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Activation of the Na^+/H^+ Exchanger Gene by the Transcription Factor AP-2*

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We have isolated and characterized regions important for expression of the mouse Na^+/H^+ exchanger gene. A 1.1-kilobase fragment upstream of the 5′-untranslated region contains specific DNA motifs characteristic of promoter and enhancer elements including a TATA box, two CAAT boxes, an SP-1 site, a cyclic AMP response element-binding site, and an AP-2-like site. This 1.1-kilobase fragment directs transcription of a luciferase reporter gene in mouse fibroblasts (NIH 3T3) and human Hep G2 cells. Deletion or mutation of an AP-2-like site 100 base pairs from the start site of transcription resulted in loss of most of the reporter plasmid activity. In addition, cotransfection of an AP-2 expression plasmid and the mouse promoter/luciferase plasmid increased the amount of Na^+/H^+ exchanger-directed transcription in AP-2-deficient Hep G2 cells. Moreover, mobility shift analysis indicated that a putative AP-2-binding site is capable of binding purified AP-2 protein and a specific protein from nuclear extracts of NIH 3T3 cells. The results show that the transcription factor AP-2 may play an important role in regulation of transcription of the mouse Na^+/H^+ exchanger gene.

The Na^+/H^+ exchanger is a mammalian plasma membrane protein that mediates the exchange of intracellular protons for extracellular sodium. It is involved in pH regulation (1), control of cell volume (2), and is stimulated by growth factors (3). Several different isoforms of the protein exist which have been designated NHE-1 to NHE-4. The NHE-1 isoform is the widely distributed "housekeeping" isoform of the family present in most, if not all, mammalian cells (4). Although the mechanism of regulation of protein levels is extremely important, only a few studies have examined the human NHE-1 gene (5, 6). Miller *et al.* (5) demonstrated that the 5′-flanking region of the human NHE-1 gene contains a number of putative sites for DNA-binding transcription factors, any of which could regulate the exchanger gene transcription. Of these sites, only AP-1 has been implicated to have a possible role in activating the NHE-1 gene (7). Recently, a more in-depth study of the human NHE-1 gene has analyzed the proximal regulatory elements of the promoter (6). To date, however, there has been no direct identification of any specific transcription factor responsible for regulating the NHE-1 promoter.

Expression of the message and activity of the Na^+/H^+ exchanger has been shown to vary greatly depending on the state of differentiation (8) and a variety of other stimuli (9–11). The regulation of the NHE-1 gene, however, involves the link between DNA-binding protein motifs located in promoter and enhancer sequences and the general transcription machinery. Various cis elements of the gene may be able to activate transcription through responses to certain extracellular messages. One such element is the transcription factor AP-2. Transcriptional activation by AP-2 involves the 52-kDa AP-2 protein binding to a specific DNA motif found in the cis-regulatory region of the gene (12). AP-2 activity is regulated in a cell type-specific manner (13) and is induced by phorbol esters, retinoic acid, and cAMP (12–14). Additionally, mRNA levels of AP-2 have been shown to increase dramatically upon differentiation indicating that the expression of AP-2 is regulated during differentiation (14).

In this study we demonstrate, for the first time, a specific region within the Na^+/H^+ exchanger gene that is involved in regulating transcription. Moreover, we have shown that this DNA motif interacts with AP-2 or an AP-2-like transcription factor. We report the sequence of the upstream region of the mouse NHE-1 gene, the identification and characterization of an AP-2 site, and the importance of this region for NHE-1 gene transcription.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonuclease and DNA-modifying enzymes were obtained from Boehringer Mannheim (Laval, Quebec, Canada) and Life Technologies, Inc. The pBluescript plasmids were from Stratagene (La Jolla, CA). Plasmid pXP-1 was a gift from Dr. M. Nemer of the Institute de recherches cliniques de Montreal, Montreal, Quebec, Canada. pRSVAP2 was a generous gift of Dr. R. Gaynor from the University of Texas Southwestern Medical School, Dallas, TX. Hybond-N^+ nylon membranes were obtained from Amersham (Oakville, Ontario, Canada). The Gel Shift Assay System was purchased from Promega (Madison, WI). [γ-^{32}P]dCTP, [α-^{32}P]dATP, [γ-^{32}P]dATP, and [α-^{35}-S]dATP were from ICN. All other chemicals were of analytical or molecular biology grade and were purchased from Fisher Scientific (Ottawa, On, Canada), Sigma, or BDH (Toronto, Ontario, Canada).

Screening and Sequencing of Genomic Clones—Screening of a mouse λ-Gem-11, genomic DNA library (Promega, Madison, WI) was with a fragment of the rabbit cardiac Na^+/H^+ exchanger (NHE-1) cDNA clone (1–691 bp,[1] 15) and a fragment of the 5′-untranslated and coding region (1–900) of the human Na^+/H^+ exchanger as described earlier (16). Filters were routinely washed with 1 × SSC at 42 °C. This resulted in the isolation of two genomic DNA clones that were excised by digestion with Sfi-1. Fragments of each clone were subcloned into pBluescript (KS-). For sequencing reactions a progressive series of deletions was constructed with exonuclease III in both directions. Double-stranded DNA sequencing of fragments and plasmids was by the DNA Sequencing Laboratory in the Department of Biochemistry, using an Applied Biosystems, model 373A DNA sequencer. Synthetic oligonucleotides were made at the same location using an Applied Biosystems, model 392 DNA/RNA synthesizer.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L37525.

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[1] The abbreviations used are: bp, base pair(s); kb, kilobase(s).

Primer Extension—Primer extension analysis was with a 20-bp oligonucleotide of the sequence 5′-TCC TTC GGT CAG CTC CAG CT-3′. The primer was end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase to a specific activity of 1–3 × 10^9 counts/min/μg. Unincorporated [γ-^{32}P]ATP was removed by purification with a Sephadex G-50 column. One pmol of primer was hybridized to 15 μg of mouse kidney poly(A)$^+$ RNA in a primer extension solution containing 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM dNTP, 0.5 mM spermidine. Mixtures were annealed at 58° C × 20 min and then at room temperature for 10 min. The sample was added to a reverse transcription buffer containing the same solution plus 2.8 mM sodium pyrophosphate and 8 units of avian myeloblastosid virus reverse transcriptase. After 30 min at 42 °C the reaction was terminated by the addition of loading dye and the samples were analyzed on a 7% polyacrylamide/urea gel.

Cell Culture—NIH 3T3 cells were obtained from Dr. J. Stone of the Department of Biochemistry, University of Alberta. They were maintained in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum. Hep G2 cells were provided by Dr. Z. Yao of the Department of Biochemistry, University of Alberta. These cells were propagated in Dulbecco's modified Eagle's media with 2 mM L-glutamine, 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin and a pH of 7.2.

Reporter Plasmid Construction—pXP-1MP was constructed by digesting a 2.2-kb fragment containing the promoter/enhancer region with *Pst*I and *Sma*I. An intermediate plasmid that contained a *Pst*I site flanked by a *Hin*dIII and *Sal*I was used to transfer the *Pst*I*sma*I 1.1-kb fragment into the *Hin*dIII and *Sal*I site of pXP-1. The 1.1-kb fragment contains base pairs −1085 to +22 of NHE-1. To construct pMP+AP2 plasmid, two oligonucleotides were used as primers for polymerase chain reaction (no. 1, ttgg atc CGT GAC ACT TCC TTC CCT and no. 3, cc ttc gaa GGG TCC CGC GGT AGC GGA) were synthesized to amplify base pairs −125 to +22 of the gene. The polymerase chain reaction product had the restriction enzyme sites *Bam*HI and *Sma*I generated on either end and was inserted directionally into pXP-1. Similarly, pMP-AP2 plasmid was made using the product of the primers no. 3 and no. 2 (no. 2, ttgg atc CTG CAC CGC GCG GGC GCT) and the 114-bp product (−92 to +22) was inserted into pXP-1. Mutations in pMP(MUT)AP2 were generated by polymerase chain reaction, using a mismatched oligonucleotide (no. 1-mut, ttgg atc CGT GAC ACT TCC TTT TTT AAG TAA TAT AAG CCG CTG CAC CG) similar to oligonucleotide no. 1 above. Other mutations in the AP-2 containing region were made using a similar procedure. pRSV-AP2 was constructed by digesting pRSVAP2 with *Eco*RI and removing the AP2 gene. All plasmids were sequenced to verify proper orientation and fidelity of polymerase chain reaction.

Transfection and Reporter Assays—Cells were plated onto 35-mm dishes. Each dish received 5 μg of luciferase reporter plasmid and 5 μg of pSV-β-galactosidase plasmid as an internal control. In some cases (*i.e.* Hep G2 cells), 5 μg of a third plasmid was also cotransfected (*i.e.* pSV±AP2). Both NIH 3T3 cells and Hep G2 cells were transiently transfected using the $CaPO_4$ precipitation technique (17). After 36 h, cells were harvested and the cell lysate was assayed for both luciferase activity and β-galactosidase activity. Each luciferase assay contained 30 μl of the cell lysate and 100 μl of the luciferase assay reagent (20 mM Tricine, 1.07 mM magnesium carbonate, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 470 μM luciferin, 530 μM ATP, 270 μM coenzyme A, and 1 ng/ml bovine serum albumin). The β-galactosidase assay included 5 μl of cell lysate, 95 μl of H_20, and 20 μl of *o*-nitrophenyl-β-D-galactopyranoside, incubated at 37 °C for 60 min. Luciferase activity was assayed with an LKB luminometer and normalized to β-galactosidase for efficiency of transfection. Results are reported as mean ± S.E.; where not shown the S.E. was to small to be displayed.

DNase I Footprinting—The 206-bp probe for DNase I footprinting was isolated from pXP-1MP using the restriction enzymes *Rsa*I and *Hin*dIII. The DNA probe was gel purified and was radiolabeled by filling in the protruding 5′-end generated by the *Hin*dIII. The reaction mixture contained dATP, dGTP, dTTP, [γ-^{32}P]dCTP, and the Klenow fragment of *Escherichia coli* DNA polymerase I. The fragment contained the mouse NHE-1 promoter region −171 to + 22 along with 13 bp of the 5′-end of the pXP-1 plasmid. The DNA (30,000 counts/min) was incubated with 50 μg of NIH 3T3 nuclear extracts at 4 °C overnight and then treated with DNase I at room temperature for 10 s to 2 min. It was terminated with 2.5 μl of stop solution (125 mM Tris-HCl, pH 8.0, 125 mM EDTA, 3% SDS) and proteins removed by phenol/chloroform extraction. After precipitation the sample was resuspended in 98% formamide dye and electrophoresed on a 6% acrylamide, 7 M urea sequencing gel.

DNA Binding Assays—Nuclear extracts were prepared from NIH 3T3 cells according to the procedure of Schreiber *et al.* (18). The synthetic oligonucleotides of the sequence 5′-TTC CTT CCC TGG GCG ACA GGG GCC-3′ (MPAP2a) and 5′-GGC CCC TGT CGC CCA GGG AAG GAA-3′ (MPAP2b) were made corresponding to the mouse promoter AP-2 region. Alternatively, the mutant oligonucleotides of the sequence 5′-TTC CTT TTT TAA GTA ATA TAA GCC-3′ (MUTAP2a) and 5′-GGC TTA TAT TAC TTA AAA AAG GAA-3′ (MUTAP2b) were also used for analysis. The oligonucleotides were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. They were heated to 95 °C for 2 min and cooled to room temperature overnight for annealing. DNA binding reactions were for 10 min at room temperature and contained: 30,000 CPM of ^{32}P-labeled oligonucleotides was mixed with purified AP-2 (1.4 μg) or NIH 3T3 nuclear extract (5 μg) in a binding buffer (4% glycerol, 1.25 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 0.05 mg/ml poly(dI-dC). After electrophoresis on 4% polyacrylamide gels, gels were dried and exposed to x-ray film for 16 h. The non-competing oligonucleotide SP-1 was 5′-ATT CGA TCG GGG CGG GGC GAG C-3′. In some experiments nuclear extracts were treated to specifically remove AP-2 protein before gel mobility shift assays were performed. 20 μl of a 10% solution of Protein A was incubated with 2.5 μg of AP-2 antibody (IgG, Santa Cruz Biotechnology Inc.) for 1 h at 4 °C. After 1 h the solution was washed three times (20 mM Tris, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride), mixed with 5 μl of NIH 3T3 nuclear extracts, and incubated at 4 °C for 16 h. The mixture was then spun at 6,500 revolutions/min for 10 min, and the supernatants were collected. Control experiments contained equal amounts of irrelevant IgG.

To examine if the Na^+/H^+ exchanger gene could directly bind purified AP-2 protein, we isolated and end labeled a DNA fragment of the gene from bp −171 to +22 of the promoter. This fragment contained the putative AP-2-binding site and the surrounding nucleotides. Gel mobility shift assay was performed as described above.

RESULTS

Two positive clones were obtained by library screening with NHE-1 probes. Clone 3–1 contained most of the coding region along with the entire 3′-untranslated region, and clone 3–3 included a section of the coding region, the 5′-untranslated region, and approximately 10 kb upstream from the 5′-untranslated region. A 2.2-kb fragment from clone 3–3 was sequenced in both directions using exonuclease digestions. The deduced sequence of the first 40 amino acids of the mouse 5′-coding region was compared with other Na^+/H^+ exchangers (NHE 1–4) from a variety of species. It was 90 to 83% identical to the other NHE-1 isoforms with the hamster showing the greatest identity. No significant identity was seen with the other isoforms of the exchanger (NHE 2-NHE 4, not shown). The sequence of the mouse promoter/enhancer region is shown in Fig. 1. It contains putative recognition sites for several transcription factors. This includes a TATA box, two CAAT boxes, an SP-1 site, a CREB site, and an AP-2 site (Fig. 1). The sequence TATAAA in the mouse NHE-1 gene is identical to the well characterized consensus sequence for the mammalian TATA box. Two CAAT boxes are present at sites −407 to −401 and −568 to −562 along with an SP-1 site at position −600 to −592. Both CAAT and SP-1 are characteristic binding sites for transcriptional regulatory proteins seen in many eukaryotic promoters (19, 20). The CREB site, at position −789 to −784, is a putative cAMP response element that can activate transcription upon cAMP or Ca^{2+} stimulation (21). The site may be of significance since it has been shown that cAMP can modulate exchanger activity acutely (22). Finally, a putative binding site for one of the more well known transcription factors, AP-2, is located −111 to −94 and was also a candidate for transcriptional regulation.

The start sites for transcription were demonstrated to occur at base pairs +1 and +5 (Fig. 1) and occur at nucleotides that are 26 and 30 bp from the TATA box. The two start sites and their distance from the TATA box are identical to that reported for the human promoter (5). Our initial experiments tested whether a 1.1-kb sequence upstream from the start site was indeed a promoter, and if so its relative ability to direct transcription in mouse NIH 3T3 cells. When the level of luciferase

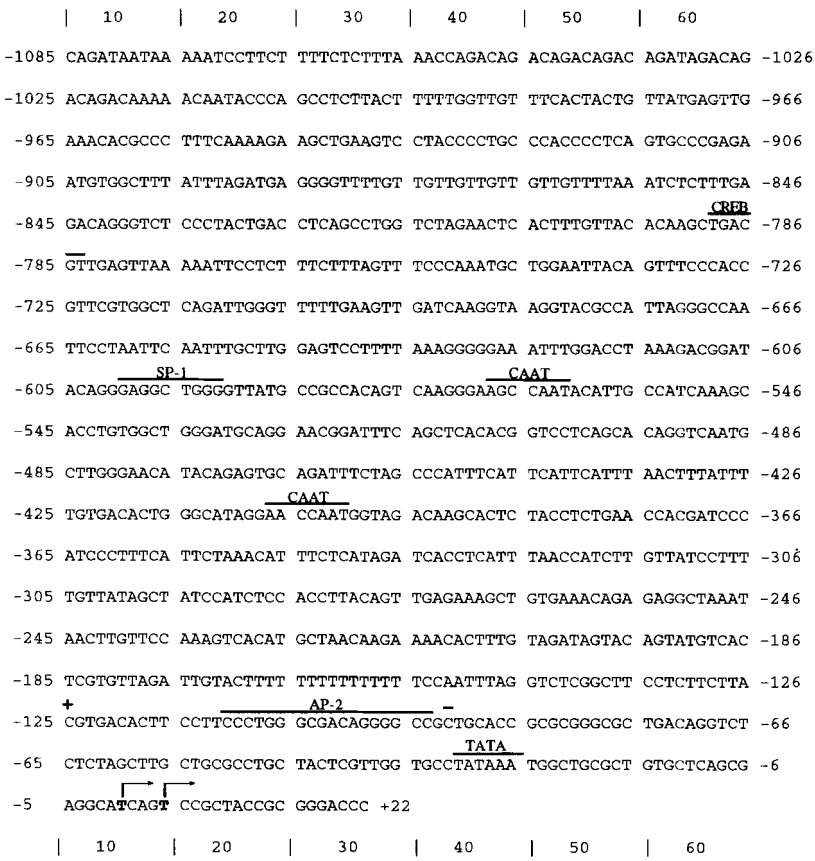

FIG. 1. **Nucleotide sequence of the promoter/enhancer region of the mouse NHE-1 isoform**. Nucleotides for the coding strand are *numbered* beginning with the first start site of transcription, shown as *+1*. Start sites for transcription are denoted with *arrows* at positions +1 and +5. *Horizontal lines* indicate putative binding sites for DNA-binding proteins, SP-1, CAAT, CREB, TATA, and AP-2. The first base pairs of the plasmids pMP+AP2 and pMP-AP2 are indicated by the + and −, respectively.

activity from pXP-1MP was compared to pXP-1, a 73-fold increase in activity was seen (Fig. 2*A*). Transfection experiments also (Fig. 2*B*) showed that pXP-1MP induced 23.8-fold less luciferase activity than the Rous sarcoma virus RSV promoter located 5′ to the luciferase gene (pRSVLUC). This indicates that the promoter for the mouse NHE-1 gene is relatively weak at directing transcription in comparison to pRSVLUC. These results could provide an explanation for the low levels of exchanger protein and mRNA present in mammalian tissues (4). Another set of experiments examined the relative ability of an 8-kb fragment of the NHE-1 promoter/enhancer region to direct transcription. This fragment did not stimulate transcription greater than the 1.1-kb fragment (not shown).

To examine which of the identified consensus sequences are involved in regulation of NHE-1 transcription, we constructed several different plasmids containing varying fragments of the promoter/enhancer region. We initially removed all base pairs upstream of the AP-2 site from pXP-1MP. This construct was named pMP+AP2. The second construct, pMP-AP2, was identical to pMP+AP2 except for the further deletion of the AP-2 site. We then examined the ability of these two plasmids to direct transcription of the luciferase gene. They were transfected into NIH 3T3 cells along with pSV-β-galactosidase which was used to normalize for any differences in transfection efficiency. *Panels C* and *D* in Fig. 2 are comparisons between the plasmid containing the entire sequenced region (pXP-1MP), the plasmid containing the AP-2-binding site (pMP+AP2) and the plasmid with the AP-2-binding site removed (pMP-AP2). Deleting all the putative transcription factor binding sites except AP-2 caused some decrease in NHE-1 transcription (Fig. 2*D*). Further removal of the AP-2 containing region, however, decreased transcription 6-fold (Fig. 2*D*).

To confirm the importance of the 33-bp deleted region, we introduced several mutations into the AP-2 consensus sequence of the pMP+AP2 construct. The substitutions are shown in Fig. 3*A*. When the mutant construct no. 1 (pMP-(MUT)AP2) is transfected into NIH 3T3 cells we see a 7.9-fold decrease in the level of luciferase activity (Fig. 3*B*). This decrease is comparable to the levels seen when the entire AP-2 region is deleted. To localize the nucleotides involved in this activity, two other mutations were generated (Fig. 3*B*). Mutation no. 2 involved a 5 base pair modification of the putative AP-2 site but had little effect on the activity of the promoter. When another two mutations were added to these existing five (mutation no. 3) a small decrease in luciferase activity was seen. The mutational analysis indicates that the region at approximately −106 to −95 may be important for regulating transcription in NIH 3T3 cells. To confirm the data from the mutational analysis, we performed DNase I footprinting experiments to determine if the specific protein from NIH 3T3 nuclear extracts could interact with the DNA sequence containing this AP-2 consensus site. A nuclear protein protected a region corresponding to −106 to −95 of the mouse NHE-1 promoter region (Fig. 4). This region lies within the AP-2 consensus site which is located in the 33-bp fragment of DNA.

NIH 3T3 cells have one of the most abundant AP-2 levels of a number of murine cell lines and tissues (13, 23). Hep G2 are human liver hepatoma cells which have been shown to be deficient in AP-2 mRNA and have little, if any, AP-2 binding and transcription activity (14). To confirm that AP-2 protein or an AP-2-like protein regulates transcription of the mouse NHE-1 gene Hep G2 cells were transfected with pMP+AP2 and cotransfected with either a plasmid expressing AP-2 protein (pRSVAP2) or the identical plasmid that cannot express AP-2 protein (pRSV-AP2). The results (Fig. 5) show that cotransfection with an AP-2 expression plasmid causes a large 2-fold increase in the activity of the NHE-1 promoter. When the mutated construct pMP(MUT)AP2 is transfected into Hep G2

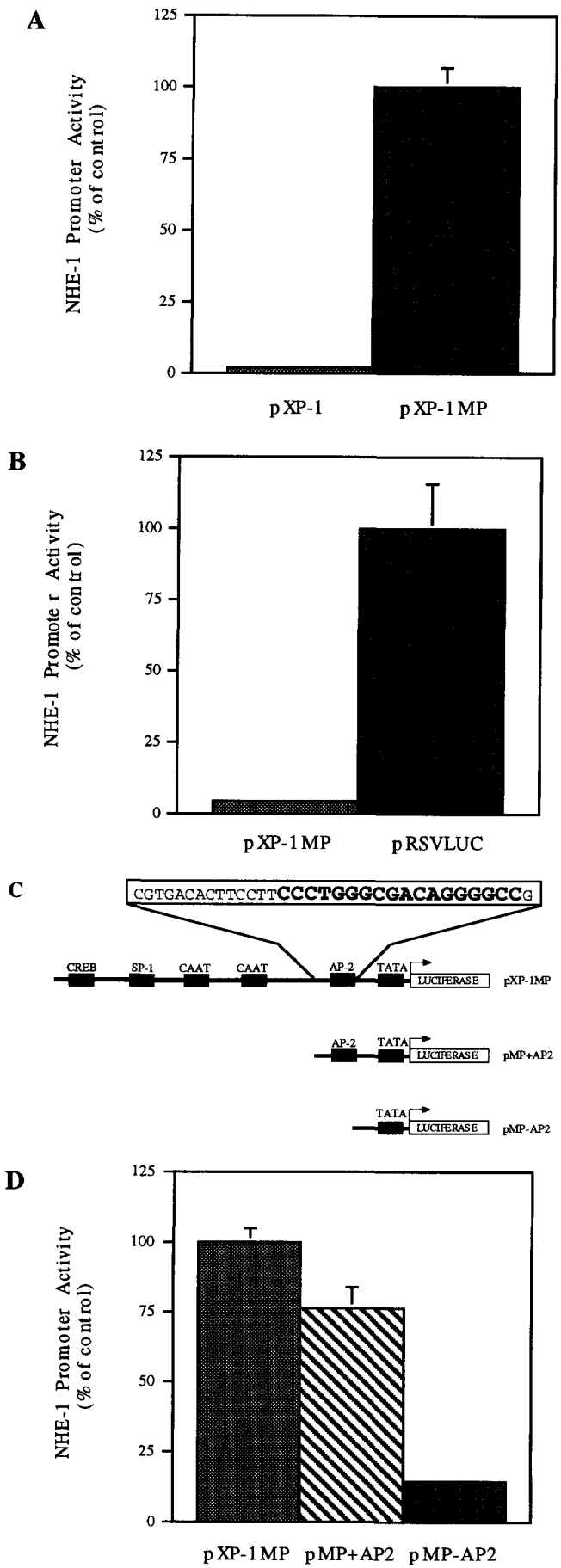

FIG. 2. **NHE-1 promoter activity in mouse NIH 3T3 fibroblasts.** *A*, mouse fibroblasts were transiently transfected with either a 1.1-kb fragment of the mouse Na^+/H^+ exchanger (NHE-1) promoter/enhancer region linked to a luciferase reporter gene (*pXP-1MP*) or with the reporter plasmid without the 1.1-kb insert (*pXP-1*). *B*, fibroblasts were transfected with either pXP-1MP as in *A* or with pRSVLUC. *C*, fragments of the promoter were inserted upstream of luciferase gene in the vector pXP-1. pXP-1MP contains from −1085 to +22 of the mouse promoter/enhancer region. pMP+AP2 contains the region between −125 and +22, and pMP-AP2 contains the region between −92 and +22. The *boxed* sequence indicates the region containing the putative AP-2 site that is deleted in pMP-AP2, and the *bold letters* denote the putative AP-2 site. *D*, mouse fibroblasts were transiently transfected with either pXP-1MP, pMP+AP2, or pMP-AP2. pSV-β-galactosidase was used as an internal control. For all transfection experiments the results reported were obtained from at least three independent experiments each carried out in triplicate using at least two different DNA preparations for each plasmid. pSV-β-galactosidase was used as an internal control.

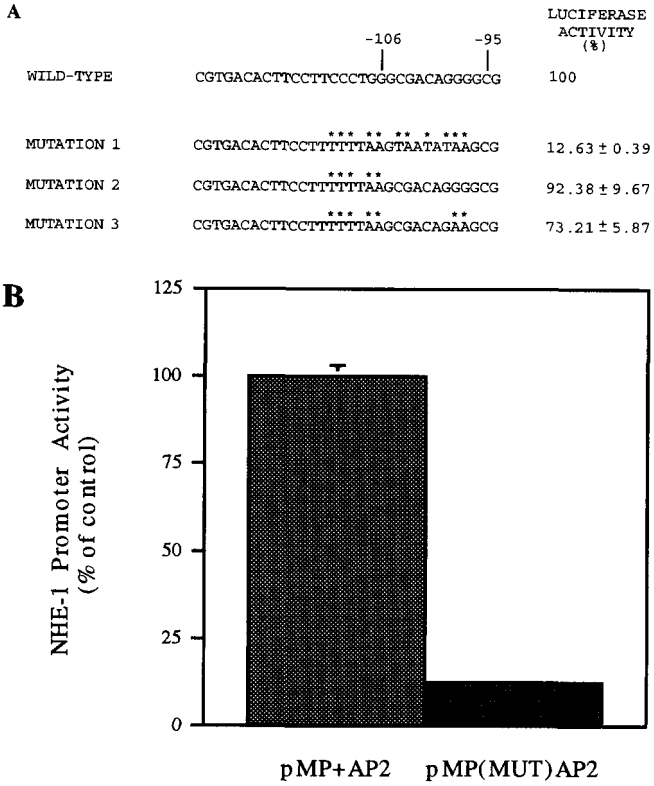

FIG. 3. **Effect of mutant constructs on NHE-1 promoter activity in NIH 3T3 cells.** *A*, comparison of the wild-type and three mutant constructs of the 33-bp sequence containing the AP-2 site. All the mutations are indicated by *asterisks*, and the *numbers* correspond to the position of the nucleotides relative to the first transcription initiation site. The *numbers* also represent the region of DNA which is protected by AP-2 protein from nuclear extracts of NIH 3T3 cells during DNase I footprint analysis. The level of luciferase activity in NIH 3T3 cells for the wild-type and the mutations are shown to the right and are expressed as percent of the pMP+AP2 construct. *B*, mouse fibroblasts were transiently transfected with either pMP+AP2 or pMP(MUT)AP2. Harvest and transfections were as described for Fig. 2.

cells along with pRSVAP2 there is no increase in activity of the mouse NHE-1 promoter as compared to the wild-type containing plasmid (Fig. 5).

To investigate the role of AP-2 protein as an enhancer of NHE-1 transcription in more detail, we examined the ability of the AP-2 motif to bind AP-2 protein. A short double-stranded oligonucleotide (MPAP2a, b) of 24 base pairs was synthesized that consisted of the sequence 5′-TTC CTT CCC TGG GCG ACA GGG GCC-3′. Gel retardation assays showed that this oligonucleotide bound to the purified AP-2 protein (Fig. 6*A*,

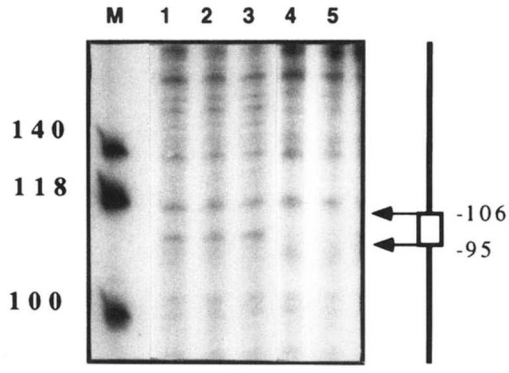

FIG. 4. **DNase I footprinting analysis of the mouse NHE-1 promoter.** DNase I footprint analysis was performed with the −171 to +22 fragment of the NHE-1 promoter (*lanes 1–5*). The naked fragment of DNA was treated with DNase I (0.1 units (*lane 1*) and 0.2 units (*lanes 2* and *3*)) for 10 s at room temperature. The fragment of DNA was also treated with DNase I (1.0 units (*lane 4*) and 2.0 units (*lane 5*)) for 2 min at room temperature after incubation with nuclear extracts from NIH 3T3 cells. *Lane M* represents the [γ-^{32}P]dATP end-labeled molecular weight markers whose lengths are shown to the left. The protected region of DNA is indicated to the right as an *open box*, and the *numbers* correspond to the position of the nucleotides in the DNA sequence.

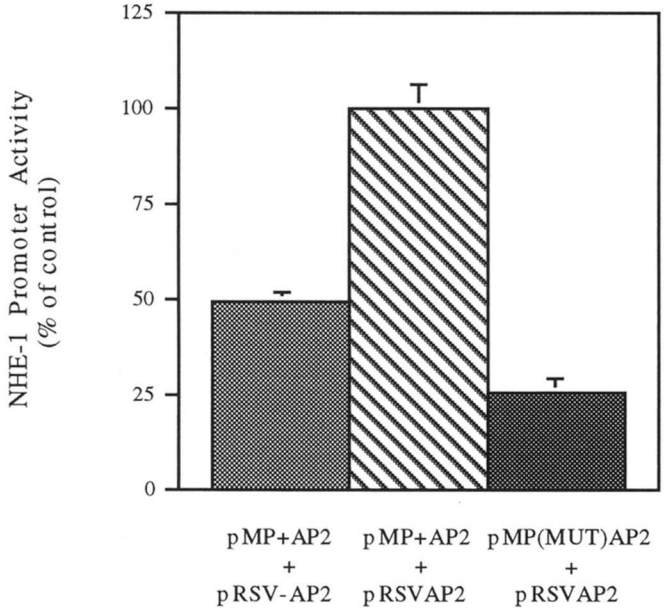

FIG. 5. **Effects of AP-2 on NHE-1 promoter activity in human Hep G2 cells.** Human liver hepatoma cells (Hep G2) were transiently transfected with either pMP+AP2 or pMP(MUT)AP2 and cotransfected with either an AP-2 expression plasmid (*pRSVAP2*) or the same expression plasmid with the AP-2 gene deleted (*pRSV-AP2*) as indicated. Harvest and transfections were as described for Fig. 2.

lane 1). Competition experiments were done to show specific binding of AP-2, using unlabeled MPAP2a, b oligonucleotide as the competitor and an unlabeled SP-1 oligonucleotide as a non-competitor. Competitor concentrations of 6, 12, and 50 times blocked the protein-DNA interaction while 100 times excess of non-competitor had no effect (Fig. 6*A*, *lanes 2–5*, respectively). To further characterize the protein binding ability of the mouse AP-2 consensus site, nuclear extracts from NIH 3T3 cells were used. The results with purified AP-2 protein were similar to those seen in experiments with nuclear extracts. Nuclear extracts of NIH 3T3 cells bound to the AP-2 containing synthetic oligonucleotide (MPAP2a, b) and 12 and 25 times excess unlabeled competitor oligonucleotide decreased the binding (Fig. 6*B*, *lanes 2* and *3*), while 100 times excess of non-competitor did not (*lane 4*). Some binding of smaller size was apparent, but competition experiments revealed that the interaction was nonspecific (*lanes 2* and *3*). We also examined whether a 193-bp fragment cut directly from the promoter could bind purified AP-2 protein. The results are shown in Fig. 6*C*. An intense band was seen indicative of AP-2 binding to the labeled fragment of the gene. In the absence of the protein, the band was not apparent. The synthetic oligonucleotides containing the sequence for the mutated mouse putative AP-2 binding site (MUTAP2a, b) were used in another bandshift assay. The oligonucleotides MUTAP2a, b only bound small amounts of protein from nuclear extracts of NIH 3T3 cells (Fig. 6*D*, *lane 2*) in comparison to MPAP2a, b (Fig. 6*D*, *lane 1*). In addition, the unlabeled mutated oligonucleotides were unable to compete with MPAP2a, b confirming their reduced ability to bind to the protein. Finally, AP-2 was specifically removed from the nuclear extract by immunoprecipitation with anti-AP-2 antibody. This resulted in greatly reduced binding tc MPAP2a, b by the nuclear extracts (Fig. 6*E*).

DISCUSSION

Previous studies have shown that the level of Na^+/H^+ exchanger mRNA and transcription itself can be increased due to a wide variety of treatments including chronic acid loading (9–11) and treatments causing cell differentiation (8). To date, however, there have been few studies on the NHE-1 promoter. One of these studies identifies the regions of the human promoter/enhancer region which are capable of binding nuclear factors (6), while the second indirectly examines the role of AP-1 (7). It was suggested that AP-1 could play a role in activating the antiporter especially during acidosis mediated increases in antiporter activity. Alternatively, it was suggested that some other protein kinase C-dependent pathway could mediate the effects of acidosis through AP-1 (7). To date, however, there has been no direct examination of the role of specific transcription factors on the actual NHE-1 promoter.

Our analysis suggests that base pairs −106 to −95 play an important role in regulation of expression. Mutation of these base pairs reduced both *in vivo* transcription and binding of nuclear extracts to this region. DNA footprinting analysis also localized the binding of a protein to this region. It is likely that the transcription factor AP-2 or a closely related AP-2-like protein is involved in binding to this region. The evidence supporting this is that cotransfection of Hep G2 with an AP-2 expression plasmid increased NHE-1 transcription levels. In addition purified AP-2 protein bound to this sequence and removal of AP-2 protein from nuclear extracts by immunoprecipitation greatly reduced their ability to bind to the AP-2 consensus sequence. However, it is possible that an AP-2-like protein could be responsible for some of the observed effects. Future experiments will attempt to purify the protein binding to this region of the gene. It is of note that base pairs −95 to −105 are perfectly conserved in sequence and location in the human gene (5) while more upstream distal regions are not. This may indicate an important regulatory function. It should be noted that AP-2 is not the only transcription factor involved in regulation of NHE-1 expression, and other regions have also recently been suggested to be of importance (6).

We examined the mouse NHE-1 promoter because of the widespread availability of a number of useful mouse cell lines. Our results show that we have isolated the NHE-1 isoform since our probes for screening the genomic library originated from the NHE-1 isoform and the clone hybridized under relatively high stringency. We also show that the promoter for the housekeeping isoform of the Na^+/H^+ exchanger family is activated by the transcription factor AP-2 or an AP-2-like tran-

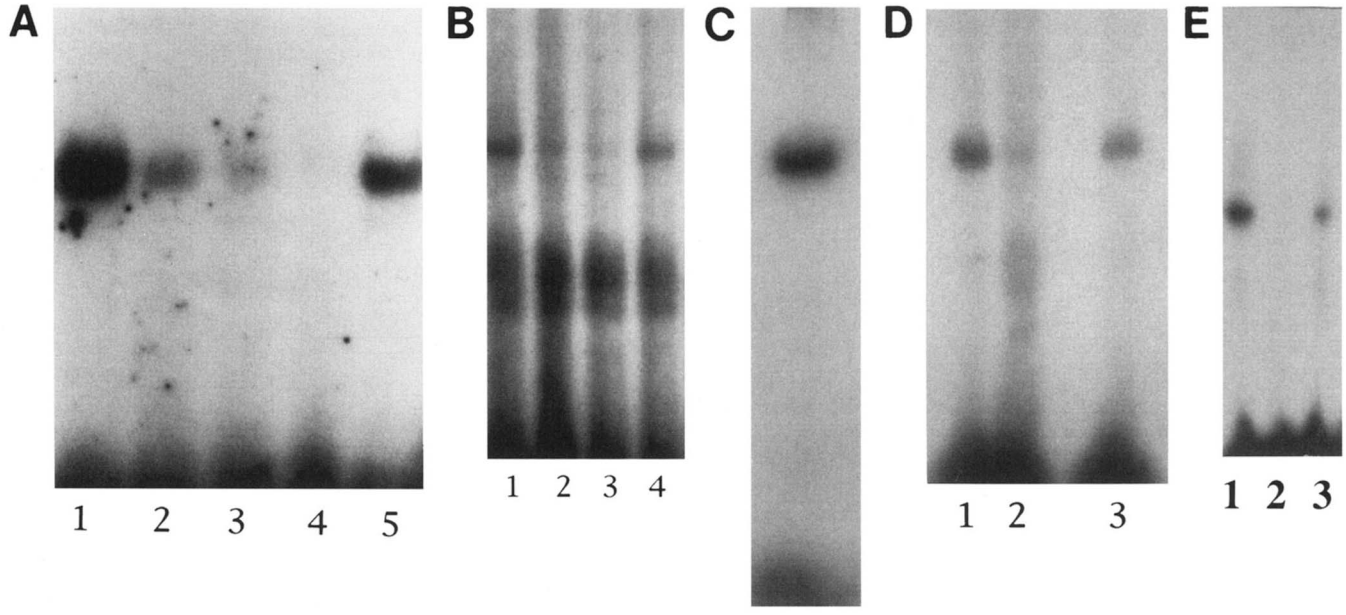

FIG. 6. **DNA-mobility shift binding assay and competition analysis of the mouse NHE-1 AP-2 site.** The labeled 24-base pair oligonucleotide MPAP2a, b (positions −117 to −94) was incubated with purified human AP-2 protein or nuclear extracts for 10 min at room temperature. The binding mixtures were analyzed by electrophoresis on 4% polyacrylamide gels as described under "Experimental Procedures." *A*, *lane 1*, purified AP-2 protein (1.4 μg) alone added to the binding reactions; *lanes 2–4*, competitions with 6-, 12-, and 50-fold excess of unlabeled MPAP2a, b respectively; *lane 5*, competition with 100-fold excess of a nonspecific sequence competitor, SP-1. *B*, nuclear extracts from NIH 3T3 were used for mobility shift binding assay with MPAP2a, b and 5 μg of NIH 3T3 cell nuclear extract as described in *A*. *Lane 1*, nuclear extract alone added to the binding reactions; *lanes 2* and *3*, competitions with 12- and 25-fold unlabeled MPAP2a, b, respectively; *lane 4* shows competition with 100-fold excess of a nonspecific sequence competitor SP-1. *C*, a fragment of Na^+/H^+ exchanger genomic DNA containing the AP-2 consensus sequence was isolated and labeled with [γ-^{32}P]ATP as described under "Experimental Procedures." Purified AP-2 protein was incubated with the fragment, and the results were analyzed as described in *A*. *D*, the labeled 24-base pair oligonucleotide MUTAP2a, b or MPAP2a, b (positions −117 to −94) were incubated with 5 μg of NIH 3T3 nuclear extract and analyzed as above. *Lane 1* is the wild-type oligonucleotide MPAP2a, b, while *lane 2* is the mutant oligonucleotide. *Lane 3* is a competition assay containing labeled MPAP2a, b and 25-fold excess of cold MUTAP2a, b. *E*, DNA mobility shift of MPAP2a, b with untreated nuclear extracts (*lane 1*), without nuclear extracts (*lane 2*), or with AP-2 immunodepleted nuclear extracts (*lane 3*).

scription factor. Although housekeeping genes such as NHE-1 are normally not acutely regulated, there are examples of such promoters that are activated by cis elements. One such example is the second promoter of the acetyl-CoA carboxylase gene (24) whose activation is mediated through an AP-2-like sequence (25). This example may be analogous to the NHE-1 promoter. Both AP-2 and NHE-1 transcription rate increase in some models of cellular differentiation. For example, during retinoic acid induced differentiation of human leukemic cells (HL-60), there is an 8.3-fold increase in NHE-1 transcription (8). Additionally, retinoic acid-induced differentiation of human NT2 teratocarcinoma cells shows increased AP-2 mRNA and protein expression levels (14). The relationship between increased AP-2 levels during differentiation induced by retinoic acid and the increase in NHE-1 transcription seems to be an important issue. Experiments are currently underway which may help to explain this relationship.

The results from this paper have, for the first time, directly examined the role of a specific transcription factor in the regulation of the Na^+/H^+ exchanger gene. We have suggested that this transcription factor is AP-2 or an AP-2-like protein, and we have located the region of the gene where it is able to bind. While the extent to which this protein regulates the Na^+/H^+ exchanger in specific cellular events is not yet known, the identification of a DNA binding protein motif has provided enough information to warrant further interest. AP-2 is involved in the regulation of a number of cellular events including growth and differentiation (14). The involvement in these events is an interesting characteristic AP-2 has in common with the Na^+/H^+ antiporter (4, 8). Future studies will focus on these specific cellular events and how they work together to control regulation of Na^+/H^+ exchanger levels and growth and differentiation of the cell.

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