Genomic Organization and Glucocorticoid Transcriptional Activation of the Rat Na⁺/H⁺ Exchanger Nhe3 Gene*

(Received for publication, February 8, 1996)

Ramani A. Kandasamy and John Orlowski‡
From the Department of Physiology, McGill University, Montréal, Québec H3G 1Y6, Canada

The activity of the apical membrane Na⁺/H⁺ exchanger NHE3 isoform of renal or intestinal epithelial cells is chronically regulated by a wide variety of stimuli, including acidosis, cAMP, glucocorticoids, and thyroid hormone. To understand the molecular mechanisms responsible for long-term regulation of this cation transporter, we have isolated and determined the structure of this gene from a rat genomic library. The Nhe3 gene spans >40 kilobases and contains 17 exons that are flanked by typical splice donor and acceptor sequences at the exon-intron boundaries. The transcription initiation site was mapped by S1 nuclease protection analyses of mRNA from rat kidney and intestine. Multiple start sites were clustered between nucleotides –100 and –96 relative to the translation initiation codon. An atypical TATA-box and CCAAT-box are centered 30 and 147 nucleotides, respectively, upstream of the predominant transcription initiation site. Sequence analysis of approximately 1.4 kilobases of the 5′-flanking promoter region also revealed the presence of other putative cis-acting elements recognized by various transcription factors (e.g. AP-1, AP-2, C/EBP, NF-1, OCT-1/OTF-1, PEA3, Sp1, glucocorticoid, and thyroid hormone receptors), some of which may participate in the chronic regulation of this gene. The glucocorticoid responsiveness of the Nhe3 gene was assessed by fusing its 5′ regulatory region to the firefly luciferase reporter gene and then by measuring the expression of the chimeric gene in transiently transfected renal epithelial OK and LLC-PK1 cells. Glucocorticoid treatment significantly increased the luciferase activity of the chimeric gene in both cell lines, thereby indicating that glucocorticoid regulation of Nhe3 is mediated primarily by a transcriptional mechanism.

The Na⁺/H⁺ exchanger (NHE)1 is an integral membrane protein present in all mammalian cells. Multiple isoforms (NHE1 to NHE5) have been identified by molecular cloning techniques (1–7). They range in size between ~81 and 93 kDa and appear to exist in the membrane as homodimers, at least in the cases of NHE1 and NHE3 (8). These isoforms exhibit differences in their patterns of tissue expression, biochemical and pharmacological characteristics, and physiological functions (reviewed in Refs. 9 and 10).

In addition, Na⁺/H⁺ exchanger activity is influenced by a wide variety of molecular signals (e.g. neurotransmitters, growth factors, peptide hormones, phorbol esters, cAMP, chemotactic factors, lectins, osmotic shrinkage, acidosis, glucocorticoids, and thyroid hormone) that act either rapidly (seconds to minutes) or following a considerable latent period (hours to days) before the effects on the rate of transport are manifested (reviewed in Refs. 11 and 12). Recent studies have begun to identify the stimuli and signaling pathways that acutely modulate the individual NHE isoforms (13–20). The mechanisms responsible for these alterations, although not fully resolved, appear to involve both phosphorylation-dependent and -independent processes.

Considerably less is known about the molecular mechanisms involved in the chronic regulation of the NHE isoforms, although recent investigations are beginning to provide some insight. Prolonged exposure of cultured renal epithelial cells to acidic medium, which serves as a paradigm for studying physiological adaptations to chronic metabolic and respiratory acidosis, elevates the activities and/or mRNA abundances of NHE1 (21, 22) and NHE3 (22, 23), but inhibits those of NHE2 (24). Interestingly, acid-mediated stimulation of NHE1 in renal MCT cells is associated with activation of protein kinase C and the transcription factor AP-1 (25), whereas acid induction of NHE3 in renal OKP cells is linked to the c-src family of non-receptor protein-tyrosine kinases (26). Aside from acidosis, chronic incubation of renal IMCD cells in hyperosmotic media also stimulates Na⁺/H⁺ exchanger activity, which is associated with increased NHE2 and reduced NHE1 mRNA abundances (24). Last, long-term administration of glucocorticoids to rabbits or sheep selectively elevates NHE3 activity and mRNA in renal proximal convoluted tubules (27, 28) and in ileum (29), but has no effect on NHE1 or NHE2. At present, the underlying mechanisms for acid, hyperosmolality, and glucocorticoid regulation of specific isoforms mRNA abundances have yet to be resolved in detail, but are likely to involve altered rates of gene transcription and/or mRNA stability. Further progress in this area will require the isolation and characterization of the NHE genes and their associated cis-acting DNA regulatory elements.

Chromosomal mapping analyses have revealed that the members of the Na⁺/H⁺ exchanger gene family are dispersed in the mammalian genome (4, 30–32). To date, the complete gene encoding human NHE1 has been characterized (33) and shown to bind several transcription factors in its 5′-flanking region (34), although their functional importance was not established. However, more recent studies have shown that binding of the transcription factor AP-2 to the mouse Nhe1 gene promoter increases gene transcription (35). No information is...
available regarding the other isoforms.

In order to understand the molecular mechanisms involved in the tissue-specific, developmental, and hormonal regulation of the NHE isoforms, we have characterized the genomic organization of the rat NHE3 gene. Furthermore, we demonstrate that the transcriptional activity of the 5′-flanking promoter region is significantly elevated in response to glucocorticoid stimulation of transiently transfected renal cell lines.

**EXPERIMENTAL PROCEDURES**

**Materials—**Restriction endonucleases, DNA-modifying enzymes, and reagents for dideoxy sequencing using T7 DNA polymerase were purchased from New England Biolabs or Pharmacia Biotech Inc. [α-32P]dATP, [α-32P]dCTP, and [γ-32P]ATP were purchased from NEN Research Products (Du Pont Canada Inc., Mississauga, Ontario). All other chemicals and reagents used in these experiments were purchased from British Drug House Inc. (St. Laurent, Quebec) or Fisher Scientific and were of the highest grade available.

**Isolation and Characterization of Rat Genomic Clones—**Approximately 1 × 10^6 phage recombinants containing partially digested (MboI) rat genomic DNA (15–20 kb) inserted into the BamHI site of the phage vector EMBL3 (a gift of Dr. Donald Back, Queen’s University, Kingston, Ontario) were screened with 10-μl NHE3 cDNA probe (KpnI-Smal fragment). The rat genomic phage clones, λ6-2, λ11-2, and λ12-1, were isolated and together contained the entire coding region of the gene but lacked the 5′ promoter region. To isolate this region, a small 90-bp polymerase chain reaction fragment from the 5′ end of the cDNA was hybridized to a second rat genomic Lambda Dash II phage library (Stratagene) obtained from Stratagene. The recombinants contained partially digested (SacIII) rat genomic DNA (9–22 kb) inserted into the BamHI site of the phage vector. This screening resulted in the isolation of phage clone λ3B which contained ~12 kb of the 5′ upstream regulatory region.

Screening of both genomic libraries involved filter hybridization with rat NHE3 cDNA probes radiolabeled with [α-32P]dATP to a specific activity of 10^9 cpm/μg. Using the random primer oligonucleotide labeling kit (Pharmacia), the filters were prehybridized at 65°C for 3–5 h in 6× SSC, 5× Denhardt’s solution, 0.1% SDS, and 100 μg of denatured salmon sperm DNA/ml (see Ref. 36 for composition of SSC and Denhardt’s solution). The filters were then hybridized in the same solution with denatured cDNA probe for 48 h at 65°C. The replica filters were washed twice for 30 min each at 22°C in 2× SSC, 0.1% SDS, and then once for 60 min at 65°C in 2× SSC, 0.1% SDS. The filters were examined by autoradiography for positive hybridization signals, and the corresponding phage colonies were purified by 3 successive screenings using standard procedures.

**Rat genomic DNA from the purified positive phage clones was mapped by cleavage with different restriction endonucleases and Southern blot analysis (37).** Briefly, the digested genomic DNA was fractionated by electrophoresis on 1% agarose gels, denatured, and transferred to nylon membranes by capillary transfer. These membranes were successively hybridized to [γ-32P]ATP end-labeled, synthetic oligonucleotides corresponding to various regions of the NHE3 cDNA.

**DNA Sequence Analysis—**Restriction endonuclease-generated DNA fragments from the phage clones that hybridized to synthetic NHE3 oligonucleotides were subcloned into pBluescript. These oligonucleotides, as well as others, were used as sequencing primers to define the exon-intron boundaries. Sequencing of the genomic DNA inserts was performed by the double-stranded dideoxy chain termination technique (38) using [α-32P]dATP and T7 DNA polymerase sequencing kit from Pharmacia or U. S. Biochemical Corp. The 5′-flanking region was sequenced in both directions.

**Primer Extension Analysis—**Primer extension analysis was performed according to a previously described method (36). A synthetic oligonucleotide complementary to the 5′-untranslated sequence in the rat NHE3 cDNA, nucleotides –13 to +4 relative to the translation initiation site, was 5′-end-labeled using [γ-32P]ATP and T4 polynucleotide kinase. The labeled primer (~10^5 cpm) was annealed to 50 μg of total cellular RNA by incubating overnight at 30°C in hybridization solution (80% formamide, 40 μM PIPES, pH 6.4, 400 μM NaCl, 1 mM EDTA). After precipitation of the annealed primer and template, the hybridized primers were extended by incubating with 50 units of avian myeloblastosis virus reverse transcriptase in 20 μl of reverse transcription buffer (50 mM Tris-Cl, pH 7.6, 60 mM KCl, 10 mM MgCl2, 1 mM dithiothreitol, 1 unit/μl plasmid RNase inhibitor, 50 μg/ml actinomycin D, and 1 mM each dNTP). The reaction was terminated by the addition of 1 μl of 0.5 M EDTA and 1 μl of DNase-free pancreatic RNase (5 μg/ml) for 30 min at 37°C. The samples were precipitated by the addition of ethanol, denatured, and analyzed on a 6% polyacrylamide sequencing gel.

**5′-Nucleotide Clamping—**5′ nucleotide protection of the 5′ end of the NHE3 transcript was performed as described previously (36). Briefly, a 5′-end-labeled oligonucleotide complementary to nucleotides +3 to +4 (relative to the translation initiation site) was annealed to a denatured plasmid (SH23/6-2) containing a 1.0-kb Sal–HindIII insert isolated from λ3B which contained the putative transcription initiation site. The primer was extended using the Klenow fragment of DNA polymerase I and the resulting double-stranded product cleaved with SacI and HindIII, fractionating the DNA on a denaturing polyacrylamide gel, and visualized by autoradiography. A radiolabeled, single-stranded fragment of 416 nucleotides in length was eluted from the gel. Approximately 5 × 10^4 cpm of probe was mixed with 50 μg total of cellular RNA and incubated in 40 μl of 5′ hybridization buffer (80 mM formamide, 40 mM PIPES, pH 6.4, 400 mM NaCl, 1 mM EDTA) at 65°C for 10 min, then at 30°C for 3 h. The samples were digested with 300 units of S1 nuclease for 1 h at 30°C in 300 μl of S1 buffer (280 mM NaCl, 50 mM sodium acetate, pH 4.5, and 4.5 mM ZnSO4) containing 6 μg of denatured salmon sperm DNA. The protected fragments were precipitated by the addition of ethanol, denatured, and analyzed on a 6% polyacrylamide sequencing gel.

**Cal Culture—**Renal OK and LLC-PK1 cell lines were propagated in α-minimal essential medium supplemented with 10% fetal bovine serum, 100 μg/ml kanamycin sulfate, and 25 μg/ml of total cellular RNA and incubated in a humidified atmosphere of 95% air, 5% CO2, at 37°C.

**Plasmid Construction and Transient Gene Expression Assays—**A chimeric Nhe3-luciferase gene was constructed by isolating a 1.44-kb BamHI–KpnI genomic DNA fragment from the phage clone λ3B that contains the Nhe3 5′-flanking region (~130 to +59 relative to the transcription initiation site) and inserting it into the polylinker region of the promoterless luciferase vector pXP1 (39). This new plasmid was named pNhe3-1380.

**Following plating overnight, subconfluent monolayers of OK and LLC-PK1 cells were washed twice with phosphate-buffered saline and then changed to standard α-minimal essential medium supplemented with 10% fetal bovine serum at least 2 h before transfection. Cells were transfected using the calcium phosphate DNA coprecipitation technique of Chan and Okayama (40). The functional activity of the chimeric gene was assessed by cotransfection of 20 μg of plasmid DNA into cells. The composition of the DNA included either the promoterless pXP1 vector (negative control) or the pRSV-Luc vector (positive control) and pHG1 (expression plasmid containing the SV40 promoter and human glucocorticoid receptor; generously provided by Dr. John White, McGill University) in a weight ratio of 3:1:1, respectively. The activity of the gene was determined using firefly luciferase activity (Promega luciferase gene was constructed by isolating a 1.44-kb BamHI–KpnI genomic DNA fragment from the phage clone λ3B that contains the Nhe3 5′-flanking region (~130 to +59 relative to the transcription initiation site) and inserting it into the polylinker region of the promoterless luciferase vector pXP1 (39). This new plasmid was named pNhe3-1380.**

**RESULTS**

**Structure of the Rat Na+ / H+ Exchanger Nhe3 Gene—**The genomic organization of the rat Na+/H+ exchanger Nhe3 gene was determined by sequencing genomic DNA inserts from phage clones λ3B, λ6-2, λ11-2, and λ12-1 using synthetic oligonucleotide probes that spanned the entire published NHE3 cDNA sequence (2). As illustrated in Fig. 1, the Nhe3 gene spans ~40 kilobases and contains at least 17 exons. The exons are evenly distributed along the genomic DNA with the exception of exon 1 which is separated from the other exons by a large intron that is estimated to be >25 kilobases. The protein-coding exons generally range in size from 71 to 302 nucleotides, with the exception of exon 17 which is >1700 nucleotides and
contains the TGA stop codon (starting at position 2 of the exon) and a large segment of the 3'-untranslated region. However, the most 3' end of this exon containing the remainder of the 3'-untranslated sequence, including the polyadenylation signal, were not identified in this genomic DNA phage clone and leave open the possibility of additional exons in this region.

The sizes of the introns were determined either by DNA sequencing or were estimated by agarose gel electrophoresis of PCR-generated DNA fragments using oligonucleotide primers complementary to the flanking exons. They generally varied in length from 0.1 to 1.2 kilobases, excluding the first intron which is estimated to be >25 kilobases. This estimate is based on the sizes of the $\lambda$6-2 and $\lambda$11-2 phage clones which did not overlap. As shown in Table I, the exon-intron boundaries conform to typical splice donor AG/GT(A/G)AGT and acceptor (T/C),N(C/T)AG/G (n $\geq$ 11) consensus sequences (41). Each splice donor site begins with an invariant GT dinucleotide, whereas each splice acceptor site ends with an invariant AG dinucleotide and is preceded by a polypyrimidine tract.

It has generally been observed that members of gene families share similar genomic organizations, such as the Na$^+$,K$^+$-ATPase isozymes (42, 43). This also applies to the NHE gene family members that have been characterized to date. A comparison of the exons within the protein coding sequences of rat NHE3 and human NHE1 (33) is illustrated in Fig. 2. With the exception of exons 2 and 3 in human NHE1, which are both split by an apparent intron in rat Nhe3, the exon-intron boundaries occur in exactly the same positions within the proposed N-terminal transmembranous region and the first $\approx$50 amino acids of the C-terminal cytoplasmic region. These regions share the highest degree of amino acid identity among the isoforms. However, little similarity in exon organization exists in the remainder of the C-terminal cytoplasmic regions of the isoforms which also share minimal sequence identity and are believed to encode the diverse regulatory elements of the transporters.

Location of the Transcription Initiation Site and Nucleotide Sequence of the 5'-Flanking Region—To identify the Nhe3 transcription initiation sites, both primer extension and S1 nuclease protection analyses were performed. For primer extension analysis, an oligonucleotide primer complementary to the 5'-untranslated sequence (nucleotides -13 to +4 relative to the translation initiation site) was used to analyze total RNA isolated from adult rat kidney. A single intense band was observed that extended to within 19 nucleotides from the 5'-end of the known cDNA sequence (data not shown). Other experimental conditions and primers were unsuccessful at increasing the extension product beyond this point. This premature termination likely indicates the presence of a strong RNA secondary structure at this position which impeded the progression of the avian myeloblastosis virus reverse transcriptase.

To resolve this difficulty, S1 nuclease protection studies were performed using total RNA isolated from adult rat intestine and kidney (Fig. 3). The S1 probe consisted of a 416-nucleotide single-stranded DNA fragment that extended from position -412 to +4 relative to the translation start site. A cluster of apparent transcription initiation sites located within a 5-bp region were observed in total RNA isolated from adult rat intestine and kidney, with a major site at -97 (T nucleotide) and two minor sites at -100 (A nucleotide) and -96 (G nucleotide). Since T is the most predominant start site in rat Nhe3, it is designated as +1 and all 5' elements have been numbered relative to this site.

The nucleotide sequence of the 5' end of the Nhe3 gene is shown in Fig. 4. The center of an atypical TATA sequence, ATTAAA, is located 30 bp upstream of the major initiation site and potentially binds the multiprotein complex, TFII D (reviewed in Ref. 44). As well, although a classical CCAAT consensus sequence capable of recognizing NF-Y/CP1 (45, 46) is not observed between -200 and -50 bp of the transcription start site, an atypical sequence, CCAAG, that resembles the core binding motif is located 147 bp 5' of the major transcription initiation site. However, a classical TATA-box and CCAAT-box are positioned further upstream at -428 and -501, respectively, but are considered too far from the promoter region to be functional. This premise is based on our inability to obtain a primer extension product following hybridization of specific oligonucleotides in this region to total RNA from adult rat intestine and kidney (data not shown).

The promoter region also contains several potential GC-box motifs or Sp1-binding sites that resemble the core hexanucleotide motif, GGGCGG (47), and are located at positions -588, -350, -139, -72, and -54 (reverse orientation) upstream of the apparent cap signal. Indeed, the first 200 bp of the promoter region is highly (G + C)-rich (67%), a characteristic that is commonly observed in the immediate 5'-flanking region of housekeeping genes.

The 5'-flanking region also contains a number of other potential cis-acting elements recognized by known transcription factors that may play a role in the basal or chronic regulation of the Nhe3 gene (see “Discussion”), including four half-sites for the glucocorticoid responsive element (GRE) (TGTCTT; posi-
Glucocorticoid Transcription Control of the Rat Nhe3 Gene Promoter—Glucocorticoids have been reported to elevate NHE3 activity and mRNA abundance in epithelial cells from the ileum (29), renal proximal tubules (27, 28), and cultured renal OK cells (56) which retain certain phenotypic characteristics of proximal tubule cells. In view of the observed putative GRE half-sites in the Nhe3 promoter, it was of interest to assess whether glucocorticoid induction of NHE3 expression could be accounted for by alterations in gene transcription. To this end, a BamHI-KpnI DNA fragment extending from −1380 to +59 relative to the major transcription initiation site was inserted into the promoterless luciferase expression plasmid pXP1 (called pNhe3-1380) (Fig. 5A) and transfected into two renal proximal tubule cell lines, OK and LLC-PK1. Of these cell lines, OK cells have been shown to express only native NHE3 (23, 57) whereas LLC-PK1 cells express both NHE1 (58) and NHE3 (59). Two additional plasmids were also cotransfected with pNhe3-1380: (i) pRSV110 (a vector containing the RSV promoter linked to the β-galactosidase gene) and (ii) pHG1 (pSG5 expression plasmid containing the human glucocorticoid receptor). The activity of the β-galactosidase gene served as an internal control to monitor for transfection efficiency and the human glucocorticoid receptor was transfected to provide for maximal ligand-dependent transactivation of the overexpressed Nhe3-1380 gene.

The promoterless expression plasmid, pXP1, showed negligible luciferase activity when transfected into both cell lines, whereas pNhe3-1380 exhibited a 69- and 7-fold increase in basal luciferase activity in OK and LLC-PK1 cells, respectively (Fig. 5, B and C). This indicated that the inserted 5′-flanking region of the Nhe3 gene contains a functional promoter. However, the basal activity of pNhe3-1380 in OK and LLC-PK1 cells is considerably less (691- and 16-fold, respectively) than that obtained with the expression plasmid pRSV-Luc that contains the more powerful Rous sarcoma virus promoter linked to the luciferase gene (data not shown). The weak promoter activity of the Nhe3 gene could account, at least in part, for the generally low abundance of NHE3 mRNA in rat tissues (2).

The glucocorticoid responsiveness of the pNhe3-1380 was tested by incubating the transfected cells in the absence or presence of 100 nM dexamethasone for 72 h. This treatment substantially elevated luciferase activity in OK and LLC-PK1 cells.

### Table I

| No. | EXON | 5′ DONOR SITE | INTRON (kb) | 3′ ACCEPTOR SITE |
|-----|------|---------------|-------------|-----------------|
| 1   | AKI  | gtaagcggcaggg  | (−25)       | ttctctcccag     |
| 2   | GLM  | gtaagtcgaagtt  | (−1.2)      | tacatcctgcg     |
| 3   | GTV  | gtaggtgagtcct  | (−0.1)      | ttctgtaccctc    |
| 4   | KGI  | gtagtacaggggg  | (−0.5)      | ccctctctcaca    |
| 5   | AI   | gatgccccctca   | (−0.8)      | tgctctccccca    |
| 6   | RA   | gagggtctaggag  | (−0.36)     | gaaccctgtgctc   |
| 7   | HGR  | gtaggctccgtt   | (−0.43)     | aggtgctttccta   |
| 8   | CATGGCGCGAG | gtagggagggctct | (−1.13) | tttctctgtaa     |
| 9   | RDK  | gtagtgccctaag  | (−1.0)      | cttttgtccca     |
| 10  | GTGGCCGAG | gctagaatatac  | (−1.15)     | tgtctctctcat    |
| 11  | CTATTCTCTT | gatgtgaccagtt  | (−0.65) | gtgtactccatgc   |
| 12  | CGGCAGGAG | gatgtcccatggg  | (−0.31)     | agttgctttccac   |
| 13  | KQR  | gaggaggggaggcgg| (−0.25)     | aatcttgctcacc   |
| 14  | KEG  | aagaactgttccc  | (−0.75)     | cctctcgtcactc   |
| 15  | SGA  | gtagatggagagag | (−0.4)      | agcccccttttctc  |
| 16  | CACACACAT | gatctggtctcctt | (−1.0)       | ttctctctccacag  |
| 17  | >1700 | cccggg . . .  |             |                 |
cells by 15- and 6.5-fold, respectively (Fig. 5, B and C). In contrast, transfected cells containing the promoterless pXP1 plasmid did not show a significant change in luciferase activity.

**DISCUSSION**

We have isolated and characterized the structural organization of the rat Na\(^{+}/\)H\(^{+}\) exchanger NHE3 gene and its 5'-flanking region from a λ phage genomic library. This gene, which has previously been mapped to rat chromosome 1 (32) and human chromosome 5p15.1 (31), spans ~40 kb in length and contains at least 17 exons. The locations of the exon boundaries for rat NHE3 (see Table I) and human NHE1 (33) are illustrated by arrows.
only exceptions are exons 2 and 3 of rat Nhe3 which are continuous in human NHE1 (forming exon 2) and exons 4 and 5 of rat Nhe3 which are also uninterrupted in human NHE1 (forming exon 3). The N-terminal transmembranous segments share high amino acid identity (55–95%) among the NHE isoforms and are believed to comprise the structural domains necessary for ion translocation. In contrast, the organization of exons 10 to 17 of the Nhe3 gene is dissimilar to the exon arrangement of the comparable region (exons 8 to 12) of human NHE1. This C-terminal region in both isoforms, which is proposed to reside on the cytoplasmic side of the plasma membrane, shares minimal amino acid identity (25–35%) and contains numerous putative regulatory motifs that respond in a distinctive manner to a variety of stimuli (14, 16–18, 60).

To begin addressing questions concerning the regulation of Nhe3 gene expression at the transcriptional level, the transcription initiation sites were determined. In addition, the 5′-flanking sequence was examined for potential promoter elements that are fixed close to the start of transcription (i.e. TATA-box and cap signal) and enhancer motifs (e.g. CCAAT-box, Sp1, AP-1, AP-2, C/EBP, OCT-1/O/TF-1, and NF-1 binding sites) and hormone receptor responsive elements (e.g. triiodothyronine and glucocorticoid receptor binding sites) that are less restricted in both position and orientation. Only those potential sites that may be of physiological relevance to Nhe3 are discussed below.

Multiple transcription initiation sites were identified for the Nhe3 gene using S1 nuclease protection analyses from rat kidney and intestine, the two major tissues where the gene is expressed. Although three initiation sites were found to cluster between nucleotides –100 and –96 relative to the translation ATG start codon, the predominant site used in both tissues occurred at position –97 (thymidine) with minor sites located at –100 (adenine) and –96 (guanine). The cap signal CCT,G closely matches the consensus cap signal NCA,G (G/C/T) (61). Although the site of initiation is frequently an A preceded by an invariant C nucleotide, the use of T (but never C or G) is observed in a minority of cases. However, this motif is only present in approximately 60% of all promoters, suggesting that this sequence is not essential for promoter function (61). Multiple start sites scattered around the promoter region have also been reported for several genes (62, 63) that lack a canonical TATA sequence. The presence of an atypical TATA-box (discussed below) in rat Nhe3 may also account for the multiple start sites. Consequently, multiple transcription initiation sites may be characteristic of genes that lack a TATA-box, or a well defined TATA motif, in the promoter region.

The promoter/enhancer region of the rat Nhe3 gene contains an atypical TATA-box and CCAAT-box that are centered 30 and 147 nucleotides, respectively, upstream of the major transcription initiation start site. The core and flanking residues of the atypical TATA-box, GATT-AAGG, differ from the extended canonical sequence (G/T)AT-AAA(A/T)G(A)/G by having an A in the –2-position, a T in the –1-position, and a G in the +4 position. Nonetheless, a recent analysis of the promoter region of ~400 genes has revealed that A, T, and G can be present in these positions, respectively, but at low frequencies (4%, 9%, and 11%, respectively) (61). The atypical CCAAT-box, AACCAAGTAG, is centered at position –147 and exhibits similarity (8/10 match) to the extended CCAAT consensus sequence (A/G)/(A/C)CCAAT(C/G)/(A/G)G (45, 46, 61). This pentanucleotide core sequence is recognized by the transcription factor NF-Y/C/P1 and is typically located between 50 and 200 nucleotides upstream of the transcription start site. The atypical CCAAT-box in Nhe3 differs from the canonical sequence by substitution of G and T in the +2 and +3 positions, respectively. The presence of G and T in these positions is rare, being observed in only 9% and 2%, respectively, of genes examined (61). However, since 6 out of 7 nucleotides that were identified to contact the NF-Y protein (based on methylation interference analysis) (45) are conserved in the proposed CCAAT-box, it is reasonable to suspect that the observed motif may be transcriptionally relevant. However, functional studies are required to verify this possibility.

In addition, other CCAAT-like elements are known to exist, such as the palindromic consensus sequence TTGCG-(N)2-GCCCA that is recognized by nuclear factor I (NF-I/TF) (46, 55). One potential NF-I binding site in the Nhe3 promoter region is located between nucleotides –271 and –257 and maintains all 6 essential contact points in the NF-I binding site that, based on methylation interference analysis, seem to contact the NF-I protein (46). A third class of nuclear transcription factors that is able to bind to CCAAT-box and related enhancer motifs belongs to the CCAAT-box enhancer binding protein (C/EBP) gene family (52, 64). C/EBP DNA-binding proteins recognize the optimal dyad-symmetrical sequence ATTGGCAAT and regulate the expression of genes during cellular differentiation (52, 64) and in response to cAMP (65, 66). Two potential C/EBP elements that match at least 7 out of 10 nucleotides of the optimal motif are located in the Nhe3 gene at positions –979 and –948.

The promoter region also contains five potential binding sites for Sp1 that are positioned at nucleotides –588 (TTGCGG-GAG), –350 (AAGGGCGGAG), –139 (GGGGCGTGAG), –72 (GGGGCGGAAAA), and –54 (GGCCCGCCT; reverse orientation) upstream of the apparent cap signal. These sites closely match the consensus decanucleotide sequence for Sp1, (G/T)GGGGCGG/G/A(G/A)(C/T), which contains an optimized core hexanucleotide sequence GGGCGG and 5′- and 3′-flanking nucleotides that, although degenerate, can significantly influence Sp1 binding (47, 61). Sp1 sites, which can function in either orientation, are often found within 200 nucleotides upstream of the start of transcription and can act in conjunction with NF-I to increase the rate of transcription (47). It is also noted that the sequence of the promoter region is (G + C)-rich (67%) from nucleotides –1 to –201 which is frequently found in
The 5′ promoter region of housekeeping genes. Thus, it appears that the Nhe3 promoter region is a mosaic of potential elements that are characteristic of both cell-specific and housekeeping genes.

Since the mRNA and/or activity of Na+/H+ exchangers in renal or intestinal epithelial cells is elevated following prolonged exposure to various stimuli, including phorbol esters (67), cAMP (68), acidic medium (22, 23, 25, 26, 69, 70), triiodothyronine (71), and glucocorticoids (27–29, 56), we examined the 5′-flanking region of the Nhe3 gene for potential cis-acting DNA response elements that may be involved in some of these responses.

The trans-acting factor AP-1 is a heterodimer composed of the c-jun and c-fos proto-oncogenes that influences basal transcription and is also required for induction of transcription by phorbol esters (50, 51). The 5′-flanking region of the Nhe3 gene contains four potential AP-1 sequences; three match 6 out of 7 nucleotides of the AP-1 consensus sequence, TGA(G/C)T(C/A)A, and the other exhibits a 5/7 match. In addition to the AP-1 site, other phorbol ester responsive elements have been identified (reviewed in Ref. 72). Insertion of an oligonucleotide containing the PEA3 consensus sequence, AGGAAGT, upstream of a heterologous promoter can confer responsiveness to phorbol esters (49). Furthermore, PEA3 can act synergistically with AP-1 to achieve maximal induction of transcription by phorbol esters (73). A PEA3 site (in the reverse orientation) that precisely matches the consensus sequence and is in close proximity to the AP-1 sites is located at nucleotide −1309 of the Nhe3 gene.

Last, the regulatory region of this gene contains four potential sites at positions −412, −295, −48, and −4 that share homology with AP-2 elements, CCCC(A/G)(G/C)(G/C)C, that act as basal transcription enhancers but are also responsive to both phorbol esters and cAMP (54, 74). The sequence of individual sites, however, can vary considerably from the canonical binding motif. The responsiveness of AP-2 sites, as well as C/EBP sites (66), to cAMP is of particular relevance since cAMP chronically up-regulates NHE3 activity in renal OK cells (68). Moreover, a consensus site for the classical cAMP-responsive binding protein CREB, TGACGT(A/C)A (74), was not identified in the 1.4-kb 5′-flanking region, although potential CREB-binding sites may be present further upstream.

Triiodothyronine, estrogen, retinoids, and vitamin D bind to specific intracellular receptors that are part of a large family of ligand-dependent transcription factors. Interestingly, the DNA recognition site for each of these receptors, which may bind as monomers, homodimers, or heterodimers, is a variant of the optimal hexanucleotide half-site AGGTCA (reviewed in Ref. 48). However, the specificity of hormone receptor binding to the cognate response element and the magnitude of transcriptional activity is highly dependent upon the number, orientation, and spacing between half-sites (75–77). For example, direct repeats separated by 3, 4, and 5 bp are selectively responsive to vitamin D, triiodothyronine, and retinoic acid, respectively (76). Two clusters of AGGTCA-like elements (each containing 3 core binding motifs) are present in the rat Nhe3 gene at positions −1025 to −1002 and −239 to −210. Each cluster contains two direct repeats in one orientation and a single half-site in the reverse orientation. The selective hormone responsiveness, if any, awaits further characterization.

The glucocorticoid hormone receptor complex binds to an inverted palindromic sequence AGAACA(N)3TGTTCT that is highly conserved and is a target of the glucocorticoid receptor (78). It is located at nucleotide −1303 of the Nhe3 gene (79). In addition to these cognate response elements, the glucocorticoid receptor can also bind to a variety of other DNA sequences that are not conserved and are not specific to the glucocorticoid receptor (80). These sequences include binding sites for other transcription factors, such as AP-1 and AP-2, which are involved in the regulation of gene expression in response to a variety of stimuli.

The glucocorticoid hormone receptor complex binds to an inverted palindromic sequence AGAACA(N)3TGTTCT that is located at nucleotide −1303 of the Nhe3 gene. This site is highly conserved and is a target of the glucocorticoid receptor (78). It is located at nucleotide −1303 of the Nhe3 gene (79). In addition to these cognate response elements, the glucocorticoid receptor can also bind to a variety of other DNA sequences that are not conserved and are not specific to the glucocorticoid receptor (80). These sequences include binding sites for other transcription factors, such as AP-1 and AP-2, which are involved in the regulation of gene expression in response to a variety of stimuli.
that the maximal activity of many GREs is greatly influenced by the presence of additional cis-elements/trans-acting factors. For example, both NF-1 (78) and the ubiquitous transcription factor OCT-1/OTF-1 (53) participate in the glucocorticoid regulation of the mouse mammary tumor virus promoter. In addition, the AP-1 complex has been found to influence the ability of the glucocorticoid receptor to stimulate or inhibit gene transcription (79). In many respects, these factors can be viewed as components of a larger composite hormone responsive unit that creates the potential for greater flexibility in the hormonal regulation of gene transcription. Interestingly, potential NF-1, OCT-1/OTF-1, and AP-1 binding sites are also located in the general vicinity of the GRE clusters in the rat Nhe3 gene, although their functional importance, if any, remains to be determined.

As mentioned previously, glucocorticoids elevate Na\(^+\)/H\(^+\) exchanger NHE3 activity and/or mRNA abundance in ileum (29), renal proximal tubules (27, 28), and OK cells (56). The studies described in this report extend these observations by demonstrating that the transcriptional activity of the 5'-flanking region of the rat Nhe3 gene fused to the luciferase reporter gene is activated by glucocorticoids in transiently transfected OK and LLC-PK\(_1\) cells. This suggests that the observed in vivo induction of native NHE3 mRNA by glucocorticoids is most probably mediated primarily at the transcriptional level. More detailed studies will be required to precisely delineate the specific cis-acting elements involved.

In summary, we have isolated and characterized the 5'-flanking promoter region and exon-intron organization of the rat Nhe3 gene. In addition, we have demonstrated that this gene can be transcriptionally activated by glucocorticoids. This information provides the framework for further investigations on the mechanisms involved in the chronic regulation of this gene by glucocorticoids as well as other stimuli and its possible involvement in pathophysiological conditions such as systemic acidosis (69), hypertension (80, 81), and congenital secretory diarrhea (82).

Acknowledgment—We thank Dr. John White (McGill University) for critical reading of the manuscript.

REFERENCES

1. Sardet, C., Franchi, A., and Pouyssegur, J. (1989) Cell 56, 271–280
2. Orlowski, J., Kandasamy, R. A., and Shull, G. E. (1992) J. Biol. Chem. 267, 9331–9339
3. Wang, Z., Orlowski, J., and Shull, G. E. (1993) J. Biol. Chem. 268, 11925–11928
4. Klancke, C. A., Su, Y. R., Callen, D. F., Wang, Z., Moneston, P., Baird, N., Kandasamy, R. A., Orlowski, J., Otterud, B. E., Leppert, M., Shull, G. E., and Menon, A. G. (1995) Genomics 25, 615–622
5. Tse, C.-M., Ma, A. Y., Yang, H., and Walker, M. S. (1995) J. Biol. Chem. 270, 9340–9346
6. Tse, C.-M., Levine, S. A., Yun, C.-H. C., Martin, J. J., Pouyssegur, J., and Donowitz, M. (1991) EMBO J. 10, 1957–1967
7. Tse, C.-M., Levine, S. A., Yun, C.-H. C., Montrose, M. H., Little, P. J., Pouyssegur, J., and Donowitz, M. (1993) J. Biol. Chem. 268, 11917–11924
8. Tse, C.-M., Brant, S. R., Walker, M. S., Pouyssegur, J., and Donowitz, M. (1992) J. Biol. Chem. 267, 9331–9339
9. Fafournoux, P., Noël, J., and Pouyssegur, J. (1994) J. Biol. Chem. 269, 2589–2596
10. Noël, J., and Pouyssegur, J. (1995) Am. J. Physiol. 268, C283–C296
11. Yoon, Y. C., Tse, C.-M., Carter, S. A., Menon, A. G., Pouyssegur, J., and Donowitz, M. (1995) Am. J. Physiol. 269, G1–G11
12. Grinstein, S., and Rothstein, A. (1986) J. Membr. Biol. 10, 1–12
13. Grinstein, S., Rotin, D., and Mason, M. J. (1989) Biochim. Biophys. Acta 988, 73–97
14. Grinstein, S., Woodside, M., Sardet, C., Pouyssegur, J., and Rotin, D. (1992) J. Biol. Chem. 267, 23823–23828
15. Wakahayashi, S., Bertrand, B., Shigekawa, M., Fafournoux, P., and Pouyssegur, J. (1994) J. Biol. Chem. 269, 5583–5588
16. Wakahayashi, S., Bertrand, B., Ikeda, T., Pouyssegur, J., and Shigekawa, M. (1994a) J. Biol. Chem. 269, 13710–13715
17. Levine, S. A., Montrose, M. H., Tse, C.-M., and Donowitz, M. (1993) J. Biol. Chem. 268, 25527–25535
18. Kapus, A., Grinstein, S., Watanabe, S., Kandasamy, R. A., and Orlowski, J. (1994) J. Biol. Chem. 269, 23544–23552
19. Kandasamy, R. A., Yu, F. H., Harris, R., Boucher, A., Hanrahan, J. W., and Orlowski, J. (1995) J. Biol. Chem. 270, 29209–29216
20. Weinman, E. J., Steplek, D., Wang, Y., and Shenolikar, S. (1995) J. Clin.
Genomic Organization and Glucocorticoid Transcriptional Activation of the Rat Na/H Exchanger Nhe3 Gene
Ramani A. Kandasamy and John Orlowski

J. Biol. Chem. 1996, 271:10551-10559.
doi: 10.1074/jbc.271.18.10551

Access the most updated version of this article at http://www.jbc.org/content/271/18/10551

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 83 references, 32 of which can be accessed free at http://www.jbc.org/content/271/18/10551.full.html#ref-list-1