5% CO_2-95% air atmosphere with media changes every 2–3 days. The cells were plated at a density of 2×10^5 cells/well onto 12-well plates. Uptake was measured at 37°C in Krebs-Ringer buffer (in mM: 133 NaCl, 4.93 KCl, 1.23 MgSO4, 0.85 $CaCl_2$, 5 glucose, 5 glutamine, 10 HEPES, and 10 MES, pH 7.4). Labeled and unlabeled ascorbic acid or thiamin was added to the incubation medium at the onset of incubation, and uptake was examined during the initial linear period (3 min) as described previously for ascorbic acid (7) or thiamin (18). Uptake of ascorbic acid and thiamin by the carrier-mediated system was determined by subtracting uptake by passive diffusion (determined from the slope of the line between uptake at a high pharmacological concentration of 1mM and the point of origin) from total uptake. In studies on the effect of deficiency and supplementation on ascorbic acid uptake cells were cultured until confluent as described above (in ascorbic acid sufficient normal culture medium), then split into plates or flasks and pretreated with no ascorbic acid (deficient), 10mM ascorbic acid (supplemented), or 10%FBS (normal/control) for 24 hours. Deficient and supplemented culture medium did not have added 10%FBS, that contained ascorbic acid concentrations of 0.7uM±0.2 when tested using an Ascorbic Acid Assay Kit as described by the manufacturer (BioVision, Inc., Mountain View, CA). The DMEM utilized in the studies did not have detectable amounts of Ascorbic Acid. Protein content of cell digests was measured in parallel wells by using a Bio-Rad DC Protein Assay kit (Bio-Rad, Richmond, VA).

Quantitative real-time PCR (qPCR) and Western blot analysis

The qPCR was performed using the Bio-Rad iCycler (Hercules, CA) and a Qiagen Quantitect SYBR green PCR kit (Valencia, CA). RNA from HepG2 cells was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and the manufacturer's procedure. The RNA was DNase treated and first strand cDNA was made from 5μg of the isolated total RNA primed with oligo dT using an Invitrogen Superscript synthesis system. The RT products were then used in the subsequent qPCR with primers specific for the human SVCT1 (F-5′-TCATCCTCTTCTCCCAGTACCT-3′, R-5′-AGAGCAGCCACACGGTCAT-3′) human SVCT2 (F-5′-TCTTTGTGCTTGGATTTTCGAT-3′, R-5′-ACGTTCAACACTTGATCGATTC-3′) human biotin transporter SMVT (F-5′-TGTCTACCTTCTCCATCATGGA-3′, R-5′-TAGAGCCCAATGGCAAGAGA-3′) or the human β-actin (F-5′-AGCCAGACCGTCTCCTTGTA-3′, R-5′-TAGAGAGGGCCCACCACAC-3′). The qPCR consisted of a 15 second 95°C melt followed by 40 cycles of 95°C melt for 30 sec, 58°C annealing for 30 sec, and 72°C extension and data collection for 1 min. Melt curve analysis with plasmid DNA was performed for the generation of standard curves, and negative controls without RT were used with every reaction. To compare the relative relationship between hSVCT levels, we utilized a calculation method provided by the iCycler manufacturer (Biorad, Hercules, CA) described previously (18). Western analysis was performed using 20μg of protein isolated from HepG2 cells and the Invitrogen XCell SureLock™ system (Invitrogen, Carlsbad, CA) with antibodies (1:200 dilution) designed to be specific for the human SVCT proteins or β-actin (Santa Cruz Biotech, Santa Cruz, CA) and detected using the ECL-System. Protein samples were obtained from HepG2 cells using Trizol (Invitrogen, Carlsbad, CA) and the manufacturer's procedure.

Preparation of promoter-luciferase and deletion constructs of the SVCT1 and 2 promoters

The *hSVCT2* promoter-luciferase constructs utilized in this study, including their primer sequences, were described previously (7). The *hSVCT1* promoter-luciferase constructs used in this study were obtained using gene specific primers (Table I) on the previously cloned *hSVCT1* promoter (7) to generate deletion constructs. The PCR products were purified and subsequently subcloned into the NheI and XhoI sites of the pGL3-Basic vector (Promega, Madison, WI). Allμ constructs used in this study were verified with DNA sequencing (Laragen, Los Angeles, CA).

Cell Culture, transfection, and luciferase assay

Four micrograms of each of the promoter-luciferase constructs for *hSVCT1* and *2* were transfected separately into HepG2 cells using the Lipofectamine 2000 reagent (Invitrogen, San Diego, CA) and the manufacturer's procedure. As a control in deficiency and supplementation experiments a pGL3-Control Vector from Promega that contains SV40 promoter and enhancer sequences, resulting in strong expression of luc+ in many types of mammalian cells was utilized. The plasmid is a convenient internal standard for promoter and enhancer activities expressed by pGL3 recombinants. To normalize for transfection efficiency, the cells were co-transfected with 100ng of pRL-TK (Promega, Madison, WI) plasmid along with the promoter constructs. Total cell lysate was prepared from cells 24 hours post transfection and firefly luciferase activity was assayed using the Dual Luciferase kit (Promega) and a Turner Design 20/20 Luminometer (Sunnyvale, CA). The activity was normalized to the Renilla luciferase activity from pRL-TK in the same extract. Data presented are mean +/−SEM of at least three independent experiments and given as fold-expression over pGL3-Basic expression set arbitrarily at one.

Mutational analysis

Mutations were introduced into the *hSVCT1* and *2* minimal promoter-luciferase constructs individually at the sites indicated in Figure 1C using a Stratagene site directed mutagenesis kit (La Jolla, CA) and the manufacturer's protocols. The sequences were verified using the Laragen DNA sequencing facility (Los Angeles, CA). The mutated constructs were then transiently transfected into HepG2 cells using the identical method described above.

Statistical analysis

Transport data presented in this paper are the result of three separate experiments and are expressed as mean ± SEM in pmol/mg protein/3min. Western blot analysis and qPCR assays were all performed on at least three separate occasions. Differences between the means of control and treated cells for various outcome parameters were tested for significance using ANOVA analysis followed by post hoc testing with the critical value p chosen as 0.05. Kinetic parameters of the saturable ascorbic acid uptake process (determined by subtracting the diffusing component, determined from the slope of the uptake line between a high pharmacological concentration of ascorbic acid of 1 mM and the point of origin, from total uptake at each concentration) were calculated using a computerized model of the Michaelis-Menten equation as described previously by Wilkinson (19).

RESULTS

Transfected hSVCT1 and 2 promoter activities and minimal promoter regions in HepG2 cells

In previous preliminary studies we reported the cloning of the *hSVCT1* promoter (7) and determined the minimal region required for basal activity of the *hSVCT2* promoter in cultured human vascular smooth muscle cells (20). To gain an understanding of the

regulation of these genes in the liver, we characterized these promoters and defined the minimal regions for basal activity in cultured human liver epithelial cells (HepG2). Using a series of deletion constructs (sizes described in Table I) we found the minimal region for promoter activity for *hSVCT1* to be contained within a 204 base pair fragment (Fig. 1A). For the *hSVCT2* promoter we established that the P2 promoter is the predominantly active promoter in HepG2 cells (Fig. 1B) and the minimal region for activity within the P2 promoter is within a 199 base pair region (Fig. 1B). Note that previously published results describe the 5′-regulatory region of the *hSVCT2* promoter including the P1 and P2 promoters (20).

Role of the putative cis-regulatory elements on the activity of the hSVCT1 and 2 minimal promoters: mutational analysis

We performed a computational analysis of the determined minimal *hSVCT1* and *2* promoters to identify potential cis-regulatory elements using MatInspector version 2.2 (http://www.genomatix.de/). Our analysis led to the identification of multiple putative cis-regulatory sites in each promoter proximal to the previously reported transcription initiation sites (20,21). Of interest were two potential Hepatic Nuclear Factor 1 (HNF-1) binding sites in the *hSVCT1* promoter region, and two general Kruppel-like Factor (KLF) sites, as well as an overlapping Stimulating Protein-1 (SP-1) site in the *hSVCT2* promoter region (Fig. 1C). To directly examine the role of these putative regulatory sites we mutated the HNF-1 sites in the *hSVCT1* promoter and the KLF/SP-1 sites (the sites have some shared core elements, see Fig. 1C) in the *hSVCT2* promoter, and then examined the effect of these mutations on the minimal promoter activity in HepG2 cells. Our results show that individual mutation of either of the HNF-1 sites in the *hSVCT1* promoter decreased the promoter activity by >90% compared to activity of the control (random CC to TT mutation) minimal *hSVCT1* promoter (Fig. 2A). For the *hSVCT2* promoter, mutations at either of the two overlapping KLF/SP-1 sites caused a >50% reduction in promoter activity (Fig. 2B).

Effect of ascorbic acid deficiency or supplementation on ascorbic acid uptake and hSVCT1 and 2 expression in HepG2 cells

We examined and compared the effect of maintaining the cultured human-derived liver epithelial cell line HepG2 in either ascorbic acid deficient or ascorbic acid supplemented conditions with a control group grown under normal culture conditions for 24 hours. At the end of the treatment we analyzed ascorbic acid uptake and the levels of hSVCT1 and 2 mRNA and protein. A significant ($p < 0.01$ for supplemented and $p< 0.05$ for control) up-regulation in [^{14}C]-ascorbic acid (32μM) uptake was found in cells grown under ascorbic acid deficient conditions compared to those grown under supplemented or control conditions (Fig. 3A). For comparison, no changes in the uptake of the unrelated essential vitamin thiamin (15 nM) were observed (Fig. 3B). In addition, the results also show that supplementation significantly decreased ascorbic acid uptake compared to deficient ($p < 0.01$) and control ($p < 0.05$) conditions. These findings suggest that ascorbic acid uptake is adaptively regulated at the functional level during ascorbic acid deficiency and supplementation, and suggests that the regulation is specific in nature.

It was determined that the changes in ascorbic acid uptake during deficiency or supplementation were associated with a reciprocal increase/decrease in hSVCT1, but with no changes in hSVCT2 mRNA and protein levels as indicated by real-time quantitative PCR (qPCR) and by Western blot analysis (Fig. 3C and D). For comparison, no changes were observed for the mRNA level of the unrelated Sodium Dependent Multivitamin Transporter (SMVT) or the protein level of β-actin in the treated cells (Fig. 3C and D). These findings suggest the possibility that the mechanism of the observed induction may be due to transcriptional regulatory mechanism(s) that increase hSVCT1 levels.

Effect of ascorbic acid deficiency and supplementation on activity of the full length hSVCT1 and 2 promoters

To directly test the possibility that transcriptional regulation of the hSVCT1 promoter plays a role in adaptive regulation during ascorbic acid deficiency and supplementation, we examined the activity of the full length *hSVCT1* and *2* promoters in HepG2 cells maintained in ascorbic acid deficient and supplemented growth media. The results showed the activity of only the full length *hSVCT1* promoter undergoes a change (approximately 50% reduction comparing deficient to supplemented) during alterations of ascorbic acid levels (Figure 4). These findings suggest that the adaptive response to ascorbic acid levels in liver epithelial HepG2 cells may be mediated by transcriptional regulatory mechanisms involving the *hSVCT1* promoter.

Determination of an ascorbic acid responsive region in the hSVCT1 promoter

Given that the *hSVCT1* promoter responds to ascorbic acid levels in cultured liver epithelial cells, our intention was to next identify an ascorbic acid-responsive region in the *hSVCT1* promoter. Promoter activity of deletion constructs in ascorbic acid deficient and ascorbic acid supplemented HepG2 cells was examined. The results (Fig. 5) showed that an ascorbic acid-responsive region is encoded in a 204 bp sequence (see Fig. 1C for sequence) in the *hSVCT1* promoter. The basis for this observation is that progressive deletions of the promoter did not result in a decrease of the ascorbic acid-responsive effect until the deletion reached a 102 bp construct. Therefore, one of the major responsive regions is contained in the 204 bp region before this deletion.

Role of specific cis-regulatory elements in the ascorbic acid-responsive region of the hSVCT1 promoter in mediating the effect of deficiency

As described in results above, the ascorbic acid-responsive region of the *hSVCT1* promoter contains two HNF binding sites that are important for basal activity of this promoter. We investigated the possible role of these sites in mediating the ascorbic acid deficiency effect on the activity of the *hSVCT1* promoter. To attain this result we examined how the ascorbic acid-dependent response in promoter activity reacts to mutations in the sites using constructs transiently transfected into HepG2 cells. The findings demonstrated that mutating the HNF site at −187 bp nullifies the ascorbic acid deficient response (Fig. 5). On the other hand, mutating the HNF site at −113 bp had a more limited affect on the ascorbic acid deficient response of the *hSVCT1* promoter activity. These findings suggest a potential critical and specific role for the HNF sites in mediating the ascorbic acid-deficient response on the *hSVCT1* promoter.

DISCUSSION

In an attempt to understand the regulation of expression of the human ascorbic acid transporters hSVCT1 and 2 in the human liver, we characterized their 5′-regulatory regions by identifying the minimal promoter sequences required for basal activity in cultured human liver epithelial cells (HepG2). We also determined the role of putative cis-elements in regulating the promoter activities. In addition, we performed a functional study that demonstrated adaptive regulation of the expression and activity of the ascorbic acid transport system in response to altered substrate level. Furthermore, we determined that transcriptional mechanisms involving the hSVCT1 promoter participate in the observed regulatory events.

Promoter mutational analysis allowed us to confirm the importance of specific cis-regulatory elements. We found that HNF-1 sites demonstrated considerable control of *hSVCT1* minimal promoter activity, whereas KLF/SP-1 sites regulated *hSVCT2* minimal promoter activity.

Hepatic nuclear factor (HNF) transcription factors have a wide range of functions. Especially interesting to our studies with ascorbic acid and liver cells are those involved in cholesterol, bile acid, and lipoprotein metabolism (22). The fact that *hSVCT1* promoter regulation involves HNF-1 sites supports the potential importance of ascorbic acid in these liver functions. The KLF and SP1 sites we identified in the *hSVCT2* minimal promoter are somewhat general GC-rich zinc finger sites. One could speculate that hSVCT2 expression is more ubiquitous in the liver and less regulated than hSVCT1. Our results with adaptive regulation, further discussed below, may support this notion.

Results from this study confirm that ascorbic acid deficiency leads to a specific and significant up-regulation in human liver epithelial cell ascorbic acid uptake that was associated with an increase in transcript abundance and protein levels of hSVCT1. Supplementation with ascorbic acid had the inverse effect, down-regulating uptake and hSVCT1 transcript and protein levels. On the other hand, we found that hSVCT2 expression is unchanged by altering substrate level and therefore does not appear to be adaptively regulated in HepG2 cells. It is important to note that the concentration of ascorbic acid used in our supplementation studies, 10mM, exceeds the normal physiological range in the bloodstream of humans, 40–60uM (23), and is most likely only attainable during clinical situations when intravenous injection of supplements are used. However, several issues should be discussed regarding our use of this high concentration. First, a previous publication reported using a supplemental concentration of 25.5 mM to attain an effect on ascorbic acid transport in intestinal epithelial cells (Caco-2) and that lower concentrations had no effect (24). In our study with HepG2 cells we also observed that lower ascorbic acid concentrations had little or no effect (unpublished observations). In addition, a recent report suggests that some cultured cells, including hepatic Hep3B cells, under normal culturing conditions store negligible amounts of ascorbic acid (25). Yet, human liver epithelial cells have been reported to store ascorbic acid to concentrations of 1mM (23). Perhaps cultured cells, routinely maintained in low concentrations of ascorbic acid, when presented with an excess of ascorbic acid in the culture media first store the excess until a threshold is reached, then physiological changes occur in uptake and SVCT levels. While *in vivo*, liver epithelial cells may never reach the depleted state of ascorbic acid obtained with cultured cells and would therefore enact any physiological effects when presented with minor ascorbic acid concentration changes in the bloodstream. Further studies will be required to address this issue. We should also acknowledge that treating HepG2 cells with 10mM ascorbic acid for periods of time greater than 24 hrs (48 and 72 hrs) began to have a cytotoxic effect on the cells with losses of up to 50% (unpublished observations).

The increase in hSVCT1 mRNA levels during ascorbic acid deficiency suggested that transcriptional regulatory mechanisms of the *hSVCT1* gene might participate in mediating the regulatory events. To test this possibility, we examined activity of our *hSVCT1* and *2* promoter-luciferase constructs during ascorbic acid deficiency and supplementation. Ascorbic acid deficiency was associated with an increase in the activity of the *hSVCT1* promoter, but the *hSVCT2* promoter activity was not significantly altered. These results are similar to findings we reported for the thiamin transporters during adaptive regulation in intestinal epithelial cells. That is, the increase in thiamin transport during deficiency appears to be the result of transcriptional mechanisms that increase THTR2 levels but not THTR1 (26). Our hypothesis with both studies is that one transporter performs the role of bulk vitamin transport during substrate sufficient conditions, whereas the other transporter is more prone to regulation during conditions where vitamin levels are altered. More studies will be required to support this hypothesis.

To elaborate on the finding that transcriptional mechanisms are involved in the ascorbic acid-deficient response, we mapped and determined a potential cis-regulatory site in an

ascorbic acid responsive region in the *hSVCT1* promoter. We found that an ascorbic acid responsive region was located in the minimal 204 bp promoter region, and that mutation of a specific HNF-1 site caused attenuation of the ascorbic acid deficient response. However, mutation of another HNF-1 site had a more limited effect. As mentioned above, the HNF-1 transcription factor has multiple roles in liver functions including cholesterol, bile acid, and lipoprotein metabolism. Our study may be a link to ascorbic acid levels, the *hSVCT1* promoter and these important liver functions. However, more work will be necessary to understand the details.

Our results identified the minimal regions required for basal activity in the *hSVCT1* and *2* promoters. Of considerable note with respect to tissue-specific regulation of gene expression, we found different *hSVCT* promoter activities in this study when compared to our previously published work on the promoter activities of the *hSVCTs* in cultured human vascular smooth muscle cells (hVSMC) (20). First, the *hSVCT1* gene is expressed in HepG2 cells, whereas its transcript is not detectable in hVSMC, likely representing the absence of gene expression. Also, with respect to the *hSVCT2* minimal promoters (P1 and P2), both the activities we report here in HepG2 cells differ considerably from our previously published findings in hVSMC. In that study, we found significant relative activity for the P1 promoter in hVSMC (approximately half the activity of P2), whereas our current findings in HepG2 cells demonstrate that the P1 promoter has very little activity (less that one tenth the activity of P2). In addition, the minimal promoter region for P2 activity in HepG2 cells is between the P2d6–7 locus, whereas in hVSMC the more important sequence may reside in the P2d7–8 locus. This result shifts the focus for examining cis-regulatory elements of the *hSVCT2* gene in the two cell lines and supports the idea that examining promoter activity in various cell types gives a better picture of tissue-specific regulation of gene expression.

In conclusion, results of these studies further characterize the *hSVCT1* and *2* promoters, establish that ascorbic acid uptake by human liver epithelial cells is adaptively regulated, and show that transcriptional mechanisms via HNF-1 in the *hSVCT1* promoter may, in part, be involved in this regulation.

Acknowledgments

The work was supported by grants from the National Institutes of Health DK73032 and the Department of Veterans Affairs.

References

1. Packer, L.; Fuchs, J. Vitamin C in health and disease. New York: Marcel Dekker Inc; 1997.
2. Meister A. Glutathione-ascorbic acid antioxidant system in animals. J Biol Chem 1994;269:9397–9400. [PubMed: 8144521]
3. Carr AC, Frei B. Toward a new recommended dietary allowance for Vitamin C based on antioxidant and health effects in humans. Am J Clin Nutr 1999;69:1086–1107. [PubMed: 10357726]
4. Harrison SA, Torgerson S, Hayashi P, Ward J, Schenker S. Vitamin E and vitamin C treatment improves fibrosis in patients with nonalcoholic steatohepatitis. Am J Gastroenterol 2003;98:2485–2490. [PubMed: 14638353]
5. Li Y, Schellhorn HE. New developments and novel therapeutic perspectives for vitamin. C J Nutr 2007;137:2171–2184.
6. Simon JA, Hudes ES. Serum Ascorbic Acid and Gallbladder Disease Prevalence Among US Adults. Arch Intern Med 2000;160:931–936. [PubMed: 10761957]
7. Reidling JC, Subramanian VS, Dahhan T, Sadat M, Said HM. Mechanisms and regulation of vitamin C uptake: studies of the hSVCT systems in human liver epithelial cells. Am J Physiol Gastrointest Liver Physiol 2008;295:G1217–1227. [PubMed: 18845575]

8. Banhegyi G, Marcolongo P, Puskas F, Fulceri R, Mandl J. Dehydroascorbate and ascorbate transport in rat liver microsomal vesicles. J Biol Chem 1998;273:2758–2762. [PubMed: 9446582]
9. Michels AJ, Joisher N, Hagen TM. Age-related decline of sodium-dependent ascorbic acid transport in isolated rat hepatocytes. Arch Biochem Biophys 2003;410:112–120. [PubMed: 12559983]
10. Upston JM, Karjalainen A, Bygrave FL, Stocker R. Efflux of hepatic ascorbate: a potential contributor to the maintenance of plasma vitamin C. Biochem J 1999;342:49–56. [PubMed: 10432299]
11. Savini I, Rossi A, Pierro C, Avigliano L, Catani MV. SVCT1 and SVCT2: key proteins for vitamin C uptake. Amino Acids 2008;34:347–355. [PubMed: 17541511]
12. Daruwala R, Song J, Koh WS, Rumsey SC, Levine M. Cloning and functional characterization of the human sodium-dependent vitamin C transporters hSVCT1 and hSVCT2. FEBS Lett 1999;460:480–484. [PubMed: 10556521]
13. Maulen NP, Henriquez EA, Kempe S, Carcamo JG, Schmid-Kotsas A, Bachem M, Grunert A, Bustamante ME, Nualart F, Vera JC. Up-regulation and polarized expression of the sodium-ascorbic acid transporter SVCT1 in post-confluent differentiated CaCo-2 Cells. J Biol Chem 2003;278:9035–9041. [PubMed: 12381735]
14. Rajan PD, Huang W, Dutta B, Devoe LD, Leibach FH, Ganapathy V, Prasad PD. Human placental sodium dependent vitamin C transporter (SVCT2): molecular cloning and transport function. Biochem Biophys Res Commun 1999;262:762–768. [PubMed: 10471399]
15. Wang H, Dutta B, Huang W, Devoe LD, Leibach FH, Ganapathy V, Prasad PD. Human Na+-dependent vitamin C transporter 1 (hSVCT1): primary structure, functional characteristics and evidence for a non-functional splice variant. Biochim Biophys Acta 1999;1461:1–9. [PubMed: 10556483]
16. Wang Y, Mackenzie B, Tsukaguchi H, Weremowicz S, Morton CC, Hediger MA. Human vitamin C (L-ascorbic acid) transporter SVCT1. Biochem Biophys Res Commun 2000;267:488–494. [PubMed: 10631088]
17. Takanaga H, Mackenzie B, Hediger MA. Sodium-dependent ascorbic acid transporter family SLC23. Pflugers Arch 2003;447:677–682. [PubMed: 12845532]
18. Reidling JC, Nabokina SM, Balamurugan K, Said HM. Developmental maturation of intestinal and renal thiamin uptake: studies in wild-type and transgenic mice carrying human THTR-1 and 2 promoters. J Cell Physiol 2006;206:371–377. [PubMed: 16206251]
19. Wilkinson GN. Statistical estimations in enzyme kinetics. Biochem J 1961;80:324–332. [PubMed: 13785321]
20. Rubin SA, Dey S, Reidling JC. Functional analysis of two regulatory regions of the human Na+-dependent vitamin C transporter 2, SLC23A2, in human vascular smooth muscle cells. Biochim Biophys Acta 2005;1732:76–78. [PubMed: 16380174]
21. Erichsen HC, Eck P, Levine M, Chanock S. Characterization of the genomic structure of the human vitamin C transporter SVCT1 (SLC23A2). J Nutr 2001;131:2623–7. [PubMed: 11584081]
22. Armendariz AD, Krauss RM. Hepatic nuclear factor 1-alpha: inflammation, genetics, and atherosclerosis. Curr Opin Lipidol 2009 Apr;20(2):106–11. [PubMed: 19280766]
23. Harrison FE, May JM. Vitamin C function in the brain: vital role of the ascorbate transporter SVCT2. Free Rad Biol & Med 2009;46:719–730. [PubMed: 19162177]
24. MacDonald L, Thumser AE, Sharp P. Decreased expression of the vitamin C transporter SVCT1 by ascorbicacid in a human intestinal epithelial cell line. Br J of Nutr 2002;87:97–100. [PubMed: 11895172]
25. Koren E, Zverev I, Ginsburg I, Kohen R. Supplementation with antioxidants fails to increase the total antioxidant capacity of several cell lines in culture. Biomed Pharmacother 2008;62(3):179–88. [PubMed: 18249084]
26. Reidling JC, Said HM. Adaptive regulation of intestinal thiamin uptake: molecular mechanism using wild-type and transgenic mice carrying hTHTR-1 and -2 promoters. Am J Physiol Gastrointest Liver Physiol 2005;288:G1127–1134. [PubMed: 15705657]

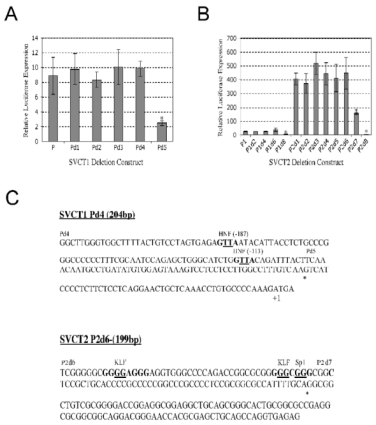

Figure 1. Functional analysis and identification of the minimal region required for basal activity of the hSVCT1 and 2 promoters

The size and position of different promoter-luciferase constructs are described in Table 1. The results of a luciferase assay for each construct following transient transfection into cultured human liver HepG2 cells is shown for the *hSVCT1* promoter (A) and *hSVCT2* promoter (B). Firefly luciferase activity was normalized relative to the activity of simultaneously expressed Renilla luciferase. The results are expressed relative to the pGL3-basic vector set at 1 and represent the average of at least three independent experiments. In panel (C) the nucleotide sequence of the minimal region required for basal activity of *hSVCT1* and *hSVCT2* is shown. Bold nucleotides indicate the positions of several identified putative cis regulatory elements. The nucleotides altered for mutational studies are underlined. Two HNF-1 sites (−187 and −113 relative to the translational start site A of ATG as number 1) were changed, the TT at position −187 was changed to CC and the TT at −113 was changed to AA in the *hSVCT1* promoter. Two KLF sites (KLF1 designates the site 5′ of the second site named KLF2) or an SP-1 site were changed from GG to TT in the *hSVCT2* promoter. Transcription initiation sites (As determined in reference 21 for SVCT1 and reference 20 for SVCT2) are designated (*). Numbers adjacent to HNF sites represent nucleotides relative to the translational start site A of ATG as number 1. The overall p-value was determined by initial ANOVA analysis followed by post hoc testing of pairs, *$p < 0.01$.

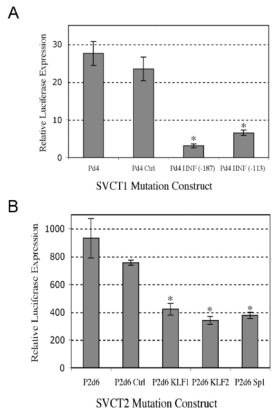

Figure 2. Mutational analysis of specific sites in the hSVCT1 and 2 minimal promoter regions

Two conserved nucleotides in the core consensus binding sites for two HNF-1 sites (−187 and −113 relative to the translational start site A of ATG as number 1) were changed in the *hSVCT1* promoter (A) and two KLF sites (KLF1 designates the site 5′ of the second site named KLF2) or an SP-1 site were changed in the *hSVCT2* promoter (B), all in separate reactions (See Figure 1C for the sites). As a control, a CC site not in any predicted binding sites, but in the same minimal region, was mutated to TT. The mutated minimal regions were then tested for promoter activity using the luciferase assay system and HepG2 cells described in Methods. The results are expressed relative to the pGL3-Basic vector set at 1 and represent the mean ± SEM of at least three independent experiments. The overall p-value was determined by initial ANOVA analysis followed by post hoc testing of pairs, *$p < 0.01$.

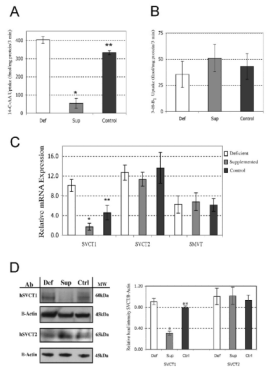

Figure 3. Adaptive regulation of the ascorbic acid uptake process

A) Initial rate of carrier-mediated ascorbic acid uptake by HepG2 cells maintained in ascorbic acid-deficient, supplemented and control growth media. Cells were incubated for 3-minute in buffer with A) [^{14}C]-Ascorbic acid (32μM) or B) [^{3}H]-Thiamin (15nM) added to the incubation medium. Data are mean ± SEM of 3 to 5 separate uptake determinations. C) Effect of ascorbic acid deficiency and supplementation on the level of hSVCT1, hSVCT2 or hSMVT RNA expression. Real-time PCR was performed on cDNA from ascorbic acid-deficient, supplemented and control HepG2 cells. Data are from 3 different experiments and expressed relative to β-actin as mean ± SEM. D) Expression of the hSVCT1 and 2 protein in HepG2 cells grown under ascorbic acid-deficient (Def), supplemented (Sup) and control (Ctrl) conditions. The antibodies utilized are shown on the left (Ab) and the estimated molecular weight of the bands based on proximity to standard markers is shown on the right (MW). Densitometry was determined using the ImageJ scanning system (http://rsbweb.nih.gov/ij/index.html) and data is presented as normalized to the intensity of β-actin for each sample. The overall p-values were determined by initial ANOVA analysis followed by post hoc testing of pairs (*$p < 0.01$, **$p < 0.05$).

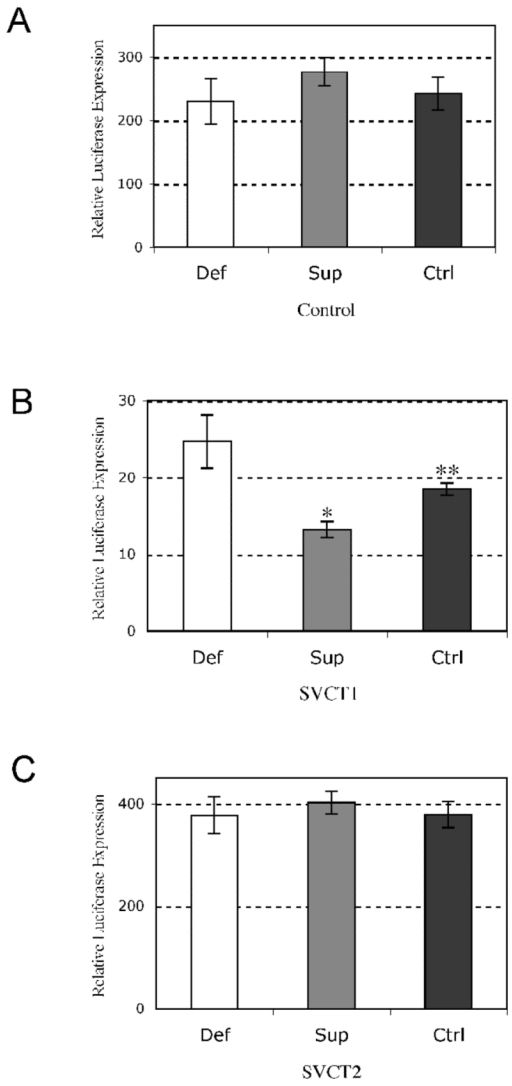

Figure 4. Effect of ascorbic acid deficiency or supplementation on the activity of the hSVCT1 and 2 promoters in HepG2 cells

Cells were transiently transfected with the control (Promega, described in Methods) (A), or full length *hSVCT1* (B) or *hSVCT2* (C) promoters and then maintained in ascorbic acid deficient (Def), supplemented (Sup) or control (Ctrl) growth medium for 24 hours. Cell lysate was isolated and assayed for Firefly luciferase activity. Luciferase activity was normalized relative to the activity of simultaneously expressed Renilla luciferase. The results are expressed relative to the pGL3-Basic vector set at 1 and represent the mean ± SEM of at least three independent experiments. The overall p-values were determined by initial ANOVA analysis followed by post hoc testing of pairs (*$p < 0.01$, **$p < 0.05$).

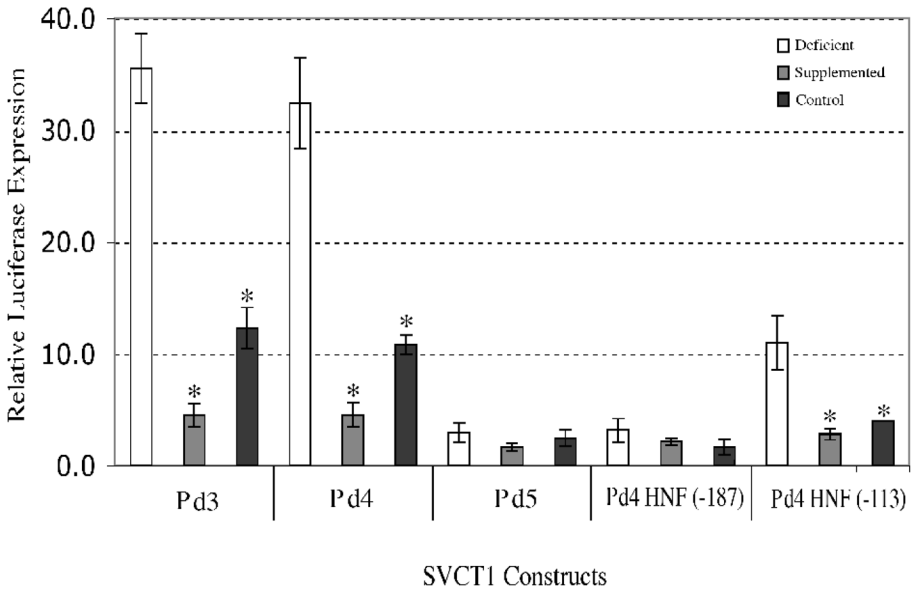

Figure 5. Effect of ascorbic acid deficiency or supplementation on the activity of the hSVCT1 promoter in HepG2 cells: determination of the ascorbic acid responsive region and mutational analysis in HepG2 cells

Cells were transiently transfected with the *hSVCT1* promoter deletion constructs Pd3, Pd4, Pd5 or the constructs mutated for HNF-1 at −187 bp or HNF-1 at −113 bp (See figure 1C) and then maintained in ascorbic acid deficient (Def), supplemented (Sup) or control (Ctrl) growth medium for 24 hours. Cell lysate was isolated and assayed for Firefly luciferase activity. Luciferase activity was normalized relative to the activity of simultaneously expressed Renilla luciferase. The results are expressed relative to the pGL3-Basic vector set at 1 and represent the mean ± SEM of at least three independent experiments. Note that, because of the small value, some statistical standard errors of the mean bars are not visible on the scale of the graph presented. The overall p-value was determined by initial ANOVA analysis followed by post hoc testing of pairs, *$p < 0.01$.

Table I

A) Gene specific primers used for generating deletions of the *hSVCT1* promoter.		
Primer Name	**Sequence (5′ to 3′)**	**Construct length (bp)**
P forward	**ctagctag**ctggtgtggtgatgtaatcgcc	3065
Pd1 forward	**ctagctagc**cctgggagagagacagaacat	1980
Pd2 forward	**ctagctagc**caaggcagctcagaccaacct	631
Pd3 forward	**ctagctagc**tggggcttggtaaacttctgg	319
Pd4 forward	**ctagctagc**ggcttgggtggcttttactgt	204
Pd5 forward	**ctagctagc**caaacaatgcctgatatgtgg	102
P reverse	**ccgctcgag**ctcatctttgggggcacaggtt	

B) Deletion constructs of the *hSVCT2* promoter utilized in this study.			
P1 Promoter		**P2 Promoter**	
Construct	**Construct length (bp)**	**Construct**	**Construct length (bp)**
P1	2249	P2	2049
P1d2	1479	P2d1	507
P1d4	899	P2d2	423
P1d6	481	P2d3	369
P1d8	266	P2d4	316
		P2d5	251
		P2d6	199
		P2d7	150
		P2d8	108

P reverse is the 3′-primer used to generate each construct.

Bold indicates restriction site sequences for NheI in the forward primers and XhoI in the reverse primer used for subcloning.