Regulation of Na⁺/H⁺ Exchanger Gene Expression

ROLE OF A NOVEL POLY(dA·dT) ELEMENT IN REGULATION OF THE NHE1 PROMOTER*

(Received for publication, September 14, 1995, and in revised form, May 22, 1996)

Weidong Yang, Huayan Wang, and Larry Fliegel‡

From the Departments of Pediatrics and Biochemistry, Faculty of Medicine, University of Alberta, Cardiovascular Disease Research Group, Edmonton, Alberta T6G 2S2, Canada

In this study we examine regulation of expression of the Na⁺/H⁺ exchanger promoter in L6 and NIH 3T3 cells. We have identified a highly conserved poly(dA·dT)-rich region that appears to be important in regulation of expression of the NHE1 gene. Deletion or mutation of this region results in dramatic decreases in promoter activity in both L6 and NIH 3T3 cells. In addition, DNase I footprinting experiments demonstrated that this region is protected by nuclear extracts from both cell types, and gel mobility shift assays showed that a protein or proteins specifically binds to the poly(dA·dT)rich element. Using Southwestern blotting, we determined that a 33-kDa protein binds to the poly(dA·dT)containing region. Mutations that abolished protein binding to this element diminished activity of the promoter. Insertion of the poly(dA·dT)-rich element into a plasmid containing the SV40 promoter demonstrated that this element can also enhance the activity of a foreign promoter. Together, the results we have presented here show that the poly(dA·dT)-rich region is important in regulation of NHE1 expression in different cell types.

The Na⁺/H⁺ exchanger is a mammalian plasma membrane protein that removes one intracellular proton in exchange for an extracellular sodium. It is involved in pH regulation (1) and control of cell volume and is stimulated by growth factors (2). Several isoforms of the protein have been identified (NHE1 to NHE5), of which NHE1 is the most widely distributed, being present in most if not all mammalian cells (3). Studies have shown that Na⁺/H⁺ exchanger mRNA levels are increased by a number of experimental procedures, including chronic acid loading and treatments that result in cellular differentiation (4-6). For example, during retinoic acid-induced differentiation of human leukemic cells (HL-60) (4) and of P19 cells (7), there is an increase in the level of NHE1 transcription. An increased antiporter activity may be important for differentiation to occur in some cell types (8, 9), but increased transcription of NHE1 has not been shown to occur universally during differentiation, and therefore, the role of the Na⁺/H⁺ exchanger may vary between cell types (10, 11).

There have been few studies on regulation of expression of

the NHE1 isoform of the Na⁺/H⁺ exchanger. Miller and coworkers (12) were the first group to isolate the upstream region of the human NHE1 gene. They identified the intron-exon boundaries and the start sites of transcription and provided the sequence of the 5'-untranslated region along with approximately 1.3 kb¹ of the promoter/enhancer region (12). Recently, another group has identified regions of the promoter that can bind nuclear proteins (13). In our studies, we have isolated and characterized a 1.1-kb fragment of the mouse NHE1 gene that is upstream of the 5'-untranslated region (14). We identified the transcription factor AP-2 or an AP-2-like protein as being involved in the regulation of this gene during differentiation of P19 cells (7, 14). We have also recently shown that serum stimulates expression of the NHE1 promoter in some cell types (15). Another group has recently isolated the rabbit NHE1 promoter (16). The sequence of the clone was similar to the human sequence, and the 708 proximal bp exhibited orientationdependent activity.

Evidence indicates that a number of regions of the NHE1 promoter contribute to the basal expression of the gene. Several studies (7, 13–15) have shown that a stepwise reduction in the 5' end of the NHE1 promoter results in a reduction in promoter activity. This effect varies between cell types (7, 13, 15). In addition, DNA footprinting experiments have suggested that several different regions of the gene bind proteins of nuclear extracts (13, 14). In previous work, we have noted the presence of a highly conserved poly(dA·dT)-rich region in the NHE1 promoter. In this study we examine the function of this region in L6 and NIH 3T3 cells. The results suggest that this conserved region plays an important role in regulation of NHE1 expression.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonuclease and DNA-modifying enzymes were obtained from Boehringer Mannheim and Life Technologies, Inc. The pBluescript plasmids used for subcloning were from Stratagene (La Jolla, CA). Plasmid pXP-1 was a gift from Dr. M. Nemer of the Institut de Recherches Cliniques de Montreal (Montreal, Quebec, Canada). Other chemicals were of analytical grade or molecular biology grade and were purchased from Fisher Scientific, Sigma, or BDH.

Reporter Plasmid Constructs—The isolation and characterization of the Na⁺/H⁺ exchanger promoter was as described earlier. pXP-1.1MP was also constructed as described earlier (14). The plasmid pXP-0.2MP was made by a modification of pXP-1.1MP. The insert was excised with *Smal* and *Hin*dIII and digested with *Rsal*. This resulted in the production of a 0.2-kb *Rsal-Hin*dIII fragment that was subcloned into the *Smal-Hin*dIII site of pXP-1 to form pXP-0.2MP. All plasmids were sequenced to verify proper orientation and fidelity of PCR. The plasmid pXP-0.18MP was made using primer 4 (cccggaTCCAATTTAGGTCTCG-GCTTCC) with primer 3 (ccttcgaaGGGTCCCGCGGTAGCGGA). The PCR product had flanking restriction enzyme sites of *Bam*HI and *Hin*dIII and was inserted directionally into pXP-1.

To construct the plasmid pMut7-0.2MP we used PCR. The primer

^{*} This work was supported in part by the Medical Research Council Program Grant in the Molecular Biology of Membranes PG-11440. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[‡] Supported by a Senior Scholarship of the Alberta Heritage Foundation for Medical Research. To whom correspondence should be addressed: Depts. of Pediatrics and Biochemistry, Faculty of Medicine, University of Alberta, 417 Heritage Medical Research Center, Edmonton, Alberta T6G 2S2, Canada. Tel.: 403-492-1848; Fax: 403-492-9753; E-mail: Ifliegel@gpu.srv.ualberta.ca.

¹ The abbreviations used are: kb, kilobase(s); bp, base pair(s); PCR, polymerase chain reaction; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymeth-yl)ethyl]glycine; HMG-I, high mobility group I.

mutPolyT 5'-cacggatccACTgTTTgcggTTggTTCCAATTTAGGTCTCGG-CTTCCTCTTC-3' was used with the primer 3 (ccttcgaaGGGTCCCG-CGGTAGCGGA) to construct a mutated form of the 0.2-kb insert. (Mutations within the poly(dA·dT) containing region are indicated by lowercase letters.) The PCR product was digested with *Bam*HI and *Hin*dIII and cloned into the plasmid pXP1 as described above. The sequence was verified to confirm the presence of the insert and the mutations. A similar procedure was used to construct pMut2–0.2MP, which had only two mutations of the poly(dA·dT) region. The primers used were cacggatccACTTTTTgTTTTTgTTCCAATTTAGGTCTCGG-CTTCCTCTTC and primer 3. To construct the plasmid pMut2a-0.2MP we used the primers cacggatccACTTTTTaTTTTTTTTTTTTTTCCAATTTA-GGTCTCGGCTTCCTCTTC and primer 3. In this plasmid the mutations were T to A instead of T to G.

To construct pXP-T-SV40, the oligonucleotides poly T (5'-GATCCT-GTACTTTTTTTTTTTTTTTTCCAATTCGA-3') and poly(A) (5'-GATCTC-GAATTGGAAAAAAAAAAAAAAAGTACAG-3') were annealed together and then digested with restriction enzymes BamHI and Bg/II. The product was then size fractionated on a 9% acrylamide gel and purified and ligated into the BamHI site of pBluescript SK- (Stratagene, La Jolla, CA). The resulting plasmid (pBS-T) was digested with EcoRI and HindIII. The SV40 promoter was digested from pCAT-promoter plasmid (Promega) using EcoRI and HindIII and was ligated into the corresponding sites of pBluescript SK⁻. The total T-SV40 promoter fragment was removed from pBluescript SK⁻ with the restriction enzymes SstI and HindIII. The plasmid pXP-1 was digested with HindIII and SstI and ligated with the SstI to HindIII fragment. This resulting plasmid pXP-T-SV40 contained one copy of the poly(dA·dT) site of the mouse NHE1 promoter located 5' to the SV40 promoter. The final construct was sequenced to verify proper orientation and fidelity of PCR. The plasmid pSVLuc contained the SV40 promoter inserted into pXP-1 and was without the poly(dA·dT)-rich insert. It was constructed as reported earlier (7).

Growth and Maintenance of Cell Lines—L6 cells were propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum essentially as described earlier (17). NIH 3T3 cells were obtained from Dr. J. Stone of the Department of Biochemistry, University of Alberta. They were also maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Transfection and Reporter Assays-Cells were plated onto 35-mm dishes at a density of 1×10^5 cells/cm². Each dish received 2.0 μ g of luciferase reporter plasmid and 2.0 μ g of pSV- β -galactosidase as an internal control. L6 and NIH 3T3 cells were transiently transfected using the CaPO₄ precipitation technique (14). After transfection the cells were allowed to incubate at 37 °C for 5 h before being washed with fresh medium and left for 36 h. After 36 h. the cells were harvested, and the cell lysate was assayed for luciferase activity and β -galactosidase activity. The medium was aspirated, and the cells were washed in 1 imesphosphate-buffered saline, 1 mM EDTA. The cells were then allowed to remain in 1 ml of phosphate-buffered saline, 1 mM EDTA for 15 min. Cells were then scraped, transferred to a microcentrifuge tube, and pelleted at 14,000 rpm for 5 s. The supernatant was removed, and the pellet was resuspended in 50 µl of ice-cold lysis buffer (Tris 100 mM, pH 7.8, 1.0% Nonidet P-40 and 1 mM dithiothreitol) for 15 min. The solution was then pelleted at 14,000 rpm for 5 min at room temperature. The supernatant was removed and then assayed for luciferase and β -galactosidase activity. Each luciferase assay contained 20 μ l of the cell lysate and 100 µl of the luciferase assay reagent (20 mM Tricine, 1.07 mM magnesium carbonate, 2.67 mM MgSO₄, 0.1 mM EDTA, 33.3 mM dithiothreitol, 470 μM luciferin, 530 μM ATP, 270 μM coenzyme A 270 μM, and 1 ng/ml bovine serum albumin). The β -galactosidase assays were by two methods. In the first method, 20 μ l of cell lysate was mixed with 80 μ l of H₂O and 20 μ l of *o*-nitrophenyl- β -D-galactopyranoside incubated at 37 °C for 60 min. After 60 min, 60 µl of 1 M Na₂CO₃ was added to stop the reaction, and the optical density was measured at a wavelength of 420. The second method for β -galactosidase assays was by luminometry. 20 μ l of cell lysate was mixed with 100 μ l of galacto reaction buffer (Tropix) and incubated at room temperature for 60 min. 15 s prior to measurement, 100 µl of chemiluminescence accelerator (ClonTech) was added. Luciferase activity was assayed with an LKB luminometer and was normalized to β -galactosidase for efficiency of transfection.

DNA Binding Assays—Nuclear extracts were prepared from L6 and NIH 3T3 cells as reported by Schreiber *et al.* (18). The synthetic oligonucleotides of the sequences 5'-gatcGTACTTTTTTTTTTTTTTTTTCC-3' (Poly T 5') and 5'-gatcGGAAAAAAAAAAAAAAAAAAAACTAC-3' (Poly T 3') were made that correspond to the pyrimidine-rich region of the mouse Na⁺/H⁺ exchanger promoter (base pairs –173 to –153). The oligonucleotides were end-labeled with [γ -³²P]ATP using T4 polynucleotide FIG. 1. Alignment of poly(dA·dT) containing regions of the human (12), mouse (14), and rabbit (16) NHE1 promoters. The alignment was made using the DNA analysis program MacVector[®]. Numbering is based on the original publications and begins from first start site of transcription. *Asterisks* indicate identity with the mouse NHE1 promoter.

kinase. They were heated to 95 °C for 2 min and cooled to room temperature overnight for annealing. DNA binding reactions were with L6 or NIH 3T3 nuclear extracts (5 μ g) in binding buffer (5% glycerol, 1.0 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 0.02-2,500 µg/ml poly(dI·dC)) and contained 30,000-40,000 cpm of γ -³²P-labeled oligonucleotides mixed for 20 min at room temperature. Binding assays were in a volume of 10 μ l. Some assays contained cold competitor at concentrations from 9.2 ng/ml to 612.7 µg/ml. After electrophoresis on 6% polyacrylamide gels, the gels were dried and exposed to x-ray film for 16 h at -70 °C. In some DNA binding assays pairs of oligonucleotides were used that contained either seven or two mutations. The oligonucleotides containing seven mutations were 5'-GATCGTACTgTTTgcggTTggTTCC-3' and 5'-gatcGGAAccAAccgcAAAcAGTAC-3'. The oligonucleotides containing two mutations were 5'-gatcGGAAcAAAAAAAAAAGTAC-3' and 5'gatcGTACTTTTgTTTTgTTCC-3'. Oligonucleotides with two mutations from T to A were 5'-gatcGGAAtAAAAAAAAAAAGTAC-3' and 5'-gatcGTACTTTTTTTTTTTTTTTTTTTCC-3'

DNase I Footprinting-The probes for DNase I footprinting were isolated from the plasmid pXP-0.2MP. The insert was removed using the restriction enzymes BamHI and HindIII, and it was inserted into pBluescript that had been digested with the same enzymes. For 5' end labeling, the resulting plasmid was cut with XbaI, and the 3' recessed end was filled in with the Klenow fragment of Escherichia coli DNA polymerase I in a reaction containing $[\tilde{\alpha}^{-32}P]dCTP$. The DNA was then cut with HindIII and gel purified using a 9% acrylamide gel. The resulting 248-bp fragment was used for footprinting. For a 3' end label, the pBluescript plasmid containing the insert was initially digested with Sall. The 3' recessed end was filled in using a reaction mixture that contained $[\alpha^{-32}P]dCTP$ and the Klenow fragment of *E. coli* DNA polymerase I. The DNA was then cut with BamHI, and the 251-bp fragment was gel purified. For DNase I footprinting, the 251-bp fragment or the 248-bp fragment (about 30 000 cpm) were incubated with $5-40\,\mu g$ of L6 nuclear extracts at room temperature for 25 min and then treated with DNase I (0.003–1 units) for 10 s to 2 min. The reaction was terminated by phenol/chloroform extraction. After precipitation the sample was resuspended in 3 µl of 10 mM Tris, pH 7.4, 1 mM EDTA plus 2 µl of running buffer (95% formamide dye, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol) and was electrophoresed on a 6% acrylamide/7 M urea sequencing gel.

Southwestern blotting—Southwestern blotting was essentially as described by others (19, 20). Nuclear extracts from L6 cells (25 μ g) were run on 12% SDS-polyacrylamide gel electrophoresis. The gels were then renatured for 2 h in a buffer consisting of 10 mM Tris, pH 7.4, 50 mM NaCl, 2 mM EDTA, 0.5 mM dithiothreitol, and 4 M urea. Following renaturation, the proteins were transferred onto nitrocellulose membranes, and the membranes were blocked for 60 min in a binding buffer consisting of 5% skim milk powder, 10 mM Tris, pH 8.0, 2 mM MgCl₂, 1 mM β -mercaptoethanol, 50 mM NaCl, plus 25 μ g/ml dI/dC. After blocking, ³²P-labeled probe (0.5 × 10⁶/ml) was added for 2 h at room temperature. The probe consisted of end labeled, annealed synthetic oligonucleotides corresponding to the poly(dA·dt) region as described above. After hybridization the membrane was then air dried and examined by autoradiography on x-ray film.

RESULTS

Recently, the human (12), mouse (14), and rabbit (16) isoforms of the NHE1 promoter have been isolated and cloned. In previous studies, we and others have noticed that progressive deletion of the promoter results in reduced activity of a reporter in a number of cell types (7, 14, 16). The effects are greatest in the more proximal regions of the gene. A comparison of the homology of these clones shows that only some short proximal regions are highly conserved between these species. One of these regions is illustrated in Fig. 1. It consists predominantly of a stretch of unbroken dT residues. In the rabbit gene there is



FIG. 2. Activity of the NHE1 promoter in L6 and NIH 3T3 cells. L6 and NIH 3T3 cells were transiently transfected with the plasmids pXP-1.1MP, pXP-0.2MP, and pXP-0.18MP as described under "Experimental Procedures." The relative activity of the constructs was compared with that of the pXP-1.1MP plasmid. *, significantly different from the plasmid pXP-0.18MP at p < 0.001. The poly(dA-dT)-containing region is indicated by *T. Shaded bars*, L6 cells; *hatched bars*, NIH 3T3 cells.

a single dA residue that interrupts the dT string.

To examine the role of this poly(dA·dT) region in the NHE1 promoter, we constructed the vectors shown in Fig. 2. pXP-1.1MP contains the entire sequence of the mouse NHE1 promoter. pXP-0.2MP contains up to bp -171, and pXP-0.18MP contains up to bp -155. To examine the relative activities of these constructs, we used transient transfections of L6 and NIH 3T3 cells (Fig. 2). There was a slight but insignificant reduction in activity of the NHE1 promoter when comparing pXP-1.1MP with pXP-0.2MP. This was more pronounced in L6 cells than in NIH 3T3 cells. In contrast, the removal of bp 171–156 resulted in a large, significant reduction in activity of the section in activity of the promoter. Again, this effect was more pronounced in L6 cells than in NIH 3T3 cells.

Next, we used DNase I footprinting analysis to determine whether this region of the gene can interact with a protein or proteins in nuclear extracts from L6 and NIH 3T3 cells (Fig. 3). Fig. 3A shows DNase footprinting of a promoter fragment that was labeled at the 5' end before digestion. *Lanes 1* and 8 show the DNase I-treated sample without nuclear extract, and lanes 2. 3. and 4 show the effect of an increasing amount of L6 nuclear extract. There is clearly a region between bp - 169 and -152 of the promoter that is protected, and this protection increases with increasing concentration of the nuclear extract. Note that the protected region corresponds to the poly(dA·dT) site. Lanes 5-7 show the same experiment but with nuclear extract from NIH 3T3 cells. The results were similar, again showing increasing protection of this region with increasing amounts of nuclear extract. Similar results were also obtained with nuclear extracts from mouse kidney proximal tubule (MCT) cells (results not shown). Fig. 3B shows an examination of the same region of the promoter but with labeling of the 3' end of the fragment. Lanes 1 and 6 show DNase I treatment of the promoter region in the absence of nuclear extract, and *lanes* 2-5 show the same treatment in the presence of increasing amounts of NIH 3T3 nuclear extract. Again, there is a protected region of the promoter corresponding to poly(dA·dT) site (bp -169 to -154). In both experiments, significant protection of this strand was evident even at low concentrations of nuclear extract

To confirm that a protein or proteins in the nuclear extract can bind to the poly(dA·dT) region of the promoter, we used DNA mobility shift binding assays and competition analysis. The results are shown in Fig. 4. Competition was with unlabeled poly(dA·dT) oligonucleotides or with commercially obtained poly dI·dC. *Lanes 2–7* show that increasing amounts of unlabeled poly(dA·dT) reduced the amount of shifted DNA. In contrast, there was no effect of even relatively large amounts of noncompetitor poly(dI·dC) on the amount of the principal band of shifted DNA (*lanes 8–14*). There was a reduction in the amount of some larger shifted complexes that may represent nonspecific interactions with the poly(dA·dT) oligonucleotide.

To further examine the interaction between proteins in the nuclear extracts and the NHE1 promoter, we used a DNA mobility shift binding assay with wild type and mutated synthetic oligonucleotides corresponding to this promoter region. The mutated oligonucleotides had either seven or two dT to dG mutations in the poly(dA·dT) region (Fig. 5). Components in nuclear extracts from both L6 and NIH 3T3 cells bound to promoter oligonucleotides (bp -173 to -153). Competition with unlabeled poly(dA·dT) (specific competitor) reduced the binding, whereas nonspecific competitor (poly(dI·dC)) had no effect (Fig. 5). The oligonucleotides with seven mutations and two mutations did not bind protein from nuclear extracts of either cell type.

Next, we examined whether dT to dA mutations in synthetic oligonucleotides of the poly(dA·dT) region of the promoter could disrupt the protein-DNA interactions. In the synthetic oligonucleotide, residues representing -164 and -157 of the promoter were charged from dT to dA. The ability of protein(s) from nuclear extracts to bind these oligonucleotides was then examined (see Fig. 6). We found that despite the two mutations, there remained a protein shift of the same size, though it was reduced in amount. In addition, some smaller and larger complexes appeared. The amount of the smaller complex was increased, not decreased, by specific competitors, suggesting that nonspecific binding by the mutated oligonucleotides had increased. Overall, the oligonucleotides with dT to dA mutations showed an intermediate binding to the same protein(s) as the nonmutated oligonucleotides and an increased nonspecific binding of proteins.

To examine the effect of mutations in the poly(dA·dT) region on promoter activity, we constructed a series of reporter plasmids with deletions or mutations in this region and compared the relative activity of the luciferase reporter. These experiments were carried out in both L6 and NIH 3T3 cells, and the results are shown in Fig. 7. Deletion of the poly(dA·dT) region markedly reduced the activity of the promoter, and this effect was slightly greater in the L6 cells than in NIH 3T3 cells. For both cell types, mutation of either two or seven dT residues to dG residues resulted in a reduction in promoter activity that was equivalent to that seen when the poly(dA·dT) region was deleted (Fig. 7). In a final experiment we mutated two dT residues to dA. The mutated residues were the same ones mutated to dG in mobility shift experiments (Fig. 6). In this case the activity of the promoter was only slightly depressed compared with that of the wild type. These results show that the effects of deletion of this region on activity of the gene are not due to simple reduction in the size of the promoter. Clearly the specific sequence of the region is important for promoter activity. There was a general trend for the importance of this region to be more pronounced in L6 cells in comparison with NIH 3T3 cells.

To further investigate the role of the poly(dA·dT) site of the NHE1 gene, we inserted it into another promoter (see Fig. 8). The plasmid pXP-T-SV40 contains the oligonucleotides -174 to -149 of the mouse NHE1 promoter. Mean relative luciferase values were over 18,000 for control cells transfected with pS-



FIG. 3. DNase I footprinting analysis of the mouse NHE1 promoter. DNase I footprint analysis was performed with the mouse promoter using bp -171 to +22 of the promoter fragment labeled on either strand at the 5' or 3' end. *A*, footprint analysis of the fragment with labeling on the 5' end. *Lanes 1* and 8, naked DNA fragment treated with DNase I (1 unit for 60 s at room temperature). *Lanes 2–4*, the fragments were incubated with DNase I (1 unit for 60 s at room temperature) in the presence of increasing amounts of L6 nuclear extracts (0.2, 0.7, and 3 μ g, respectively). *Lanes 5–7*, NIH 3T3 nuclear extracts (0.2, 0.7, and 3 μ g, respectively). *B*, footprint analysis of the fragment with labeling on the 3' end. *Lanes 1* and 6, naked DNA fragments treated with DNase I (0.003 units for 60 s at room temperature). *Lanes 2–5*, the fragments were incubated with DNase I (0.003 units for 60 s at room temperature) in the presence of increasing amounts of NIH 3T3 nuclear extracts (0.1, 0.2, 0.3, and 0.64 μ g, respectively).



FIG. 4. DNA mobility shift binding assay and competition analysis of the mouse NHE1 poly(dA·dT) site. The labeled oligonucleotides corresponding to the positions -173 to -153 were incubated with nuclear extracts from L6 cells for 20 min at room temperature. The binding mixtures were analyzed by electrophoresis on 6% polyacryl-amide gels as described under "Experimental Procedures." *Lane 1*, nuclear extract alone (5 μ g) added to the binding reaction. *Lane 7*, no nuclear extract added. *Lanes 2–6*, reaction mixture containing nuclear extract and increasing amounts of unlabeled competitor oligonucleotide (0.919, 15.3, 61.3, 61.3, and 6, 127 ng, respectively). *Lanes 8–14*, reaction mixture containing nuclear extract and increasing amounts of nonspecific sequence competitor poly(dI·dC) (0.2, 1.0, 3.3, 10, 60, 250, and 2,500 ng, respectively).



FIG. 5. DNA mobility shift binding assay and analysis of dT to dG mutations of the mouse NHE1 poly(dA·dT) site. Labeled oligonucleotides corresponding to the positions -173 to -153 were incubated with nuclear extracts from L6 or NIH 3T3 cells for 20 min at room temperature. The binding mixtures were analyzed by electrophoresis on 6% polyacrylamide gels as described under "Experimental Procedures." A *plus sign* indicates addition of the following; *L6*, L6 nuclear extract, 5 µg; *NIH*, NIH 3T3 nuclear extract, 5 µg; *Wt*, wild type synthetic oligonucleotides with no mutations; *7Mt*, synthetic oligonucleotides with seven dT to dG mutations; *SC*, addition of 625 ng of specific competitor; *NSC*, addition of 625 ng of nonspecific competitor (poly(dI·dC)).

VLuc. We found that insertion of the poly(dA·dT) site resulted in a doubling of luciferase activity from the SV40 promoter (Fig. 8).

In our earlier experiments we have shown an interaction between the poly(dA·dT) region of the NHE1 promoter and proteins in nuclear extracts. To identify these proteins we used Southwestern blotting (see Fig. 9). Proteins in two distinct molecular weight ranges of the nuclear extract bound to the synthetic oligonucleotide. The first was a diffuse band of high molecular weight, the second a sharp band of 33 kDa. When an identical membrane was probed with the synthetic oligonucleotide containing seven dT to dG mutations, neither of these



FIG. 6. DNA mobility shift binding assay and analysis of dT to dA mutations of the mouse NHE1 poly(dA·dT) site. Labeled oligonucleotides corresponding to the positions -173 to -153 were incubated with nuclear extracts from L6 or NIH 3T3 cells for 10 min at room temperature. The binding mixtures were analyzed by electrophoresis on 6% polyacrylamide gels as described under "Experimental Procedures." A plus sign indicates addition of the following; L6, L6 nuclear extract, 5 μ g; *NIH*, NIH 3T3 nuclear extract, 5 μ g; *Wt*, wild type synthetic oligonucleotides with no mutations; 2MtA, synthetic oligonucleotides with two dT to dA mutations; SC, addition of 625 ng of specific competitor; NSC, addition of 625 ng of nonspecific competitor (poly(dI·dC)).

bands was present (not shown). These results confirm that a protein component(s) of L6 nuclear extracts binds to the poly(dA·dT) region of the NHE1 promoter and that this binding is dependent on the integrity of the nucleotide sequence.

DISCUSSION

Previous studies have shown that there are several regions of potential importance in the NHE1 promoter (7, 13–15). We have shown that the AP-2-containing region is important in NHE1 expression, especially during the process of cellular differentiation (7, 14), and we noted (14) that essential features of the AP-2 containing region are conserved between different species. We therefore examined the NHE1 promoters of various species for other regions of homology, and we noticed a highly conserved region (Fig. 1) consisting predominantly of poly(dA·dT). To examine the possible importance of this region we constructed a series of reporter plasmids. One contained the entire 1.1-kb mouse NHE1 promoter; a second contained a partial promoter to just past the poly(dA·dT) containing region; the third did not contain the poly(dA·dT) region. Deletion of most of the promoter but not the poly(dA·dT) region resulted in only a moderate loss of activity in L6 cells and a smaller loss in NIH 3T3 cells. However, removal of the poly(dA·dT) region resulted in a much greater reduction in promoter activity, and this effect was more pronounced in L6 cells than in NIH 3T3 cells. These results suggest that this region of the NHE1 gene is important for its basal expression in both cell types. In addition there was a trend for a more significant role in L6 cells in comparison with NIH 3T3 cells.

Several of our experiments indicate that the poly(dA·dT) element may have an important regulatory role in NHE1 expression. Using DNase I footprinting (Fig. 3) we found that a protein or proteins in nuclear extracts from L6 and NIH 3T3 cells binds to this region. Gel mobility shift analysis further supported this conclusion. The binding was specific because it was removed by competitor oligonucleotide but not by noncom-



0.2 0.18 7Mt 2Mt 2MtA

25

n

FIG. 7. Effect of disruption of the poly(dA·dT) site on activity of the NHE1 promoter. A comparison was made of the wild type promoter with an intact poly(dA·dT) site with the promoter with the poly(dA·dT) site deleted or mutated. A, comparison of the relevant regions of the promoter. The numbers correspond to the positions of the nucleotides relative to the first transcription initiation site. Asterisks indicate difference from the wild type sequence. B, the levels of luciferase activity are shown for L6 and NIH 3T3 cells transiently transfected with the plasmids pXP-0.2MP (0.2), pMut7-0.2MP (7Mt), pMut2-MP (2Mt), pMut2a-MP (2MtA), and pXP-0.18MP (0.18). Results are shown relative to the values obtained for pXP-0.2MP. * or †, significantly different from the plasmid pXP-0.2MP at p < 0.05 or p < 0.01, respectively.



FIG. 8. Effects of insertion of the poly(dA·dT)-rich region into the SV40 promoter. The poly($dA \cdot dT$)-rich region (nucleotides -174 to -149) was inserted into the SV40 promoter as described under "Experimental Procedures." The plasmids pXP-T-SV40 and pSVLuc were transiently transfected into L6 cells. Corrected luciferase values are shown. *, significantly different from the plasmid pSVLuc at p < 0.05.

petitor oligonucleotide. In a further experiment, we also examined whether the poly(dA·dT) region could activate a foreign promoter by inserting it into the SV40 promoter. We found (Fig. 9) that the poly(dA·dT) site could activate the SV40 promoter, and because the two plasmids, pXP-T-SV40 and pSV-Luc, differed only in the presence of the poly(dA·dT) region, it was clear that this region plays a significant role in regulation of promoter activity.

Finally, to confirm that the sequence of the poly(dA·dT) region is important in regulating expression of the NHE1 promoter, we mutated several residues of the poly(dA·dT) sequence. Mutation of only two residues in this sequence was sufficient to reduce expression regulated by the NHE1 promoter (Fig. 7). We also found a strong correlation between promoter activity and protein binding to the poly(dA·dT) sequence. Specifically, mutation of either two or seven residues



FIG. 9. Detection of poly(dA·dT) binding proteins by Southwestern (DNA protein) hybridization. Nuclear extracts from L6 cells were separated on SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. They were probed with a double stranded synthetic oligonucleotide corresponding to the wild type poly(dA·dT) region as described under "Experimental Procedures."

abolished protein binding to the poly(dA·dT) region (Fig. 5) and reduced promoter activity (Fig. 7). It was most interesting that mutation of the dT residues to dA instead of to dG resulted in partial restoration of protein binding to the poly(dA·dT) region (Fig. 6) and in partial restoration of promoter activity. Clearly, T to A substitution does not disrupt the binding site of putative transcription factor(s) to the same extent as a T to G substitution. This regult is consistent with naturally occurring variation in this region, because the rabbit NHE1 promoter contains one T to A substitution compared with the mouse and human sequence (Fig. 1).

Although there are poly(dA·dT) elements in a number of genes, there have been few studies on such elements. It has been noted that the actin gene of *Dictyostelium* contains one such element that is important in its regulation (21). Apparently, the element is 45 consecutive residues and functions to promote the level of expression of the promoter because its removal results in a 12-fold reduction in promoter activity (21). As yet, however, this region has not been analyzed in detail and the binding of nuclear proteins has not been investigated. Similarly, deletion of an A/T element in the rice actin gene reduced activity, but again no specific protein binding was examined (22). Another study, however, has noted that the LuxR protein binds to a poly(dA·dT) region of the Vibrio harveyi lux gene (23). The LuxR protein is a single polypeptide of M_r 23,000 and binds to (A + T)-rich regions of the *luxC* gene. Whether this protein is similar to the proteins that bind to the poly(dA·dT) region of the NHE1 promoter is not yet known. However, it is unlikely to be very closely related to the proteins regulating the NHE1 gene in this region because the poly(dA·dT) region of the lux gene is markedly different from the homo poly(dA·dT) region of the NHE1 gene (23). In addition, our results with Southwestern blotting suggest that the protein(s) that bind to the poly(dA·dT) region of the NHE1 gene are larger than that 23,000 M_r protein that binds to the *lux* gene.

Another protein, HMG-I, is a possible candidate for binding to the poly(dA·dT) region of the NHE1 gene. This protein has been shown to be important in controlling expression of lymphotoxin in PD and PD31 cells. The upstream activating sequence to which HMG-I specifically binds is, essentially, a homopolymeric A + T-rich region (24). The HMG-I(Y) isoform of this protein binds to AT-rich regions, can facilitate both protein-DNA and protein-protein interactions, and can promote transcription by promoting assembly of multiprotein complexes (24). Whether HMG-I or one of its related isoforms is involved in the poly(dA·dT) region of the NHE1 promoter remains to be determined.

It has recently been shown that a poly(dA·dT) structure modulates expression of the yeast his3 promoter (25). Specifically, the authors found that insertion of a $poly(dA \cdot dT)$ element resulted in stimulation of promoter activity and that this stimulation varied inversely with activity of the promoter. That is, a more active promoter resulted in less stimulation by the addition of the element. The protein datin was identified as a potential protein mediator of the effect. Analysis of the accessibility of the chromatin structure suggested that the yeast poly(dA·dT) site functions to improve the accessibility of the promoter, possibly by destabilization of nucleosomes covering this region (25). It is not yet known if the poly(dA·dT) sequence in mammalian cells functions similarly. However, there are many long poly(dA·dT) tracts in a variety of genes that have this potential. In this study, we examined one such tract in the Na⁺/H⁺ exchanger promoter, and our results show that the poly(dA·dT) element modulates expression of this promoter. Future experiments are necessary to determine whether the mechanism behind this modulation is analogous to those described in other systems.

Acknowledgments—We are grateful to Dr. Rachel E. Milner for critical reading of the manuscript.

REFERENCES

- 1. Boron, W. F., and Boulpaep, E. L. (1983) J. Gen. Physiol. 81, 29-52
- Grinstein, S., Clarke, C. A., and Rothstein, A. (1983) J. Gen. Physiol. 82, 619–638
- 3. Pouyssegur, J. (1985) Trends Biochem. Sci. 10, 453-455
- Rao, G. N., Sardet, C., Pouyssegur, J., and Berk, B. C. (1992) J. Cell. Physiol. 151, 361–366
- Horie, S., Moe, O., Miller, R. T., and Alpern, R. J. (1992) J. Clin. Invest. 89, 365–372
- Krapf, R., Pearce, D., Lynch, C., Xi, X. P., Reudelhuber, T. L., Pouyssegur, J., and Rector, F. C. Jr. (1991) *J. Clin. Invest.* 87, 747–751
- Dyck, J. R. B., and Fliegel, L. (1995) *J. Biol. Chem.* 270, 10420–10427
 Alvarez, J., Garcia-Sancho, J., Mollinedo, F., and Sanchez, A. (1989) *Eur. J. Biochem.* 183, 709–714
- 9. Hazav, P., Shany, S., Moran, A., and Levy, R. (1989) *Cancer Res.* 49, 72–75
- 10. Vairo, G., and Hamilton, J. A. (1993) Cell Growth & Differ. 4, 461-466
- Ladoux, A., Cragoe, E. J., Geny, B., Abita, J. P., and Frelin, C. (1987) J. Biol. Chem. 262, 811–816
- Miller, R. T., Counillon, L., Pages, G., Lifton, R. P., Sardet, C., and Pouysségur, J. (1991) J. Biol. Chem. 266, 10813–10819
- Kolyada, A. Y., Lebedeva, T. V., Johns, C. A., and Madias, N. E. (1994) Biochim. Biophys. Acta 1217, 54–64
- Dyck, J. R. B., Silva, N. L. C. L., and Fliegel, L. (1995) J. Biol. Chem. 270, 1375–1381
- Yang, W., Dyck, J. R. B., Wang, H., and Fliegel, L. (1995) Am. J. Physiol. 270, H259–H266
- Blaurock, M. C., Rebouças, N. A., Kusnezov, J. L., and Igarashi, P. (1995) Biochim. Biophys. Acta 1262, 159–163
- Altenberg, G. A., Aristomuno, P. C., Amorena, C. E., and Taquini, A. C. (1989) *Clin. Sci.* 76, 649–652
- Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) Nucleic Acids Res. 17, 6419
- Silva, C. M., Tully, D. B., Petch, L. A., Jewell, C. M., and Cidlowski, J. A. (1987) *Proc. Natl. Acad. Sci. U. S. A.* 84, 1744–1748
- Kwast-Welfeld, J., de Belle, I., Walker, P. R., Whitfield, J. F., and Sikorska, M. (1993) *J. Biol. Chem.* 268, 19581–19585
- 21. Hori, R., and Firtel, R. A. (1994) Nucleic Acids Res. 22, 5099-5111
- Wang, Y., Zhang, W., Cao, J., McElroy, D., and Wu, R. (1992) *Mol. Cell. Biol.* 12, 3399–3406
- 23. Swartzman, E., and Meighen, E. A. (1993) *J. Biol. Chem.* 268, 16706–16716
- Fashena, S. J., Reeves, R., and Ruddle, N. R. (1992) Mol. Cell. Biol. 12, 894–903
- 25. Iyer, V., and Struhl. K. (1995) EMBO J. 14, 2570-2579

Regulation of Na⁺/H⁺ Exchanger Gene Expression: ROLE OF A NOVEL POLY(dA·dT) ELEMENT IN REGULATION OF THE NHE1 PROMOTER Weidong Yang, Huayan Wang and Larry Fliegel

J. Biol. Chem. 1996, 271:20444-20449. doi: 10.1074/jbc.271.34.20444

Access the most updated version of this article at http://www.jbc.org/content/271/34/20444

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 25 references, 13 of which can be accessed free at http://www.jbc.org/content/271/34/20444.full.html#ref-list-1