Expression of sodium-hydrogen exchanger isoform 3 (NHE3) in the intestinal and renal epithelium plays a critical role in sodium absorption and acid/base homeostasis. To decipher rat NHE3 gene regulation, its cis-acting regulatory elements and associated transcription factors were characterized by transient transfection of Caco-2, IEC-6, QT6, and Drosophila SL2 cells. Deletion and mutational analyses demonstrated that the atypical TATA box located at bp −260/−31 was not necessary for promoter activity, and that a −20/+8-bp fragment represents a functional initiator. Within the 81-bp upstream region, three Sp transcription factor binding sites were critical because their mutation drastically reduced promoter activity. The roles of Sp1 and Sp3 were further demonstrated by electromobility shift assay and by transient transfection of the NHE3 promoter in SL2 cells by forced expression of Sp1 and Sp3. Both of these transcription factors were found to act synergistically with GATA-5 bound to a GATA box in exon 1 (+20/+23 bp). These studies demonstrate that rat NHE3 promoter is initiator-driven and controlled mainly by Sp1 and Sp3, which functionally interact with GATA-5. This interaction represents a novel regulatory mechanism, which is likely to participate in a gradient of intestinal gene expression along the crypt-villus axis.

NHE3 activity is regulated at different levels: phosphorylation-mediated regulation of the transporter dependent on its linkage to the cytoskeletal protein ezrin with NHERF and E3KARP (6), regulation by trafficking of NHE3 protein on and off the apical membrane via changes in endocytosis and apical membrane recycling (7), and through transcriptionally mediated changes in NHE3 mRNA levels. An array of factors has been implicated in transcriptional regulation of NHE3 gene expression including glucocorticoid hormones (9, 10), thyroid hormone (11), protein kinase C (12), and sodium butyrate (13, 14). Previous studies from our laboratory also suggested that transcriptional regulation is a critical component of maturational changes in intestinal NHE3 activity and gene expression during rat postnatal development (15). Although laboratory rodents have become a common model to study physiology and pathophysiology of Na⁺/H⁺ exchange, and the rat NHE3 gene promoter has been cloned by two independent groups of investigators (9, 16), a comprehensive analysis of its function and basal regulation has been lacking to date.

NHE3 exhibits not only a temporal but also a horizontal, differentiation-specific pattern of expression along the crypt-villus axis. NHE3 expression is very low in the small intestinal epithelium of suckling animals and drastically increases after weaning (15). Additionally, NHE3 protein is not expressed in undifferentiated crypt cells of the small intestine or colon, while it is present in high abundance in differentiated absorptive enterocytes with a detectable gradient of expression directed toward the tips of the intestinal villi (17–19). Due to its physiological importance and complex expression patterns, NHE3 may be considered a marker gene suitable for studying the role of specific transcription factors and their interactions during enterocyte differentiation as well as during postnatal intestinal development, similar to the already established models of lactase-phlorizin hydrolase (LPH), sucrase-isomaltase (SI), and intestinal fatty acid-binding protein (fABP). The body of knowledge provided by studying regulation of intestinal gene expression provided by studies of the three latter genes is already significant, but despite this fact, a complete understanding of the genetic programming of mammalian intestinal differentiation and maturation is still far from being achieved.

In recent years, a concept of combinatorial gene regulation has become a paradigm in understanding complex regulation of gene transcription in eukaryotes. The activation of eukaryotic genes in vivo often requires the coordinated binding of multiple transcription factors to promoter-enhancer regions of genes. Many of these transacting factors are not only expressed in tissue- and differentiation-specific manners, but they are also regulated by distinct signaling pathways. Furthermore, in many cases, cooperative binding of multiple transcription fac-
tors to gene promoter requires a unique composition and spatial arrangement of transcription factor binding sites. These facts add to the complexity of transcriptional networks participating in regulation of gene expression.

In this article, we describe the first comprehensive analysis of NHE3 promoter function. We confirm previously disputed promoter function, whereas DNase I-treated RNA 

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**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human colon adenocarcinoma (Caco-2) cells, normal rat small intestinal epithelial (IEC-6) cells, NIH-3T3 mouse fibroblasts, quail fibro sarcoma (Q6) cells, and Drosophila SL2 cells were obtained from American Type Culture Collection (Manassas, VA). Caco-2 and IEC-6 cells were maintained in high-glucose Dulbecco’s Modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. QT-6 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum and antibiotics. QT-6 cells were maintained in F12K medium supplemented with 10% (v/v) fetal bovine serum and antibiotics. Tobacco acid pyrophosphatase (TAP) was generously provided by Drs. R. Tjian and G. Suske, respectively; pAc-GATA-5 construct was made by subcloning a Small/EcoRV fragment of mouse GATA-5 into the EcoRV site of pAc5.1A plasmid (Invitrogen). β-galactosidase activity was expressed as relative light units (RLU) per μg of protein. The protein concentration was determined by BCA protein assay (Pierce, Rockford, IL).

**Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays**—Nuclear extracts were prepared using a modification of the method by Dignam (21) as described by Dent and Latchman (22). The obtained nuclear protein was dialyzed in 10,000 MWCO mini dialysis units (Amicon, Bellefonte, PA) against redistilled water at 4°C and applied to a Drosophila SL2 cells were maintained in Schneider’s insect medium supplemented with 10% (v/v) fetal bovine serum and antibiotics. All of the aforementioned cells were grown at 37°C in a 5% CO2, 95% air incubator. Drosophila SL2 cells were maintained in Schneider’s insect medium supplemented with 10% (v/v) fetal bovine serum and antibiotics, and were grown at 25°C without CO2.

**Plasmid Constructs**—Rat NHE3 promoter constructs were prepared in pGL-3 basic luciferase reporter vector (Promega, Madison, WI) as described earlier (14). Since overexpression of Sp or GATA transcription factors activated promoter-less pGL3-basic but not pGAL-basic vector (Clontech, Palo Alto, CA) in transfected cells, transcriptional experiments with these factors were performed with NHE3 promoter fragments subcloned into pGAL-basic. Site-directed mutations were introduced by standard PCR with mutant forward primers utilizing a high fidelity DNA polymerase (Pfx, Invitrogen, Carlsbad, CA) or by two-step overlap PCR as previously described (20). A construct spanning ~20 bp to ~8 bp of the NHE3 gene was created by subcloning a double-stranded synthetic oligonucleotide into XmaI/XhoI sites of pGL3-basic. All constructs were confirmed by DNA sequencing.

**5’-RLM-RACE**—Since two previous reports (9, 16) described different transcription initiation sites within the rat NHE3 gene, RNA ligase-dependent rapid amplification of cDNA ends (5’-RLM-RACE) was employed to determine the 5’-end of the NHE3 transcript utilizing components of FirstChoice RLM-RACE kit (Ambion, Austin, TX). High quality total RNA was isolated with trizol reagent (Invitrogen) followed by extraction with acid phenol. RNA from the rat small intestine and colon was used to confirm the transcription initiation site in the endogenous NHE3 gene, whereas DNase I-treated RNA isolated from Caco-2 cells transfected with NHE3 gene reporter plasmid (~118/55Luc) was used to confirm the transcription initiation site utilized in synthetic reporter gene constructs. Briefly, 10 μg of RNA was dephosphorylated to remove the 5′-phosphate group from RNA or contaminating DNA molecules. Tobacco acid pyrophosphatase (TAP) was then used to specifically remove the cap structure from mRNA molecules. An RNA oligonucleotide was next ligated to newly de-capped mRNA using T4 RNA ligase and the resulting RNA was reverse-transcribed using SuperScript III (Invitrogen) and random primers. Oligo and nested hot-start PCR reactions were performed using proofreading DNA polymerase (Platinum Taq Hi Fidelity, Invitrogen) with adapter- and gene-specific primers. 1 μl of the nested PCR product was subcloned into the pCR4Blunt-TOPO vector (Invitrogen). 25–35 independent clones were sequenced for each 5′-RLM-RACE reaction.

**Transfections and Reporter Gene Assays**—Caco-2 cells were transfected in 24-well plates at ~80% confluence with 200 ng of NHE3 promoter constructs and 2 ng of promoter-less pRL-null vector containing the Renilla luciferase reporter gene as an internal control. IEC-6, NHE3 promoter constructs and 10 ng of pRL-null. In experiments with forced expression of GATA transcription factors, 0.2 or 1 μg of pcDNA3 (Invitrogen) or pcDNA3-based expression vectors with mouse GATA-4, GATA-5, or GATA-6 cDNAs (generously provided by Dr. E. Morrissey) were co-transfected along with NHE3 promoter constructs into respective cell lines. All mammalian cell lines were transfected with LipofectAMINE (Invitrogen) and Q76 cells were transfected with FuGENE 6 (Roche; Indianapolis, IN) according to manufacturer’s instructions. 24 h post-transfection, cells were harvested and assayed for reporter gene activity using a dual luciferase assay (Promega; for constructs made in pGL3-basic) or with Galacto-Light Plus (Applied Biosystems, Foster City, CA) and Renilla luciferase assay system (Promega; for constructs in pGal-basic). Endogenous β-galactosidase activity in mammalian cell lines was reduced by incubating cell lysates at 48°C for 50 min prior to performing the β-galactosidase assay.

SL-2 cells were seeded at 500,000 cells per well in 24-well plates 24 h prior to transfection with 200 ng of pβGal–118/58 and various amounts of SP1 or Sp3 and GATA-5 expression constructs utilizing the Drosophila actin 5 promoter (pPacSp1 and pPacUSp3 were generously provided by Drs. R. Tjian and G. Suske, respectively; pAc-GATA-5 construct was made by subcloning a Small/EcoRV fragment of mouse GATA-5 into the EcoRV site of pAc5.1A plasmid (Invitrogen)). β-galactosidase activity was expressed as relative light units (RLU) per μg of protein. The protein concentration was determined by BCA protein assay (Pierce, Rockford, IL).

**Southwestern Blotting**—All procedures were carried out at 4°C. 50 μg of Caco-2 and IEC-6 cell nuclear extract was fractionated on a 4–20% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane in a buffer containing 25 mM Tris and 190 mM glycine for 30 min at 100 V. The membrane was then incubated with blocking buffer (2% nonfat dry milk, 1% bovine serum albumin, 50 mM HEPES (pH 7.9), 75 mM MgCl2, 40 mM KCl, 0.05 mM EDTA, 5% glycero, 140 mM β-mercaptoethanol, and 16 μg/ml sonicated salmon sperm DNA) for 2 h and then incubated with 10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 4% glycero, 1 μg/reaction poly(dI-dC) and 1 μg/reaction polylsine.

**Statistical Analysis**—One-way analysis of variance or the Student’s t test were employed for statistical analyses utilizing StatView software (v. 4.0; SAS Institute, Inc., Cary, NC). Post-hoc multiple comparisons were carried out with Fisher’s PLSD test.

**RESULTS**

**Transcription Initiation Site Mapping**—Since previous reports utilizing primer extension (9) and S1 nuclelease protection assay (16) techniques produced contradictory results, we employed 5’-RLM-RACE as an alternative method of mapping the 5’-end of the NHE3 transcript. This technique identified multiple transcription initiation sites (TIS), overlapping those detected by Kandasamy and Orlowski (16) by S1 nuclelease protection assay and localized within the -3’ACCTG-5′ sequence (Fig. 1). Interestingly, the major transcription initiation site reported by Kandasamy and Orlowski (16), which is used for nucleotide numbering throughout this study, was not seen in the jejunal RNA pool or in the heterologous NHE3/Luc gene, and only 4% of colonic NHE3 transcripts started at this position. Utilization of TIS in different tissues varied slightly, as summarized in Fig. 1. None of the sequenced clones matched...
the TIS described by Cano (9) by primer extension. Small proportion of 5′/H11032-RLM-RACE products (4% jejunum; 4% colon) started 20–70 bp downstream of the 5′/H11032-end of NHE3 transcript identified by initial cloning experiments with the cDNA library (23). Since cDNA libraries rarely contain full-length 5′/H11032-untranslated regions, these results were dismissed as likely artifacts.

The same cluster of TIS was utilized to drive expression of the luciferase reporter gene from the 5′/H11002118/5′/H1100158Luc NHE3 promoter construct (Fig. 1). While 91% of detected transcripts originated within the same cluster of nucleotides (5′/H110023AC-CTG5′/H110012), 4% of transcripts of the reporter gene started at nt 9 and 5% at nt +21. Relevance of these sites is however, unclear.

**Functional Mapping of the Rat NHE3 Promoter**—A series of reporter plasmids containing various lengths of the NHE3 5′-flanking regions (from nt 1360 to nt 20) upstream of the firefly luciferase (Luc) gene was transfected into Caco-2 cells. The levels of Luc activity were normalized to Renilla luciferase activity. A short promoter fragment spanning nt 118 to 58bp conferred 82% of the activity of the longest cloned promoter fragment (p0.015 for 1360/158 versus 118/58) (Fig. 2).

Minor fluctuations in activity were observed among constructs including more than 118 bp of the promoter sequence, suggesting the presence of enhancing or repressive elements, which conceivably play a role in fine-tuning transcriptional activity. Also, further deletion to 81 bp only slightly altered the promoter activity (18% decrease; p = 0.03 for 118/58 versus 81/58). Surprisingly, a short construct spanning 20/58...
58 bp exhibited significant activity (4.9-fold above background; p = 0.008 for −20/+8 versus pGL3-basic), whereas extending the sequence further upstream (−35/+58 bp) to include the reported atypical TATA box (ATTAAA; Ref. 16) resulted in a decrease to levels not statistically different from the promoterless vector (Fig. 2). Since extension of the promoter past the TATA-like sequence and initiator in NHE3 promoter activity, we set out to address its relevance by mutating it (ATTAAA → ATGCAA; mutated bases in bold) in the −118/+58-bp construct and testing its activity in transiently transfected Caco-2 and IEC-6 cells. Mutation of this atypical TATA box did not result in significant changes in promoter activity in either cell line (Fig. 3A). Similar results were obtained with an internal deletion construct (not shown).

Additional studies showed that 28 bp immediately surrounding the transcription start site (−20/+8 bp), were able to promote low level transcription (Fig. 3B). In gel mobility shift assays with Caco-2 cell nuclear extract and a labeled −20/+8 bp oligonucleotide as a probe, we were able to demonstrate a specific protein-DNA complex, which could be competed in a dose-dependent fashion with a consensus TATA box containing oligonucleotide, which was previously demonstrated to bind TATA-box binding protein (TBP; Ref. 24) (Fig. 3C). Additionally, in Southwestern blotting experiments with nuclear protein from Caco-2 and IEC-6 cells, we demonstrated weak binding of labeled −20/+8 bp probe to a protein of ∼40 kDa, which is consistent with the molecular weight of the p36 TFIID protein (Fig. 3D).

**Downstream Elements**—Deletion of 50 nt of exon 1 in construct −118/+8 resulted in a significant reduction in NHE3 promoter activity (Fig. 4B). Computer prediction analysis (25) of putative cis elements located in this exonic region suggested binding sites for AP4 and GATA transcription factors (Fig. 2) and an overlapping binding site for AP1 and CREB. Mutation of the AP1/CREB site resulted in no significant change in promoter activity (data not shown). We therefore focused on the sequence spanning nt +8 to +30 and performed gel mobility shift assays with this region as labeled probe and with competing double-stranded oligonucleotides with scanning mutations in blocks of four base pairs (Fig. 4A). Only mutant 4 (M4), which included a mutated GATA box was not able to compete for nuclear protein binding, suggesting that GATA transcription factors bind to this element. Furthermore, this shifted complex could also be competed with a commercially obtained consensus GATA box oligonucleotide (Geneka) from the tal-1 gene (26). The importance of this GATA-box in the NHE3 gene was further demonstrated by introducing an internal deletion (AGATA) into the −118/+58 construct. In transfected Caco-2 cells, the activity of this construct was significantly lower than that of the wild-type −118/+58-bp plasmid, and was not significantly different from the construct with 3′ deletion of 50 nt of exon 1 (Fig. 4B).

**Proteins Binding to a +20/+23 NT GATA-Box**—GATA-4, -5, and -6 subfamily proteins are expressed in an overlapping pattern in the developing heart and endoderm-derived organs of the gastrointestinal tract including the stomach, intestine,
liver, and pancreas (27). To address binding specificity of the reported GATA box, a 20-bp GATA box-containing probe (nt +10/+30) was used in gel mobility shift assays. Nuclear protein from Caco-2 (Fig. 5A) and IEC-6 cells (not shown) formed a specific complex which could be competed with excess unlabeled probe (Comp. A) and a tal-1 consensus GATA box (Comp. B), but not with a probe containing a mutation in the GATA box from the NHE3 gene (Comp. C). Also, the complex could be supershifted with an antibody specific for GATA-6 (Geneka), the GATA isoform predominantly expressed in Caco-2 cells. Gel shift experiments with IEC-6 cell nuclear protein showed identical results (data not shown). Although IEC-6 cells were found by RT-PCR to express GATA-4, -5, and -6 (data not shown), we were not able to demonstrate their binding in supershift experiments due to lack of reliable antibodies for GATA-4 and GATA-5. Instead, we used nuclear protein from Qt6 cell nuclear extract confirmed that these cells lack the endogenous nuclear proteins, which bind to mammalian GATA sequences. Moreover, nuclear protein from GATA-transfected Qt6 cells formed specific complexes with the NHE3 GATA probe (+10/+30 nt), which upon longer separation corresponded with differences in molecular weight of respective GATA proteins (Fig. 5B). This experiment indicated that all three intestinally expressed GATA transcription factors were capable of binding to the NHE3 GATA probe.

Transactivation of NHE3 Promoter by Overexpressed GATA Transcription Factors—Forced expression of GATA-4, -5, or -6 in transiently transfected Caco-2 and IEC-6 cells resulted in increased NHE3 promoter activity (Fig. 6A), with GATA-5 being the strongest stimulator. Similar results were obtained in the non-intestinal cell lines, NIH-3T3 (data not shown) and Qt6 fibroblasts (Fig. 5C). Surprisingly, overexpression of GATA-5 did not affect the activity of a short promoter fragment (nt −20/−58), despite the fact that it contained an intact GATA box (nt +20/+23) (Fig. 6B), suggesting that elements upstream of nt −20 are necessary for GATA-5 stimulation of the NHE3 promoter. Extending the 5′-flanking region to nt −81 restored the transactivation of the promoter to levels observed with the −118/−58 construct. Furthermore, mutation of three putative Sp family transcription factor binding sites, with core cis elements located at nt −71/−68 (SpA), −58/−55 (SpB; complementary strand), and −46/−43 (SpC), again eliminated the stimulatory effects of GATA-5 on NHE3 promoter activity (Fig. 6B). This suggested cooperative or synergistic effects of GATA-5 and Sp proteins interacting at one or more of the three putative Sp binding sites.

The Role of the Putative Sp Binding Sites in NHE3 Promoter Activity—Gel mobility shift assays were performed with a 42-bp DNA probe spanning all three putative Sp consensus elements and nuclear protein purified from Caco-2 and IEC-6 cells. We demonstrated that this sequence forms a specific shifted complex, which could be competed with excess unlabeled probe (Fig. 7A). We also tested the ability of individual and composite mutants of the three Sp sites to compete for binding as an indirect measure of their affinity for Sp transcription factors. Single site A and C mutants effectively competed for binding with labeled wild type probe, although not as efficiently as the unlabeled wild type probe, suggesting that these sites have very low affinity for Sp proteins. Site B mutant oligonucleotide was much less effective as a competitor, which signifies the importance of site B in interacting with Sp transcription factors. Double mutation of sites A and C did not result in decreased competition; however, double mutations of sites A and B as well as B and C reduced their ability to compete for binding. Furthermore, mutation of all three Sp binding sites resulted in complete loss of competition. Identical results were obtained with Caco-2 and IEC-6 nuclear protein (Fig. 7A). These data suggest that while site B is the predominant site of Sp/DNA interaction, sites A and C may also play a supportive role, with Sp binding to site B being a prerequisite and possibly preceding event.

The relevance of site B in nuclear protein binding is further demonstrated in Fig. 7B, where individual sites A, B, and C from the NHE3 promoter were used as competitors along with
a commercial Sp consensus probe and a nonspecific doublestranded oligonucleotide (TATA-box). Competitor probes with individual Sp sites were slightly modified at their 5'- and 3'-ends to avoid overlap and to provide sufficient probe length. In this series of experiments, site B was able to compete for binding along with excess wild type unlabeled probe and Sp consensus probe. Furthermore, functional data from Caco-2 cells transfected with GATA-4, -5, and -6 expression vectors form specific complexes with the NHE3 gene GATA box (radiolabeled probe as in panel A), which upon longer separation correspond to differences in molecular weight of respective GATA transcription factors. C, forced expression of GATA transcription factors in Qt6 cells resulted in induction of co-transfected -118/+58-βGal reporter gene construct activity.

![Figure 5. Proteins binding to the GATA-box. A, in-gel mobility shift assays with Caco-2 nuclear protein, specific protein/DNA complexes (lane 2) could be supershifted with anti-GATA-6 antibody (Ab; lane 3) and could be competed with excess of unlabeled probe (Comp. A; lane 4), or a consensus GATA probe from an unrelated gene (Comp. B; lane 5), but not by a cold probe with a mutated GATA box (Comp. C, mutation underlined; lane 6). B, nuclear proteins from Qt6 cells transfected with GATA-4, -5, and -6 expression vectors form specific complexes with the NHE3 gene GATA box (radiolabeled probe as in panel A), which upon longer separation correspond to differences in molecular weight of respective GATA transcription factors. C, forced expression of GATA transcription factors in Qt6 cells resulted in induction of co-transfected -118/+58-βGal reporter gene construct activity.]

![Figure 6. Transactivation of NHE3 promoter by GATA transcription factors. A, activity of an NHE3 promoter construct (-118/+58) in Caco-2 (black bars) or IEC-6 (open bars) cells contransfected with empty vector (pCDNA3) or with respective GATA cDNA cloned into the same vector. B, in transfected Caco-2 cells, the shortest active promoter construct (-20/+58) containing an intact GATA box in exon 1 did not respond to overexpression of GATA-5 (black bars) when compared with cells contransfected with empty expression vector (gray bars). However, extension of the 5'-flanking region to include putative Sp transcription factor binding sites in construct -81/+58 restored the response, which was again abolished by mutating all three Sp binding sites (-81/+58 SpMutABC). Depicted below is a scheme representation of NHE3 promoter constructs used in this experiment. +1 indicates TIS (16). Data presented in panels A and B are means ± S.D. (n = 4–6).]

Synergistic Interaction between GATA-5 and Sp1 and Sp3—Results reported in Fig. 6 suggested that GATA-5 functionally interacts with Sp transcription factors to regulate NHE3 promoter activity. To determine if these transcription factors indeed act synergistically, we utilized Sp-deficient SL2 cells. Cotransfection of an NHE3 promoter construct (nt -118/+58 in pβGal-basic) with increasing amounts (5–200 ng) of GATA-5 specific antibody. A blocking antibody specific for Sp3 only weakly reduced the intensity of the complex. In IEC-6 cells, however, Sp3 was the predominant isoform found in the protein-DNA complex, as the band intensity was significantly reduced by anti-Sp3 antibody, while Sp1 antibody only very weakly supershifted the formed complex. Binding of Sp1 and Sp3 to this region of the NHE3 promoter was further demonstrated by gel mobility shift assay with nuclear protein from Drosophila SL2 cells transfected with empty expression vector or Sp1 or Sp3 expression constructs (Fig. 8B).
expression plasmid under control of the Drosophila actin 5 promoter, did not result in a significant increase in reporter gene activity in the absence of Sp proteins (data not shown). In order to determine the amount of transfected Sp1 or Sp3 expression plasmids that would result in submaximal stimulation of NHE3 promoter activity, we cotransfected SL2 cells with the −118/+58βGal promoter construct along with increasing amounts of pPacSp1 or pPacUSp3 vectors. The stimulatory effect of overexpressed Sp1 saturated with a lower amount of cotransfected plasmid (Fig. 9A) as compared with Sp3, which increasingly activated reporter gene expression throughout the range of transfected plasmid amount (Fig. 9B). In subsequent experiments, the −118/+58 bp NHE3 promoter construct was cotransfected into SL2 cells with 20 ng of GATA-5 expression vector, 5 ng of Sp1 or 10 ng of Sp3 vector, or with a combination of GATA-5 and Sp1 or Sp3 plasmids (Fig. 9C). In agreement
with previously obtained data, overexpression of GATA-5 alone did not stimulate NHE3 promoter activity. However, the same amount of GATA-5 expression plasmid cotransfected with Sp1 or Sp3 resulted in synergistic activation of NHE3 promoter activity. Additive, synergistic, or antagonistic interactions among transcription factors were identified by calculating the interaction response (IR), which is a measure of comparing the effect of two expression vectors cotransfected together to the additive effect of each of the expression vectors cotransfected separately (28) as shown in Equation 1.

\[
IR = \log\left[\frac{([\text{GATA-5} + \text{Sp1}]\text{GATA-5}) + [\text{Sp1}]}{[\text{GATA-5}] + [\text{Sp1}]}\right]
\]  

(Eq. 1)

Values of −0.1 to 0.1 are defined as additive effects, greater than 0.1 represent synergistic effects, and less than −0.1 represent antagonistic effects. According to these criteria, both Sp1 and Sp3 activated the NHE3 promoter synergistically with GATA-5 (IR = 0.44 and 0.19, respectively).

FIG. 8. Proteins binding to the cluster of Sp sites in the NHE3 promoter. Gel mobility shift assays were performed with Caco-2 and IEC-6 cell nuclear protein and WT sequence (see the legend to Fig. 7) as a labeled probe. Supershift analysis (sc, specific complex; ss, supershifted complex) indicated that Sp1 is the predominant Sp transcription factor binding this promoter region in Caco-2 cells, while Sp3 is the predominant protein in IEC-6 cells. B, similar analysis with nuclear protein from SL2 cells transfected with empty vector or Sp1 or Sp3 expression plasmid further confirmed that both Sp1 and Sp3 were capable of binding the probe.

DISCUSSION

The rat NHE3 promoter was cloned in 1996 by two independent laboratories (9, 16). Both reports provided genomic sequence of approximately the same region and discussed several putative, prediction analysis based, transcription factor binding sites. However, no detailed characterization of the promoter or functional mapping of cis-acting elements was presented. Moreover, different transcription initiation sites were demonstrated by both groups. Kandasamy and Orlowski (16) used S1 nuclease protection analysis and mapped it to a cluster of five nucleotides with a major site at −97 (T), and two minor sites at −100 (A) and −96 (G) nt relative to the translation start codon. Cano (29) mapped the transcription initiation site to the atypical TATA box (GGAATTA; +1 nt in bold) located at −128 nt 5′ of the AUG initiation codon. To address this discrepancy, we utilized an alternative method to map the NHE3 gene transcriptional start site, 5′-RLM-RACE. Sequencing data obtained from RACE products obtained with RNA isolated from the small intestine, colon, as well as from Caco-2 cells transiently transfected with the −118/+58 Luc construct, mapped the transcription initiation site to a cluster of nucleotides reported by Kandasamy and Orlowski (16). Also, functional data from transiently transfected Caco-2 and IEC-6 cells described in this paper, argue against the transcription initiation site reported by Cano (29). A reporter construct containing −20 to +58 bp of the NHE3 gene lacking the TATA-like sequence (positioned at nt −30 to −27) was transcriptionally active (5-fold higher then promoter-less vector). Extending the 5′-flanking region of the gene to include the only start site reported by Cano (in construct −35/+58 Luc) in fact decreased the activity of the promoter to background levels. Many A/T-rich sequences can convey TATA box activity, which is partly because TATA-box-binding protein (TBP) recognizes the minor DNA groove, where protein-DNA interactions are more often influenced by T/A content than by a specific sequence (30). Therefore, we also mutated or deleted this atypical TATA box in constructs −118/+58mTA (Fig. 3A) and −118/+58TA (not shown). Both promoter constructs exhibited the same activity as the wild-type sequence in transfected Caco-2 and IEC-6 cells. Taken together, these data suggest that transcription initiation occurs independently of the presence of this TATA-like sequence, and that sequence surrounding the transcription start site as mapped by S1 nuclease protection assays and
5'-RLM-RACE may form the actual NHE3 gene core promoter. In support of this hypothesis, we demonstrated that the sequence spanning -20/+8 bp of the NHE3 gene was able to promote low level transcription, consistent with typical low activity of isolated initiator (Inr) elements.

In mammals, the diversity of core promoters is quite significant, although precise analyses on a genome-wide scale are complicated by the lack of accurate descriptions of transcription initiation sites for the majority of genes. Promoters can be classified into those that contain a functional TATA-box, TATA-box paired with an Inr, Inr element with downstream promoter elements (DPM), and CpG island-rich promoters which apparently lack all three core elements (31). These core elements are believed to serve as recognition sites for the TFII D complex, which contains TBP and various TBP-associated factors (TAFs) (32). Although far from being conclusive, our data obtained from gel mobility shift assays as well as Southwestern blotting (Fig. 3, C and D) suggests that TBP bind to the NHE3 core promoter Inr element, despite the fact that it has a weak homology to the otherwise loose Inr consensus sequence (Py-Py-A,−N-T(A)-Py-Py) (33).

Downstream control elements located 3' of the transcription initiation site within Inr-containing promoters have been found essential for promoter activity in vivo, e.g. in the adrenoviral major late (AdML) (34) and murine terminal transferase (TdT) (35) genes. Although some studies indicated that TFII D, TFII-I, cap-binding protein (CAP), or USF proteins may bind to these regions, downstream control elements remain poorly understood. In the NHE3 promoter, deletion of nt +9/+58 resulted in a significant loss of promoter activity. Further studies unequivocally demonstrated that a GATA-box, located at position +20/+23, was critical for promoter function, since deletion of it was equivalent to the gross deletion of 50 bp of exon 1. We further demonstrated that all three GATA isoforms expressed in the intestinal epithelium, GATA-4, -5, and -6 are capable of binding their upstream cis-elements in exon 1, enhances transcription through functional interaction with Sp1 or Sp3 binding upstream of an atypical initiator sequence. Intestinal GATA-5 is believed to function during differentiation to activate genes in fully differentiated absorptive cells of the villi (40). Therefore, the cooperative activation of an intestinal promoter through GATA-5 and Sp family transcription factors likely represents a novel mechanism of regulating expression of genes along the crypt-villus axis of the intestinal epithelium.

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