Role of Sp1 and HNF1 transcription factors in SGLT1 regulation during chronic intestinal inflammation

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Kekuda R, Saha P, Sundaram U. Role of Sp1 and HNF1 transcription factors in SGLT1 regulation during chronic intestinal inflammation. Am J Physiol Gastrointest Liver Physiol 294: G1354–G1361, 2008. First published March 13, 2008; doi:10.1152/ajpgi.00080.2008.—In a rabbit model of chronic intestinal inflammation, we previously demonstrated that the activity of Na-glucose cotransporter (SGLT1), SLC5A1, is inhibited. This inhibition is secondary to a decrease in the number of cotransporters, indicating that the regulation of SGLT1 during chronic inflammation is at the level of transcription. However, the regulation of SGLT1 expression and the transcription factors involved in the regulation are not yet known. In this report, we describe the cloning and characterization of rabbit SGLT1 promoter and the identification of transcription factors affected in villus cells during chronic intestinal inflammation. The promoter sequence for SGLT1 gene was identified by using the publicly available rabbit genomic sequence. Even though rabbit SGLT1 promoter did not have considerable overall homology with other mammalian SGLT1 promoters, two specificity protein 1 (Sp1) and a hepatocyte nuclear factor 1 (HNF1) binding sites were highly conserved among the species. Rabbit SGLT1 cDNA was encoded by 15 exons. Minimal promoter region determination showed that 196 nucleotides upstream of the transcription start site were sufficient for optimal promoter activity. This region encompassed two transcription factor binding sites, Sp1 and HNF1. For maximal SGLT1 promoter activity, these two transcription factor binding sites were essential, and their effect was synergistic, indicating that two separate regulatory pathways might be involved in their regulation. Using mobility shift assays, we further demonstrated that the binding of both Sp1 and HNF1 transcription factors to SGLT1 promoter regions were affected during chronic intestinal inflammation. Thus this report demonstrates that Sp1 and HNF1 transcription factors act in concert to regulate SGLT1 expression in the chronically inflamed intestine.

Na-glucose cotransporter 1; rabbit SGLT1 promoter; transcriptional regulation

MATERIALS AND METHODS

Induction of chronic intestinal inflammation and drug treatment. New Zealand White male rabbits (2.0–2.2 kg) were utilized. Chronic intestinal inflammation was produced in rabbits as previously reported (22). Pathogen-free rabbits were intragastrically inoculated with Eimeria magna oocytes or sham inoculated with 0.9% NaCl (control animals). None of the sham inoculations and 80% of inoculations with coccidia resulted in chronic intestinal inflammation during days 13–15. Only enterocytes from those animals that had histologically confirmed chronic ileal inflammation were utilized for experiments. Animals were euthanized with an overdose of pentobarbital sodium through the ear vein according to the IACUC-approved protocols of the West Virginia University.

Cell isolation. Villus cells were isolated from the intestine by a calcium chelation technique as previously described (21, 22). Previously established criteria were utilized to validate good separation of villus and crypt cells. Some of these criteria included 1) marker enzymes (e.g., thymidine kinase and alkaline phosphatase), 2) transporter specificity e.g., Na/H on the brush-border membrane (BBM) of villus but not crypt cells), 3) differences in intracellular pH (e.g., intracellular pH is higher in crypt cells compared with villus), 4) morphological differences (e.g., villus cells are larger and better developed BBM compared with crypt cells), and 5) differing rates of protein synthesis (e.g., higher synthesis rate in crypt cells compared with villus). Previously established criteria were also utilized to study cells with good viability and to exclude cells that showed evidence of poor viability: 1) Trypan blue exclusion, 2) the demonstration of Na/H and Cl/HCO3 exchange activities, and 3) the ability of the cells to maintain a baseline pH or imposed acid or alkaline gradient and return to baseline pH after perturbations. At least one, frequently two, or all three criteria are used to assess viability of all cell preparations (21, 22).

Identification of rabbit SGLT1 promoter sequence and transcription start site. Rabbit SGLT1 promoter sequence was identified by using the genomic sequence data generated by the National Institutes of Health Intramural Sequencing Center. Genomic assembly with...
SGLT1 mRNA except the 3' end. A trace archive sequence (from National Center for Biotechnology Information, NCBI) with accession number 635823780 was used to identify the 3' end of the SGLT1 mRNA.

To determine the transcription start site, 5' rapid amplification of cDNA ends (RACE) technique was used. SMART RACE cDNA amplification kit (Clontech Laboratories, Mountainview, CA) was used to synthesize cDNA from the total RNA isolated from rabbit intestinal villus cells according to the manufacturer's protocol. With the use of the cDNA generated as template, PCR was performed with universal primer mix provided by the manufacturer and a SGLT1 gene-specific anti-sense primer with the sequence GGGCTCAAAGT-GCTGCTG. The resultant PCR product was cloned into pGEM-T Easy vector (Promega, Madison, WI). The identity of the PCR product was confirmed by the custom cDNA sequencing service provided by Agencourt Bioscience, Beverly, MA.

Rabbit SGLT1 promoter subcloning and construction of deletion variants. Using rabbit genomic DNA, a PCR product of 1,373 bp in length, which encompassed rabbit SGLT1 promoter region and part of the coding region, was generated using sense and anti-sense PCR primers designed on the basis of the sequence of AC166310.1, harboring almost all the exons corresponding to the SGLT1 cDNA. The exon/intron analysis showed except when error bars are inclusive within the symbol. All experiments were done in triplicate and repeated thrice unless otherwise specified. Student’s t-test was used for statistical analysis.

RESULTS

Promoter analysis and genomic organization of rabbit SGLT1. Since our laboratory showed that SGLT1 activity is inhibited during chronic intestinal inflammation secondary to a decrease in the number of transporters (23), identification of regulatory elements in the SGLT1 promoter region was necessary to decipher the mechanism of regulation of SGLT1 activity during chronic inflammation. A search of the genomic database with rabbit SGLT1 cDNA sequence (9) yielded a genomic assembly with accession number AC166310.1, harboring almost all the exons corresponding to the SGLT1 cDNA except the 3’ end. A trace archive sequence (from NCBI) with accession number 635823780 was used to identify the 3’ end exon of the SGLT1 cDNA. The exon/intron analysis showed that SGLT1 gene is encoded by 15 exons (Fig. 1). Since the genomic sequence of rabbit is incomplete, the total length of the SGLT1 gene could not be deciphered.

Rabbit SGLT1 promoter cloning and construction of deletion variants. Putative transcription start site for rabbit SGLT1 was identified by 5’ RACE (data not shown). Rabbit genomic assembly AC166310.1 encompassed 8.2-kb upstream sequence from the putative transcription start site. A 1,373-bp promoter fragment, extending from nucleotides −1,269 to
was first PCR amplified using rabbit genomic sequence as a template, which was subsequently used as a template for the construction of deletion variants.

For the determination of minimal promoter region necessary for SGLT1 promoter activity, seven deletion constructs were prepared. This was accomplished by performing PCR using seven upstream primers designed for the various regions of SGLT1 promoter and a common downstream primer as described in MATERIALS AND METHODS. The resultant PCR products, the length and position of which are shown in Fig. 4, were subcloned into pGL4.10(luc2) vector unidirectionally upstream of firefly luciferase reporter gene. Identity of all the seven constructs was confirmed by sequencing, and the genomic sequence between nucleotides −1,049 and +38 is shown in Fig. 2 (GenBank accession number EU414633).

To date, human (24), rat (18), and sheep SGLT1 (26) promoter sequences have been identified. Next we compared the 275-nucleotide-long rabbit SGLT1 promoter with the promoter sequences of rat (GenBank accession number AF007832), human (GenBank accession number Z74021), and sheep (GenBank accession number AJ223077) SGLT1 genes. As shown in Fig. 3, even though overall homology of the promoter sequences between the four species was insignificant, four highly conserved blocks of sequences were observed. At nucleotide position −31, TATA box was located. At position −55, a highly conserved sequence that represents HNF1 transcription factor binding site was located. At nucleotide positions starting at −253 and −69, we observed two other conserved sequences, which corresponded to the Sp1 transcription factor binding sites, also known as GC box sequences and are labeled as GC box 1 and GC box 2, respectively, in Fig. 3.

**Determination of minimal promoter region required for the function of rabbit SGLT1 promoter.** We constructed a series of deletion variants for the purpose of determining the minimal promoter sequence necessary for SGLT1 promoter activity (Fig. 4). Constructs 1–4 are systematic deletion of SGLT1 promoter sequence starting at nucleotide −1,049. Construct 5 had a deletion of GC box 1, construct 6 had a deletion of GC box 2, and construct 7 had a deletion of HNF1 transcription factor binding site. For the functional analysis of SGLT1, first we determined the transfection efficiency of intestinal epithelial cell (IEC)-18 (rat intestinal) and Caco-2 (human intestinal) epithelial cells using construct 1, the longest construct containing 1,049-nucleotide-long promoter sequence. Under the experimental conditions used, Caco-2 cells showed high transfection efficiency, whereas IEC-18 cells did not transfet well, and hence Caco-2 cells were used for the functional analysis of SGLT1 promoter (Fig. 5A).

Next, the various deletion constructs were transfected into Caco-2 cells along with pRL-TK vector, which was used to

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**Fig. 2. Rabbit SGLT1 promoter sequence.** The sequence of 1,049-nucleotide-long promoter region, upstream of the transcription start site (TSS), putative translational start site (START), TATA box (at nucleotide −31), hepatocyte nuclear factor 1 (HNF1) (between nucleotides −55 to −42), and two predicted GC-box sequences (between nucleotides −253 to −243 and −69 to −60) are highlighted.
normalize transfection efficiency. Assay for the expression of firefly gene was performed to determine SGLT1 promoter function. As can be seen from Fig. 5B, constructs 1 through 3 exhibited increasing levels of promoter activity and constructs 4 and 5 (GC box 1 deletion) showed maximal luciferase activity. However, construct 6 (GC box 2 deletion) showed 40%, and construct 7 (HNF1 transcription factor binding site deletion) showed 10% promoter activity compared with construct 5. These results showed that GC box 1 function is dispensable for SGLT1 promoter function. These results also showed that GC box 2 and HNF1 transcription factor binding sites were essential for SGLT1 promoter function and that the effect of these two transcription factors is synergistic.

Mobility shift assays using Sp1 and HNF1 transcription factor binding oligonucleotides and nuclear extracts from villus cells from the normal rabbit small intestine. Since deletion experiments showed that GC box 2 and HNF1 transcription factor binding sites were essential for SGLT1 promoter activity, we performed mobility shift assays to demonstrate that the oligonucleotides corresponding to these two sites bind their respective transcription factors. As seen in Fig. 6A, mobility of Sp1 oligonucleotide shifted in the presence of villus cell nuclear extract giving rise to two bands, a slow-migrating and fast-migrating band (Fig. 6A, lane 2). A nonspecific oligonucleotide, AP2 transcription factor binding, did not inhibit the binding of the labeled Sp1 oligo to the nuclear extract (Fig. 6A, lanes 3 and 4). On the other hand, excess cold Sp1 oligonucleotide inhibited the binding of the labeled Sp1 oligo to the nuclear extract (Fig. 6A, lanes 5 and 6).
of labeled putative Sp1 transcription factor consensus sequence (lanes 5 and 6), whereas the binding was completely inhibited by cold Sp1 oligonucleotide (lanes 3 and 4) at 50-fold molar excess.

Mobility shift assays using Sp1 and HNF1 transcription factor binding oligonucleotides and nuclear extracts from villus cells from the chronically inflamed rabbit small intestine.

To decipher whether chronic intestinal inflammation had any effect on the binding of labeled Sp1 oligonucleotide, we performed mobility shift assays with the nuclear extracts obtained from the villus cells from the normal and chronically inflamed rabbit villus cells. As seen in Fig. 7A, nuclear extract from the villus cells from the normal rabbit ileum gave the expected two shifted bands; slow-migrating and fast-migrating (lane 2). On the other hand, when nuclear extract from the villus cells from the inflamed ileum was used, the mobility of the slow-migrating band shifted upwards further, producing a diffuse band (Fig. 7B, lane 3).

Figure 7B shows mobility shift experiments performed using the nuclear extracts from the villus cells and the labeled HNF1 transcription factor binding oligonucleotide (Fig. 7B). When the nuclear extract from the villus cells from the normal rabbit ileum was used, we obtained slow- and fast-migrating shifted bands with labeled HNF1 oligonucleotide (Fig. 7B, lane 2). On the other hand, when the villus cell nuclear extract from inflamed ileum was used, the mobility of the slow-migrating band shifted upwards further, producing a diffuse band (Fig. 7B, lane 3).
Effect of LPS on the SGLT1 promoter deletion constructs.

To decipher whether SGLT1 deletion constructs containing minimal promoter regions could be used to delineate regulatory pathways during chronic intestinal inflammation, Caco-2 cells transfected with the promoter constructs were treated with LPS, and luciferase assays were performed (Fig. 8, A and B). LPS inhibited SGLT1 promoter activity to a significant extent when construct 5, where the Sp1 transcription factor binding site (GC box 2) was intact, was transfected into Caco-2 cells (Fig. 8B), whereas no inhibition of luciferase activity was observed when Sp1 transcription factor binding site was removed (Fig. 8B, construct 6). This data showed that Sp1 transcription factor is absolutely essential for the regulation of SGLT1 promoter activity upon LPS treatment.

DISCUSSION

In this study we reported the identification of rabbit SGLT1 gene promoter by using the rabbit genomic sequence available from the public sequence database. Even though the rabbit genomic sequence was incomplete, we were able to identify the genomic sequence representing all the exons representing the SGLT1 cDNA using both the draft genomic assemblies and trace archive sequences from NCBI. Rabbit SGLT1 cDNA sequence is 2,164 bp long (9). However, 5' RACE results indicated that there are 12 extra nucleotides with sequence GAGGGACGCGTT at the cDNA 5' terminus compared with the published cDNA sequence. Rabbit SGLT1, like its human ortholog, is encoded by 15 exons (24). Since the primary goal of this report is to characterize the SGLT1 promoter, lack of information on the total size of the genomic DNA encoding SGLT1 does not affect the conclusions drawn in this paper.
Even though rabbit promoter sequence did not show significant overall homology to the SGLT1 promoter sequences from other species, three transcription factor binding sites located within nucleotides −255 and −1 were highly conserved (Fig. 3; GC box 1, GC box 2, HNF1) compared with rat, sheep, and human SGLT1 promoter sequences (18, 24, 26). Since a high degree of DNA conservation underscores the functional significance of the regulatory elements (20), we mainly focused on these three regulatory elements in the present study. Even though we identified a 8.2-kb promoter region, we did not analyze the upstream promoter region since the study of a long promoter could get complicated by the presence of numerous cis- and trans-activating elements. This point is validated by the observation that when a longer 1,061-bp SGLT1 promoter sequence was used, the expression of luciferase was the lowest compared with the shorter constructs, indicating the presence of negative regulatory elements in this longer promoter construct (Fig. 5B). We observed that the construct containing −290 to −1 of the promoter sequence had maximal luciferase activity, indicating that the promoter sequence upstream of this region harbors one or more negative regulatory elements.

Minimal promoter determination studies of Martin et al. (13) showed that, for human rabbit SGLT1 promoter function, all three conserved transcription factor binding sites, GC box 1, GC box 2, and HNF1, were necessary. Contrary to these findings, as seen from Fig. 2 and Fig. 5B, rabbit SGLT1 was not regulated by GC box 1 (construct 5); deletion of GC box 1 transcription factor binding site had no effect on luciferase expression. On the other hand, when GC box 2 was deleted, luciferase activity was reduced by 60% (Figs. 2 and 4, construct 6). When HNF1 transcription factor binding site was deleted (Figs. 2 and 4, construct 7), luciferase activity was reduced by 90%. These results proved that both GC box 2 and HNF1 sites are necessary for rabbit SGLT1 promoter activity and their effect on SGLT1 promoter regulation is synergistic.

Sp1 is the first identified member of the growing number of Sp1 transcription factor family (5) that contains three zinc fingers within a highly conserved DNA-binding domain. GC-rich sequences known as GC boxes bind to Sp1 family of transcription factors and regulate gene function. Presently, in humans, six members of Sp1 family are known, Sp1 to Sp6 (11). All the Sp1 family members have very high homology in the region of the zinc finger domain (84–95%). Among these, Sp1, Sp4, and Sp6 are transcription activators, and Sp3 can act as both activator and repressor. The role of Sp2 and Sp5 are presently not known. Hence, it is possible that any member of the Sp1 transcription factor might be responsible for SGLT1 promoter activity. Using mobility supershift experiments using antisera against Sp1, Sp2, and Sp3, Martin et al. (13) showed that all three transcription factors can bind to the human GC box 1 and 2 oligonucleotides. However, since all the Sp1 transcription factor family members bind to GC-rich sequences, the mobility shift assay experiments do not prove or disprove the involvement of any of the Sp1 transcription factors. Also, antisera against Sp1 to Sp3 are available but not Sp4 through Sp6. Because of these reasons, we did not perform supershift experiments using antisera against Sp1 transcription factor members, but rather we showed the specificity using specific and nonspecific oligonucleotides corresponding to Sp1 transcription factor binding consensus sequence. Hence, which of the Sp1 transcription factor family members involved in rabbit SGLT1 gene regulation is not presently known.

HNF family of transcription factors includes HNF1-α, HNF1-β, HNF3-α, -β and -γ, HNF4, and HNF6 (15). Among these, HNF1-α and HNF1-β are expressed in polarized epithelia of different organs including liver, digestive tract, pancreas, and kidney (15). Recently it has been shown that null mutations of HNF1-α not only suffer from diabetes in mice, but also suffer from a renal Fanconi syndrome characterized by glucose loss (16). Using Northern blot studies, the authors showed that SGLT2 was inhibited by 80–90% in homozygous HNF1-α mutant mice compared with either wild-type or heterozygous mice, whereas SGLT1 levels were unaffected. Using mobility shift assay and cotransfection experiments, Martin et al. (13) also showed that both HNF1-α and HNF1-β are involved in SGLT1 gene regulation. However, the authors note that the supershifts obtained with the antisera against HNF1-β could be nonspecific. Using promoter-luciferase constructs and mobility shift and mobility supershift assays, Vayro et al. (25) showed that ovine SGLT1 gene is regulated by both HNF1-α and HNF1-β. Rhoads et al. (18) showed that rat circadian periodicity of intestinal SGLT1 mRNA is transcriptionally regulated. By analyzing the rat SGLT1 promoter sequence, the authors showed an HNF1-binding element was capable of forming different complexes with nuclear extracts depending on the time of the day. Our results in the present report show that HNF1 regulates rabbit SGLT1 gene regulation (Fig. 6B), further underscoring the importance of HNF1 transcription factors in SGLT1 gene regulation.

HNF1 is a glycosylated protein and exists as a homo- or heterodimer (15). It also interacts with an accessory protein called dimerization cofactor for HNF1 (DCoH) (14). HNF1 is highly regulated during embryonic development (15). Sp1 family of transporters is also known to be regulated by glycosylation and phosphorylation (12). Because of the functional conservation of these two transcription factors and the fact that SGLT1 is transcriptionally regulated during chronic intestinal inflammation, we speculated that SGLT1 gene regulation during inflammation is brought about at the level of the transcription factors. Hence mobility shift assays were performed using nuclear extracts obtained from villus cells from normal and chronically inflamed rabbit intestine and Sp1- and HNF1-binding oligonucleotides (Fig. 7, A and B). As can be seen from these figures, we did not see any difference in the fast-migrating bands obtained with either labeled Sp1 or HNF1 oligonucleotides, whereas the slow-migrating bands in both the cases shifted upwards, giving rise to diffuse bands (Fig. 7, A and B; lanes 2 and 3). These results demonstrated that both these transcription factors are affected during chronic intestinal inflammation. The shift in migration of the labeled oligonucleotides could be brought about by phosphorylation, glycosylation, or both of the transcription factors. It could also be brought about by association with accessory proteins; for example, association with DCoH in the case of HNF1. The exact nature of the modification of transcription factors is not known and is being investigated presently in our laboratory.

One of our aims of the present study is to find out whether we can use the minimal SGLT1 promoter constructs to study pathways of SGLT1 regulation. One of the most widely accepted theories of IBD pathogenesis is that the disease is brought about by an aggressive cell-mediated immune re-
response to commensal bacteria in the small intestine and colon (6, 7, 10). Since LPS is produced by the gram-negative bacteria, LPS treatment could be used in vitro to mimic the effect of the commensal bacteria in vivo. In fact, it was previously shown that LPS injection into mice could be used as a model for severe experimental gram-negative sepsis and that glucocorticoid treatment reduced LPS-induced cytokine production (19). It was also shown that gram-negative bacteria can activate NF-κB through LPS binding to membrane-bound toll-like receptor 4 (TLR-4, 8). Since NF-κB is one of the transcription factors upregulated during chronic intestinal inflammation, leading to the expression of inflammatory cytokines (28), we used LPS to treat Caco-2 cell lines transfected with promoter constructs as a cellular model for chronic intestinal inflammation. As can be seen from Fig. 8B, LPS significantly inhibited the expression of construct 5, whereas it did not have any effect on construct 6. The conclusions drawn from this experiment are twofold; one is that LPS acts via the regulation of Sp1 transcription factor or both Sp1 and HNF1 transcription factors, and second is that the minimal SGLT1 promoter constructs could be used for the study of inflammatory pathways. The pathways of SGLT1 transcriptional dysregulation during inflammation are not presently known. The availability of the promoter constructs now paves the way to future studies of deciphering second messenger pathways that affect SGLT1 during inflammation. Further delineation of the regulatory pathways of SGLT1 regulation using the promoter constructs is underway in our laboratory.

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