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Specific Activation of the Na^+/H^+ Exchanger Gene during Neuronal Differentiation of Embryonal Carcinoma Cells*

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We examined the regulation of the Na^+/H^+ exchanger gene during differentiation of the P19 mouse embryonal carcinoma cells. Treatment of P19 cells with retinoic acid induces the development of neurons, astroglia, and microglia cells. Upon retinoic acid-induced differentiation of P19 cells, there was an early and rapid 10-fold increase in NHE1 transcription. A proximal cis-acting AP-2 site of the NHE1 promoter was sufficient for stimulation of transcription of the gene by differentiation. Bandshift experiments demonstrated that in retinoic acid-treated cells there was an elevated level of AP-2 transcription factor binding to the AP-2 consensus site of the Na^+/H^+ exchanger gene. In the differentiation defective mutant RAC65, the effect of differentiation on Na^+/H^+ exchanger gene expression was reduced by 60%. Examination of Na^+/H^+ exchanger activity showed that retinoic acid-treated P19 cells recovered from an acid load at a rate approximately three times greater than untreated cells. The increases in gene expression and protein activity preceded major changes in cell morphology, suggesting that the initiation of differentiation is linked to NHE1 gene expression. Our findings show for the first time that the NHE1 gene is activated early in cell differentiation and that this activation may play an important role in the process of neuronal cell differentiation.

The Na^+/H^+ exchanger is a mammalian plasma membrane protein that mediates the exchange of intracellular H^+ ions for extracellular Na^+ ions with a stoichiometry of 1:1. Several isoforms of the protein have been identified which are designated NHE1–4. The NHE1 isoform is present in all mammalian cells and has been described as the housekeeping isoform of the Na^+/H^+ exchanger family (1). NHE1 is involved in pH regulation (2), control of cell volume (3), and is activated by growth factors (4). Although the mechanism of regulation of the protein has received much attention, the recent cloning of both the human and the mouse NHE1 genes have enabled more in-depth study of the promoter activity (5, 6). Specific regions of both promoters have been identified as putative cis-elements that may be responsible for controlling the regulation of the NHE1 gene (6). We have provided direct evidence of a transcription factor that can up-regulate the NHE1 gene. This trans-acting nuclear protein has been identified as the transcription factor AP-2 (5). These findings have shown that the Na^+/H^+ exchanger gene can be up-regulated and suggest that the exchanger performs specific functions separate from its normal housekeeping role.

The Na^+/H^+ exchanger is involved in cell proliferation and differentiation (7). During cell proliferation the exchanger is responsible for an elevation of intracellular pH. In some cell types this pH change has been shown to play an important permissive role in growth (8). Cell differentiation, however, results in more drastic alterations in growth patterns and involves the activation of many genes. How the Na^+/H^+ exchanger gene is regulated and involved during this process remains unknown. Rao *et al.* (7) examined the regulation of Na^+/H^+ exchanger expression and its role in retinoic acid-induced differentiation of HL60 cells. Immediately prior to differentiation into granulocyte-like cells, the activity of the Na^+/H^+ exchanger increases and remains elevated well into differentiation (7, 9). There is an 18-fold increase in NHE1 mRNA levels as well as a 7-fold increase in protein levels (7). This dramatic rise in Na^+/H^+ antiporter levels suggests that Na^+/H^+ exchanger gene expression and differentiation are closely linked.

Retinoic acid-induced differentiation is not specific to HL60 cells. Many murine cell lines can also differentiate when treated with retinoic acid (10). In tissue culture systems the acidic form of vitamin A (retinoic acid) replaces the natural morphogen present during development (11). The effects of retinoic acid are believed to be exerted through a wide variety of receptors and binding proteins. These proteins act as trans-activating factors which can regulate the expression of specific genes (12, 13). The transcription factor AP-2 is one such regulatory protein (14). Retinoic acid causes increases in AP-2 mRNA and protein levels. These increases are believed to be important in initiating differentiation in embryonal carcinoma cell lines such as P19 cells and human NT2 teratocarcinoma cells (14, 15).

P19 cells are a well-established tissue culture model for early embryonic determination and differentiation. These cells are a widely utilized pluripotent cell line able to grow continuously in serum-supplemented media. The differentiation of these embryonal carcinoma cells can be controlled by nontoxic doses of retinoic acid or dimethyl sulfoxide. Treatment with retinoic acid effectively induces the development of neurons, astroglia, and microglia cells normally derived from the neuroectoderm. Six days after initial retinoic acid treatment more than 85% of cells express neuronal markers (16). The transcription factor AP-2 has been implicated in retinoic acid-induced differentiation of P19 cells and in NT2 cells, a similar human teratocarcinoma cell line (11, 14, 15). In this study we examine regulation of the Na^+/H^+ exchanger gene during retinoic acid induced

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differentiation of P19 cells. Because AP-2 has been suggested to be important in both regulation of the Na^+/H^+ exchanger gene (6) and embryonal carcinoma cell differentiation (11, 15) we suspected that AP-2 may be involved in regulation of antiporter expression during differentiation. Our findings provide direct evidence to support the conclusion that the Na^+/H^+ exchanger gene is activated early in P19 cellular differentiation. They suggest that this AP-2-dependent activation may play a key role in the process of retinoic acid induced differentiation in this cell type.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonuclease and DNA-modifying enzymes were obtained from Boehringer Mannheim (Laval, Quebec, Canada) and Life Technologies, Inc. All trans-retinoic acid and G418 were purchased from Sigma. Hybond-N$^+$ nylon membranes were obtained from Amersham (Oakville, Ontario, Canada). [α-^{32}P]dCTP and [δ-^{32}P]dATP were from ICN. The plasmid pTZ 19 was from Pharmacia LKB Biotechnol. All other chemicals were of analytical or molecular biology grade and were purchased from Fisher Scientific (Ottawa, Ontario, Canada), Sigma, or BDH (Toronto, Ontario, Canada).

Cell Culture—P19 mouse embryonal carcinoma cells were obtained from American Type Culture Collection (Bethesda, MD). RAC65 cells were a generous gift from Dr. McBurney from the Department of Medicine, University of Ottawa, Ontario, Canada. Wild type and mutant P19 cells were maintained in α-minimum essential medium supplemented with 2.5% fetal bovine serum and 7.5% calf serum as reported earlier (17). Before transfection cells were plated in 10-cm dishes at a density of 2.5×10^6 cells/ml and incubated for 24 h. After 24 h the medium was changed, the cells transfected, and incubated for 5 h. On completion of the incubation stage, the cells were split into two 10-cm dishes, one treated with retinoic acid and the other left in control medium. For the stable cell line, a slightly different protocol was used to induce a high percentage of the cells to differentiate to neuronal cell types. The cells were aggregated for 4 days in the presence of 5 μM retinoic acid in non-adhesive plates then plated on adhesive substrates in the absence of retinoic acid for another 48 h. NIH 3T3 cells were maintained in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum as described earlier (6).

Reporter Plasmid Construction—pXP-1MP was constructed as described earlier (6). The 1.1-kilobase fragment contains base pairs −1085 to +22 of NHE1. 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 *Hin*dIII generated on either end and was inserted into the *Bam*HI and *Hin*dIII sites of pXP-1. Similarly, pMP-AP2 plasmid was made using the product of the primers 3 and 2 (no. 2, ttgg atc CTG CAC CGC GCG GGC GCT) and the 114-base pair product (−92 to +22) was inserted into pXP-1. To construct p(AP2)$_3$SV, oligonucleotides AP2R1 (5′-GAT C(CT)T TCC TTC CCT GGG CGA CAG GGG CCA-3′) and AP2R2 (5′-GAT C(CT)G GCC CCT GTC GCC CAG GGA AGG AAA-3′) were annealed, ligated together, and then digested with restriction enzymes *Bam*HI and *Bgl*II. The product was size fractionated on a 9% acrylamide gel, and the ligated product of the oligonucleotides was purified and ligated into the *Bam*HI site of PTZ 19. The DNA was removed from PTZ with the restriction enzymes *Sal*I and *Eco*RI. The SV40 promoter was digested from pCAT-promoter plasmid (Promega) using *Eco*RI and *Hin*dIII and was ligated into the corresponding sites of pBluescript SK- (Stratagene, La Jolla, CA). This fragment was removed from pBluescript SK- with the restriction enzymes *Eco*RI and *Hin*dIII. The plasmid pXP-1 was digested with *Hin*dIII and *Sal*I and ligated simultaneously with the two fragments: the *Sal*I and *Eco*RI fragment containing the AP-2 repeats and the *Eco*RI and *Hin*dIII fragment containing the SV40 promoter. The resulting plasmid (p(AP2)$_3$SV) contained three tandem copies of the AP-2 site of the mouse NHE1 promoter located 5′ to the SV40 promoter. All plasmids were sequenced to verify proper orientation and fidelity of polymerase chain reaction.

Transfection and Reporter Assays—Cells were transiently transfected using the calcium phosphate precipitation method as described earlier (6). P19 cells were plated onto 10-cm dishes, and each dish received 10 μg of luciferase reporter plasmid and 10 μg of pSV-β-galactosidase plasmid as an internal control. After cells were incubated for 5 h with the plasmid and the calcium phosphate coprecipitate, they were divided into new plates with a dilution of 1:2 and were treated with 5 μM of retinoic acid or control medium. After 40 h cells were harvested and cell lysates assayed for luciferase activity and β-galactosidase activity. NIH 3T3 cells were transfected as described earlier (6). Stably transfected cells were made using the same calcium phosphate transfection protocol with pRSVneo plasmid (1 μg) and with pMP+AP2 (20 μg). After transfection, cells were incubated for 24 h before being replated at a dilution of 1:10. After replating the cells were treated with 200 μg/ml of G418 for 18 days. G418-resistant colonies were identified, and individual colonies were repropagated until reaching a higher density. They were then assayed for the ability to express luciferase activity. Polymerase chain reaction was used to determine if the entire mouse promoter region from pMP+AP2 had been inserted. The polymerase chain reaction primers amplified a region from the 5′ end of the luciferase gene to the 5′ end of the mouse NHE1 insert. P19 cells stably transfected with the plasmid pMP+AP2 (referred to as P19A2) were used to study cell differentiation. P19A2 cells were aggregated for 4 days on 10-cm non-adhesive type Petri dishes in the presence of retinoic acid, then plated on adhesive substrates for another 48 h. The same number of cells were harvested at various time points throughout the differentiation process and were assayed for luciferase activity. Each luciferase assay contained 40 μl of the cell lysate and 100 μl of the luciferase assay reagent (20 mM Tricine,[1] 1.07 mM magnesium carbonate, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithiothreitol, 470 mM luciferin, 530 mM ATP, 270 mM coenzyme A, and 1 ng/ml bovine serum albumin). The β-galactosidase assay included 50 μl of cell lysate, 50 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 for transiently transfected cells and to the number of cells and protein content for stably transfected cells. Results are reported as mean ± S.E.; where not shown the S.E. was to small to be displayed.

Polyacrylamide Gels and Immunostaining—SDS-polyacrylamide gel electrophoresis was on 9% polyacrylamide gels as described earlier (18). Protein levels from cell extracts were determined using the Bio-Rad DC Protein Assay kit with bovine serum albumin as the standard. Equal amounts of protein were loaded onto the gels, and after separation, proteins were transferred electrophoretically on to nitrocellulose membranes. Immunostaining of nitrocellulose was carried out in the presence of 1% skim milk powder using the neuron-specific class III β-tubulin monoclonal antibody (19). The antibody was detected using the Amersham Enhanced Chemiluminescence Western blotting and Detection System as described by the manufacturer.

Internal pH Measurement—For measurement of intracellular pH P19 cells were grown for 40 h with or without retinoic acid and then trypsinized and resuspended in phosphate-buffered saline. Fluorescence measurements were made on a Shimadzu RF-5000 spectrofluorophotometer. Cells were incubated in a cuvette with constant stirring. The acetoxy methyl ester of 2′-7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM 2 μg/ml) was incubated with cells for 5 min in a solution containing 135 μM NaCl, 5 μM KCl, 1.8 μM $CaCl_2$, 1.0 μM $MgSO_4$, 5.5 μM glucose, 10 μM HEPES, pH 7.4. Intracellular pH was measured utilizing the dual excitation single emission ratio technique. Excitation wavelengths were at 452 and 500 nM with emission at 520 nM. This allows measurement of intracellular pH that is independent of cell concentration and dye loading (20). A calibration curve for intracellular dye was generated by the nigericin method (20). To measure initial rates of proton extrusion after an acid challenge the NH_4Cl prepulse method was used. Cells were incubated with buffer containing 15 μM NH_4Cl for 5 min. They were pelleted (10,000 rpm for 30 s) and then resuspended in Na^+-free buffer that contained 135 mM *N*-methyl-D-glucamine instead of NaCl. The cells were pelleted and resuspended in a solution containing 67.5 μM NaCl (and 67.5 μM *N*-methyl-D-glucamine), and the initial rate of rise of internal pH was measured during the first 40 s of recovery.

DNA Binding Assays—Nuclear extracts were prepared from P19 cells as reported by Schreiber *et al.* (21). 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 which correspond to the AP-2 region of the mouse Na^+/H^+ exchanger promoter. 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 counts/min of γ-^{32}P-labeled oligonucleotides mixed

[1] The abbreviations used are: Tricine, *N*-tris(hydroxymethyl)methylglycine; BCECF-AM, 2′-7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein.

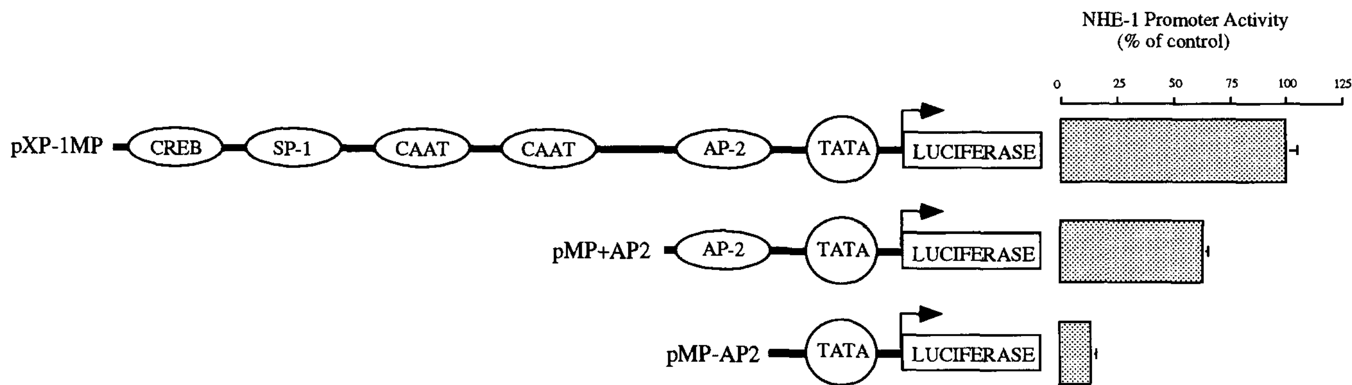

FIG. 1. **NHE-1 promoter activity in mouse P19 embryonal carcinoma cells.** Mouse P19 cells were transiently transfected with either pXP-1MP, pMP+AP2 or pMP-AP2. pXP-1MP contains base pairs −1085 to +22 of the mouse promoter/enhancer region. pMP+AP2 contains the region between −125 and +22, and pMP-AP2 contains the region between −92 and +22. *Ovals* indicate putative binding sites for DNA-binding proteins, SP-1, CAAT, CREB, and AP-2, and the *circle* represents the TATA box. Luciferase activity was corrected for transfection efficiency using cotransfection with pSV-β-galactosidase. For all transfection experiments, the results are mean ± S.E. from at least three independent experiments each carried out in triplicate utilizing at least two different DNA preparations for each plasmid.

with P19 nuclear extract (5 μg) in a binding buffer (4% glycerol, 1.25 μM $MgCl_2$, 0.5 μM EDTA, 0.5 μM dithiothreitol, 50 μM NaCl, 10 μM Tris-HCl, pH 7.5, and 0.05 μg/ml poly(dI-dC)). After electrophoresis on 4% polyacrylamide gels, the gels were dried and exposed to x-ray film for 16 h. In some experiments nuclear extracts were treated to specifically remove AP-2 protein before gel mobility shift assays were performed. Twenty μl of a 10% solution of Protein A was incubated with 1 or 2 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 μM Tris, pH 7.9, 400 μM NaCl, 1 μM EDTA, 1 μM EGTA, 1 μM dithriothreitol, and 1 μM phenylmethylsulfonyl fluoride), mixed with 5 μl of P19 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.

RESULTS

Identification of Regulatory Regions in the Mouse NHE1 Gene—A 1.1-kilobase fragment of the mouse NHE1 gene has recently been cloned, and putative regulatory sites have been identified (6). Initial studies concentrated on identifying the regions of the promoter that are involved in the regulation of the Na^+/H^+ exchanger gene in P19 cells. We constructed several different plasmids containing varying fragments of the promoter/enhancer region. Our initial construct contained the entire 1.1-kilobase mouse promoter/enhancer region located 5′ to a luciferase gene. This plasmid was termed pXP-1MP. From this initial plasmid we deleted base pairs −1085 to −126 (pMP+AP2) and −1085 to −92 (pMP-AP2) to create two new plasmids (Fig. 1). These plasmids were then transfected into P19 cells in an attempt to determine which regions were responsible for regulating the NHE1 gene. All transfection experiments also included pSV-β-galactosidase which was used to normalize for any differences in transfection efficiency. Fig. 1 is a comparison of luciferase activity between the three plasmids. When the region from −1085 to −126 was deleted from the original pXP-1MP construct there was a 37% decrease in the rate of transcription. This decrease indicates that the deleted region must contain some element(s) that contribute to the basal levels of transcription of the NHE1 gene in P19 cells. We then examined the importance of the region immediately 3′ to this deletion. When an extra 33 base pairs were deleted from pMP+AP2 the level of luciferase activity decreased another 62% from that seen with the complete promoter. These 33 base pairs have been shown earlier to contain the binding site for the transcription factor AP-2 and to be involved in NHE1 gene regulation (6).

To examine the direct involvement of the AP-2 site from the mouse NHE1 gene in P19 cells, two new reporter plasmids were constructed. The luciferase gene was linked to a wild type SV40 promoter (pSVLUC) or to the SV40 promoter which contained a triplicated AP-2-binding site derived from the NHE1 gene (*p(AP2)₃SV*, Fig. 2). These plasmids were then transfected into P19 cells with pSV-β-galactosidase. Fig. 2*A* demonstrates the importance of the AP-2 site in the regulation of transcription in P19 cells. In these experiments, $p(AP2)_3SV$ showed a 3.2-fold increase in transcription in comparison to pSVLUC (Fig. 2*A*, *lanes 1* and *2*). This indicates that in P19 cells the AP-2 site from the NHE1 gene can activate a foreign promoter and increase the luciferase levels. Because the two new plasmids differ only in the presence of the NHE1 AP-2 site, we can conclude that this site plays a significant role in the elevated expression of the exchanger gene in P19 cells. This regulation may allow for a constitutively higher level of transcription in P19 cells compared to cells with lower levels of AP-2 protein. The need for increased exchanger promoter activity may be due to the pluripotent nature of P19 cells (17). Higher levels of NHE1 transcription may lead to a parallel increase in protein expression. This overall increase in exchanger protein may be important for the P19 cell to maintain its elevated pH (22). The increase in steady state pH in P19 cells may contribute to producing a permissive environment for protein and DNA synthesis necessary for differentiation (23, 24).

Regulation of the Gene during Retinoic Acid-induced Differentiation—Our hypothesis is that the AP-2 site is involved in regulating the mouse NHE1 gene in differentiating P19 cells. To test this hypothesis, we first needed to rule out any involvement from the region(s) proximal to the AP-2 site. Because this proximal region is involved in regulating basal levels of transcription in P19 cells (Fig. 1), the same region may also be involved in regulating the gene during differentiation. P19 cells were transfected with either pXP-1MP or with pMP+AP2. The cells were then treated with 0.5 μM all-*trans*-retinoic acid for 40 h. This concentration of retinoic acid has been shown to induce differentiation of P19 cells into neurons, astrocytes, and fibroblast-like cells (25). Fig. 3*A* demonstrates the effects of differentiation on the level of transcription of the NHE1 promoter. After 40 h of treatment, the differentiating cells exhibited a 4-fold increase in luciferase activity (Fig. 3*A*). The results show that retinoic acid-induced differentiation results in an increase in transcriptional activity from the mouse NHE1 gene. In addition, because the amount of increase in luciferase activity is the same in both plasmids (*i.e.* pXP-1MP and pMP+AP2) then the region responsible for the increase must not lie within base pairs −1085 to −126. It is for this reason we concentrated further studies on the region of the gene beginning from the

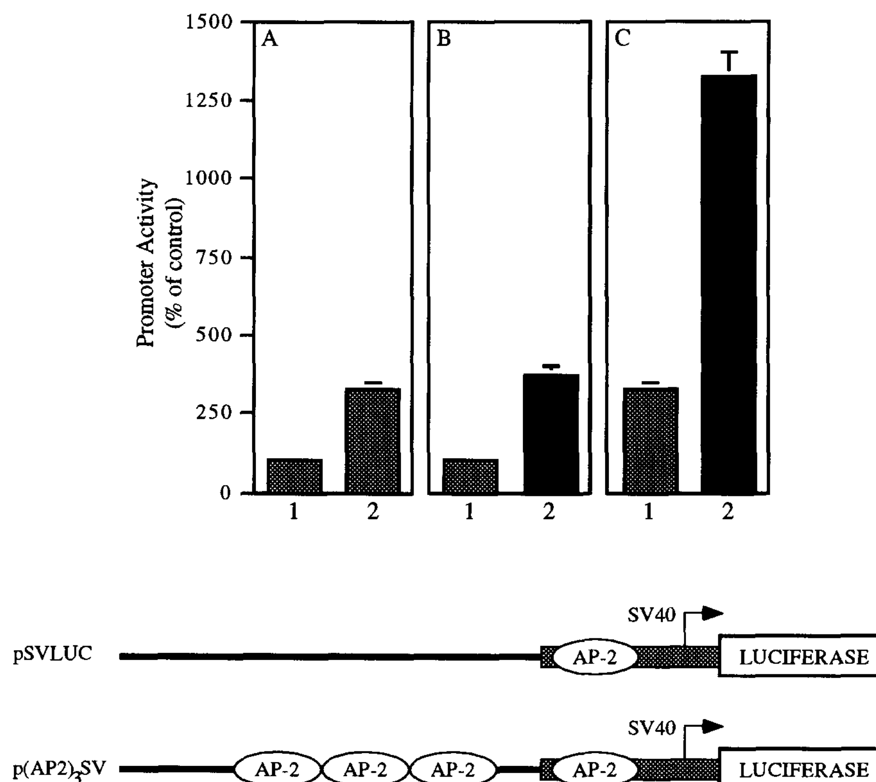

FIG. 2. **AP-2-dependent activation of the SV40 promoter.** Plasmids pSV-LUC and $p(AP2)_3SV$ were transiently transfected into P19 cells and either treated with retinoic acid to induce differentiation or untreated for controls. Corrected levels of luciferase activity are shown. *A*, promoter activity from pSV-LUC (*lane 1*) and $p(AP2)_3SV$ (*lane 2*) in P19 cells. *B*, promoter activity from pSV-LUC in P19 cells (*lane 1*) and in P19 cells that had been treated with 5 μM retinoic acid for 40 h (*lane 2*). *C*, promoter activity from $p(AP2)_3SV$ in P19 cells (*lane 1*) and in P19 cells that had been treated with 5 μM retinoic acid for 40 h (*lane 2*). pSV-β-Galactosidase was used as an internal control for all transfections. For all 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. A schematic representation of the luciferase reporter plasmids used in the experiments is shown. The *dotted box* indicates the SV40 promoter, where the *arrows* denote the direction and start sites of transcription. The *single oval* indicates the SV40 AP-2 consensus sites, and the *three linked ovals* represent three copies of the NHE1 AP-2 consensus site.

AP-2 site to the start site of transcription.

When P19 cells are induced to differentiate with retinoic acid, cells are subjected to two variables. The increase in NHE1 transcription may be directly due to retinoic acid, due to differentiation, or to a combination of the two stimuli. To ensure that a maximal stimulus has been achieved and complete neuronal differentiation has been induced, we increased the concentration of retinoic acid to 5 μM. This concentration has been shown to be sufficient to fully induce neuronal differentiation (26). With higher concentrations of retinoic acid, we observed even higher levels of NHE1 promoter activity. Fig. 3*B* demonstrates that the construct pMP+AP2 now produces a 10-fold increase in luciferase activity with retinoic acid-induced stimulation. The plasmid pMP-AP2, however, produced only a minor, insignificant increase in promoter activity compared with pMP+AP2. The results from Fig. 3*B* confirm that retinoic acid-induced effects on P19 cells are localized to the region known to interact with transcription factor AP-2 (6). Additionally, Fig. 3*B* demonstrates that with the higher concentration of retinoic acid either the cells are stimulated to further differentiate or that retinoic acid itself affects the NHE1 gene.

In an attempt to distinguish direct transcriptional effects of retinoic acid from the effects mediated through differentiation, the P19 mutant RAC65 was utilized. This cell line was derived from a clone which has been selected for its inability to differentiate when exposed to retinoic acid (27). These cells were transfected with pMP+AP2 and pSV-β-galactosidase and then treated either with or without 5 μM retinoic acid. When cells were treated with retinoic acid there was a 4-fold increase in transcription from the NHE1 promoter (Fig. 3*C*, *lanes 1* and *2*). From this, one can conclude that of the 10-fold increase in transcription seen in Fig. 3*B*, approximately 40% is due to direct or related effects of retinoic acid, and 60% is due to effects mediated via differentiation. To confirm that the effect of retinoic acid on the promoter in P19 cells is specific, NIH 3T3 cells were transfected with pMP+AP2 and treated with 5 μM retinoic acid (Fig. 3*C*). Since NIH 3T3 cells do not differentiate, any effect seen with retinoic acid would indicate a direct response of the promoter to retinoic acid. As seen in Fig. 3*C* (*lanes 3* and *4*), there is no change in NHE1 promoter activity. This indicates that the NHE1 gene is not directly responsive to retinoic acid in all cell types, but may be specific to differentiating cells.

Activation of the NHE1 Gene Occurs at an Early Stage of P19 Differentiation—When P19 cells are treated with retinoic acid, a large number of events occur which cause differentiation. Many regulatory genes are expressed in a temporal manner. However, it is not known if a cascade of gene expression occurs (13). How the Na^+/H^+ exchanger fits into the process of differentiation is also not known. Our next experiment set out to determine at what point in differentiation the transcriptional activity of the NHE1 gene is increased. We subjected P19 cells to 5 μM retinoic acid for 40 h as described above. We then analyzed the cellular extracts using Western immunoblot analysis (Fig. 4). An antibody produced against β-tubulin was used as an indicator of differentiation into neuronal cells. As seen in Fig. 4*A*, control cells and cells treated with retinoic acid for 40 h express the same low levels of β-tubulin in the early stages of differentiation. This low level of β-tubulin is produced from the small fraction of P19 cells known to differentiate spontaneously when plated at high concentrations (17). Because retinoic acid-treated cells express the same level of β-tubulin as the controls, it appears that 40 h of retinoic acid exposure is not sufficient to produce fully differentiated cells. This does not mean, however, that the retinoic acid-treated cells have not begun the differentiation process. The effects of retinoic acid have been shown to produce cellular alterations after as little as 2–4 h of exposure (16). In addition we observed dramatic stimulation of the NHE1 gene within this time (Fig. 3). It can therefore be assumed that differentiation has been initiated in retinoic acid-treated cells and it is at this point where the Na^+/H^+ exchanger gene is up-regulated. To confirm that our treatment of P19 cells does result in differentiation, we analyzed cells that had undergone the enhanced neuronal differentiation protocol

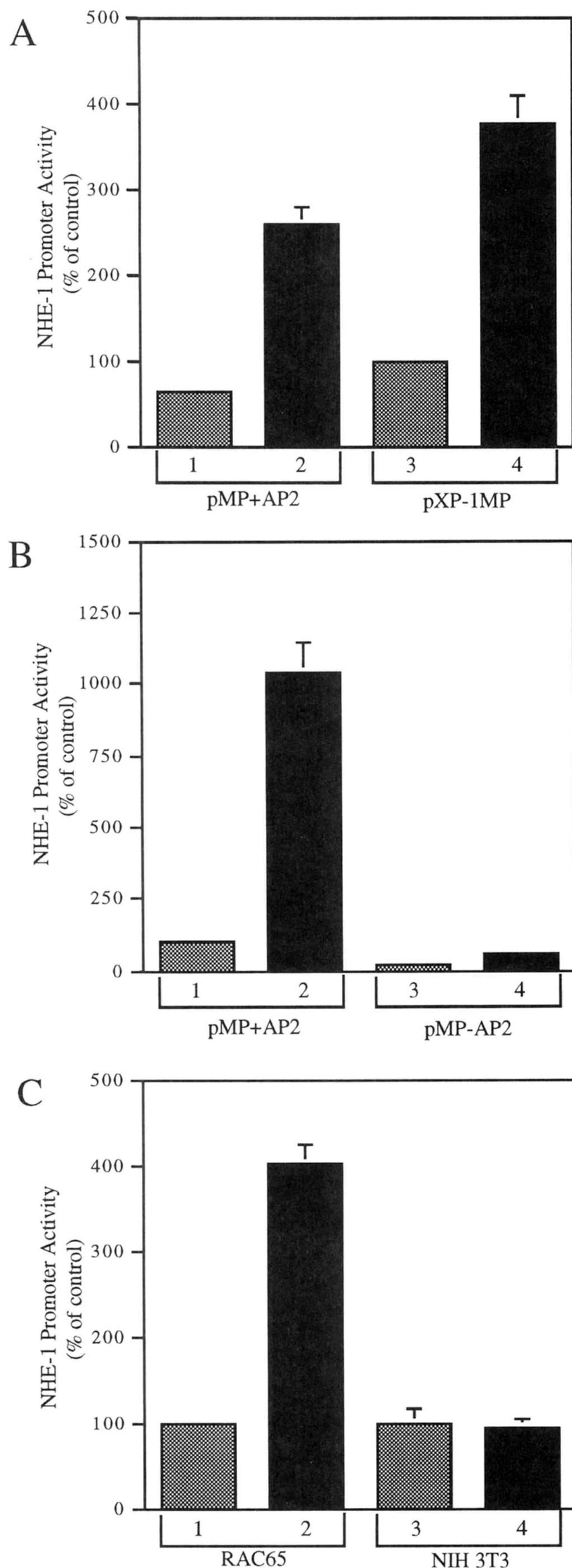

FIG. 3. **Effects of retinoic acid-induced differentiation on transcription by the NHE1 promoter in P19 and RAC65 cells.** *A*, luciferase activity from pMP+AP2 and pXP-1MP in control P19 cells (*lanes 1* and *3*) and cells induced to differentiated with 0.5 μM retinoic acid (*lanes 2* and *4*). *B*, luciferase activity from pMP+AP2 and pMP-AP2 in P19 cells (*lanes 1* and *3*) and cells induced to differentiate with 5 μM retinoic acid (*lanes 2* and *4*). *C*, luciferase activity from pMP+AP2 in untreated RAC65 and NIH 3T3 cells (*lanes 1* and *3*) and in RAC65 and NIH 3T3 cells treated with 5 μM retinoic acid (*lanes 2* and *4*). pSV-β-Galactosidase was used as an internal control. Results are from at least three independent experiments carried out in triplicate using at least two different DNA preparations for each plasmid.

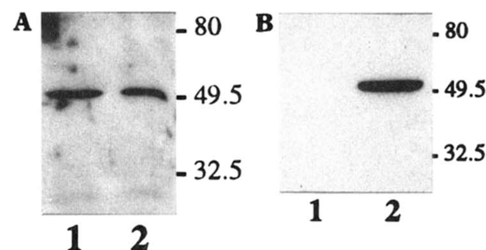

FIG. 4. **Western immunoblot analysis of P19 cells with β-tubulin antibody.** Cell extracts from control and retinoic acid-treated P19 cells (5 μM) were obtained and run on 9% SDS-polyacrylamide gel electrophoresis. After transfer to nitrocellulose, samples were immunostained with class III β-tubulin antibody. *A*, *lane 1* indicates the untreated P19 cells, and *lane 2* represents cells treated with retinoic acid for 40 h. The immunoblot was developed using the Amersham enhanced chemiluminescence kit and was exposed for 2 min to enhance detection of the weak signal. *B*, control and retinoic acid-treated P19 cells immunostained with β-tubulin antibody; *lane 1* indicates control P19 cells, and *lane 2* represents cell extracts treated with retinoic acid for 4 days using the differentiation protocol to enhance development of neuronal cells as described under "Experimental Procedures." The immunoblot was exposed for 10 s.

as described under "Experimental Procedures." The results are shown in Fig. 4*B*. Longer treatment of aggregated P19 cells with retinoic acid resulted in a strong increase in the levels of expression of β-tubulin in comparison to controls.

Because transient transfection of P19 cells only allowed for the study of events occurring early in the differentiation process, a separate procedure was used to examine regulation of the NHE1 gene during the later stages of neuronal differentiation. P19 cells were stably transfected with the plasmid pMP+AP2. This stable cell line (P19A2) was treated to induce a high percentage of the cells to differentiate to neuronal cell types. The cells were aggregated for 4 days in the presence of retinoic acid in non-adhesive plates and then plated on adhesive substrates for another 48 h. This treatment produces cells that begin to lose embryonic carcinoma antigen properties shortly after 48 h (28). By day 6, approximately 85% of the cells begin to express specific neuronal markers (16; Fig. 4*B*). Equal numbers of cells were harvested at various time points during differentiation and assayed for luciferase activity. Fig. 5 illustrates the activity of the NHE1 promoter during this differentiation procedure. Within 48 h the promoter activity increased 19.4-fold, thus confirming similar results seen with the transient transfection protocol. During days 3 and 4 a dramatic decrease in luciferase activity was observed. Since it is not until after this time that most of the cells have begun expressing neuronal markers, it can be concluded that the initial increases in NHE1 promoter activity are not due to changes in cell type. The alterations in promoter activity must therefore be a result of early events involved in differentiation. This result suggests that there may be a need to alter intracellular pH or change the capacity to regulate intracellular pH at a very early stage in differentiation.

To assess if the activity of the Na^+/H^+ exchanger protein increases during differentiation, we measured the activity of the exchanger in both control P19 cells and P19 cells treated

with retinoic acid to induce differentiation (Fig. 6). The control and the retinoic acid-treated cells were subjected to an acute acid load induced by ammonium chloride. Fig. 6A shows a tracing of a typical experiment for P19 cells. The rate of recovery from ammonium chloride-induced acid load of the retinoic acid-treated cells is superimposed. It is apparent that the retinoic acid-induced differentiating cells recovered at a much greater rate than the P19 control group. The summary of the differences in the initial rate of recovery for an acid load is shown in Fig. 6B. The retinoic acid-treated cells recovered at a rate approximately three times greater than untreated cells. The recovery from acid load was inhibited by the amiloride analog hexamethylamiloride (not shown), indicating Na^+/H^+ exchanger mediated pH changes. This suggests that there is a significant increase in Na^+/H^+ exchanger activity in differentiating P19 cells. Because the Na^+/H^+ exchanger in P19 cells has previously been shown to be constitutively active and unaffected by extracellular stimuli (22), the most plausible explanation is that increased promoter activity results in elevated exchanger levels.

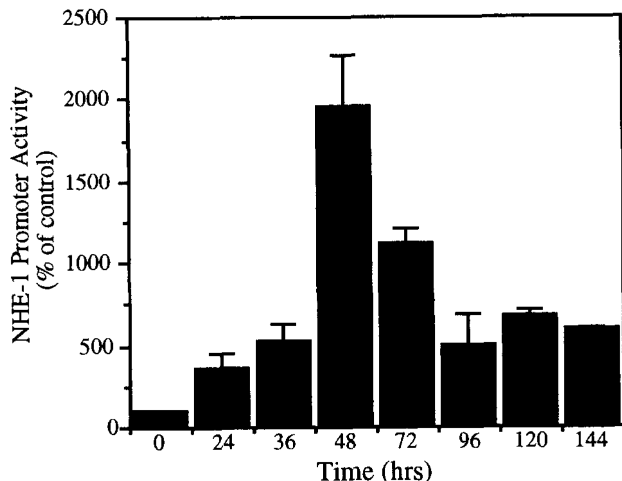

FIG. 5. **NHE1 promoter activity during neuronal differentiation of P19 cells.** P19 cells were stably transfected with the NHE1 promoter plasmid, pMP+AP2, and the neomycin gene (pSVneo). The stable transformant (P19A2) cells were then incubated with 5 μM retinoic acid for 4 days and treated to enhance neuronal differentiation as described under "Experimental Procedures." After 4 days of retinoic acid treatment, the cells were then plated and incubated for 2 days further in the absence of retinoic acid. Equal numbers of cells were harvested at time 0 and after 24, 36, 48, 72, 96 120, and 144 h.

The Role of Transcription Factor AP-2 in the Regulation of the NHE1 Gene during Retinoic Acid-induced Neuronal Differentiation—The activity of the NHE1 promoter has been shown to increase upon stimulation of P19 cells with retinoic acid. The large increase in transcription can be attributed in part to a 33-base pair region located at −125 to −93 of the mouse NHE1 gene. This region has previously been shown to bind AP-2 protein and regulate the Na^+/H^+ exchanger gene (6). Because the levels of AP-2 protein have been shown to increase in P19 cells with retinoic acid-induced differentiation (15), we investigated the role of AP-2 in regulating NHE1 during this time. The constructs pSVLUC and $p(AP2)_3SV$ were used to demonstrate the importance of AP-2 in regulating differentiation. Examination of the sequence of the SV40 promoter shows the presence of one AP-2 like site. The plasmid containing the SV40 promoter (pSVLUC) showed a 3.7-fold increase in transcription when the cells are induced to differentiate (Fig. 2B). This result indicates that an independent viral promoter that contains its own AP-2 site is also responsive to retinoic acid induced differentiation. This finding is of particular importance because it indicates that the AP-2-dependent transcriptional stimulation observed during retinoic acid-induced differentiation may not occur exclusively in the NHE1 gene but may occur in other genes. We showed that the insertion of three tandem repeats of the NHE1 AP-2 site into pSVLUC ($p(AP2)_3SV$) results in an increase in basal levels of transcription in P19 cells (Fig. 2A). This increase may be due to the increased copies of the AP-2-containing region responding to basal levels of AP-2 protein in the cell. When $p(AP2)_3SV$ is transfected into P19 cells and the cells are induced to differentiate with retinoic acid, we demonstrate a large increase in promoter activity. The increase is approximately 4-fold greater than untreated $p(AP2)_3SV$ and over 13-fold greater than the untreated levels of transcription from the plasmid pSVLUC (Fig. 2, B and C). The absolute level of stimulation of luciferase activity from $p(AP2)_3SV$ in the presence of retinoic acid is also much greater than that of pSVLUC stimulated with retinoic acid (Fig. 2, B and C). It appears that the repeated AP-2 site from the NHE1 gene can

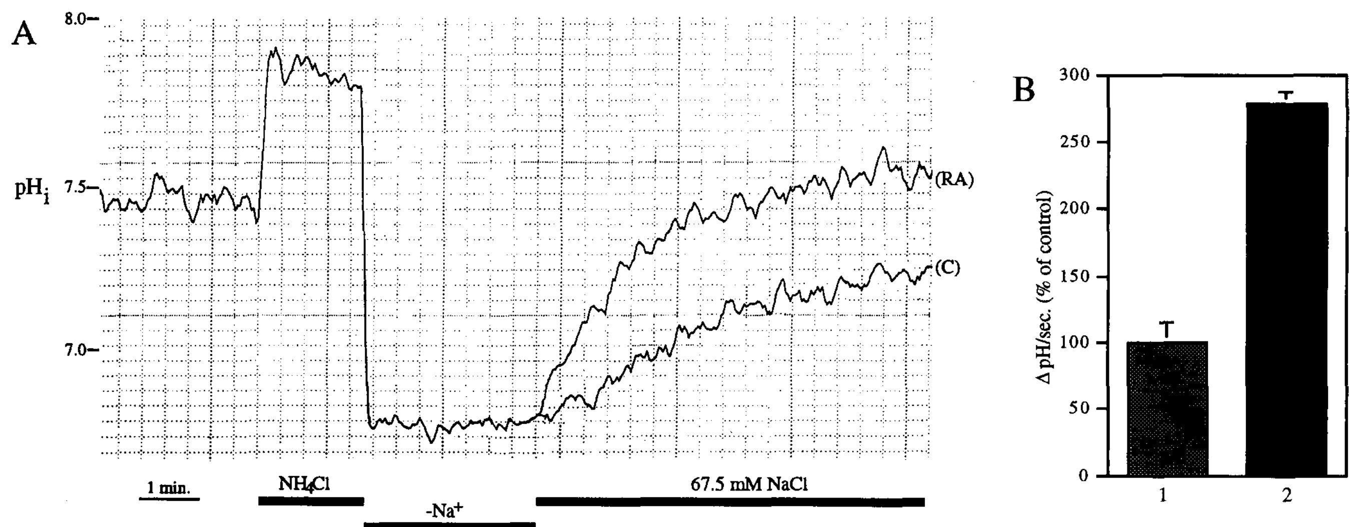

FIG. 6. **Recovery from an acute acid load by control and retinoic acid-treated P19 cells.** *A*, cells were prepared and intracellular pH was measured as described under "Experimental Procedures." Ammonium chloride prepulse was used to induce acute acidosis. The intracellular pH and recovery of control cells from an acute acid load in 67.5 mM NaCl are shown. The recovery is also shown from the retinoic acid-treated cells. *B*, P19 cells (*lane 1*) and retinoic acid-treated cells (*lane 2*) were examined as described in *A*. The initial rate of recovery in the presence of 67.5 mM NaCl and 67.5 mM *N*-methyl-D-glucamine is shown. Each group represents the means ± S.E. least six independent experiments.

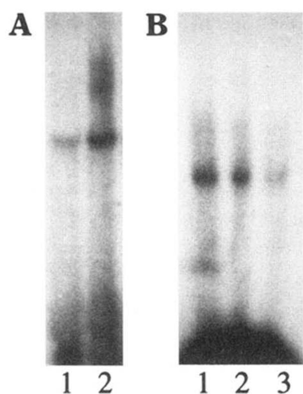

FIG. 7. **DNA mobility shift binding assay of the mouse NHE-1 AP-2 site in control and retinoic acid-differentiated P19 cells.** The labeled 24-base pair oligonucleotide MPAP2a,b (positions −117 to −94) was incubated for 10 min at room temperature with nuclear extracts from control or retinoic acid-treated P19 cells. The binding mixtures were analyzed by electrophoresis on 4% polyacrylamide gels as described under "Experimental Procedures." *A*, *lane 1* contains 5 μg of nuclear extract from control P19 cells; *lane 2* contains 5 μg of nuclear extract from retinoic acid-treated P19 cells. *B*, DNA mobility shift binding assay was performed with nuclear extracts from retinoic acid-treated P19 cells that were extracted via immunoprecipitation with either 2 μg of irrelevant IgG (*lane 1*), 1 μg of anti-AP-2 antibody (*lane 2*), or 2 μg of anti-AP-2 antibody (*lane 3*).

further stimulate transcriptional activity in P19 cells in response to retinoic acid-induced differentiation. This shows that the increased number of AP-2 sites can respond to retinoic acid stimulation with greater effectiveness in comparison to the SV40 promoter alone.

Bandshift experiments were used to confirm the identity of the transcription factor regulating the NHE1 gene during retinoic acid-induced differentiation. We used a 24-base pair double-stranded oligonucleotide (MPAP2a,b) which was previously shown to bind specifically to purified AP-2 protein and AP-2 protein from NIH 3T3 nuclear extracts (6). Nuclear extracts from both control and retinoic acid-treated P19 cells were isolated and incubated with the oligonucleotide MPAP2a,b. AP-2 protein from nuclear extracts of treated and untreated P19 cells caused a shift to occur. Nuclear extracts from the retinoic acid-induced cells, however, produced a considerably larger increase in the level of shifted oligonucleotide (Fig. 7*A*). This indicates an increase in the amount of AP-2 protein binding to the oligonucleotide compared to the amount seen with the control P19 cells. This agrees with the recent study indicating increased AP-2 protein levels in retinoic acid-induced differentiating P19 cells (15). To confirm that the shifted band was due to AP-2 protein, AP-2 was specifically removed from the nuclear extracts by immunoprecipitation with anti-AP-2 antibody (Fig. 7*B*). Nuclear extracts from retinoic acid-treated P19 cells were immunoprecipitated with either 2 μg of irrelevant IgG (*lane 1*), 1 μg of anti-AP-2-containing IgG (*lane 2*), or 2 μg of anti-AP-2-containing IgG (*lane 3*). Control IgG had no effect on the binding of AP-2 to the oligonucleotide whereas anti-AP-2 antibody greatly reduced binding to the oligonucleotide in a dose-dependent manner. This indicates that AP-2 protein from P19 cells binds to the oligonucleotide MPAP2a,b and produces the resulting shift. These results provide strong evidence to support the conclusion that the transcription factor AP-2 is increased in differentiation of P19 cells and that this increase acts to regulate the NHE1 gene during cellular differentiation.

DISCUSSION

Differentiation of mammalian cells is a complicated process involving many steps and the complex regulation of gene expression. It has been possible to study elements of neuronal differentiation with the advent of specific teratocarcinoma cell lines such as P19. In these embryonal carcinoma cells, the expression of several key regulatory genes occurs in an appropriate defined sequence during retinoic acid-induced differentiation (13). Regulatory proteins involved in the differentiation into neurons include the retinoic acid receptors, epidermal growth factor receptor, and the transcription factors Oct-3 and Brn-2. Recently, the transcription factor AP-2 has been shown to increase during differentiation of P19 cells and during differentiation of a similar human teratocarcinoma cell line (11, 14, 15). This suggests that AP-2 may play an important role in regulation of neuronal differentiation. We have earlier shown that AP-2 is important in regulation of NHE1 transcription (6). It has also been demonstrated that there is a dramatic increase in NHE1 message levels in retinoic acid-induced differentiation of HL60 cells (7). Therefore, we suspected a close relationship between retinoic acid-induced cellular differentiation, the Na^+/H^+ exchanger, and the transcription factor AP-2.

The results of our present study confirm that differentiation of P19 cells is linked with Na^+/H^+ exchanger expression. Transcription of the Na^+/H^+ exchanger is dramatically increased during neuronal differentiation of P19 cells. When RAC65 cells were stimulated with retinoic acid the response of the NHE1 promoter was attenuated by 60% in comparison to P19 cells. This cell line is believed to posses a dominant repressor mutation of the retinoic acid receptor α gene (27). The mutation blocks the process of differentiation and may block the actions of all three classes of retinoic acid receptor α, β, and γ (13). Because the NHE1 gene was only partially activated in RAC65 cells this suggests that the increase comes about as a result of two components of retinoic acid stimulation. One of these stimuli is retinoic acid, and the other is the cellular process of differentiation. Differentiation may act to activate NHE1 expression through other trans-acting elements downstream of retinoic acid. Clearly at least one of these downstream elements activated by retinoic acid is the transcription factor AP-2. The response of the NHE1 gene to retinoic acid was eliminated when the AP-2 containing region was deleted. Additionally, insertion of the AP-2 containing region into a foreign promoter resulted in a similar response to retinoic acid stimulation. With the stimulation of P19 cells by retinoic acid, we have also shown an increase in the amount of AP-2 protein binding to this region. This may be due to elevated AP-2 levels arising from increased transcription, similar to what has been reported in P19 cells and in other teratocarcinoma cell lines with retinoic acid treatment (14, 15). An alternative, yet less likely scenario, suggests that the increase in AP-2 binding may be due to increased phosphorylation of the transcription factor thus allowing for increased DNA binding properties. In either case, the net effect is a stimulation of transcription from the NHE1 promoter. It may be that short term initial effects of retinoic acid are mediated through phosphorylation of AP-2 and that other elements of stimulation require elevation of AP-2 levels. Retinoic acid has been shown to stimulate rapid phosphorylation of several proteins upon administration to HL60 cells (29, 30). In addition it has been suggested that the transcription factor AP-2 may be regulated by phosphorylation in some cell types (31). Future experiments will investigate these possibilities.

In intact RAC65 cells, the response to retinoic acid was attenuated. However, the cells still responded with a 4-fold increase in NHE-1 transcription. Because the response of the Na^+/H^+ exchanger gene was only partially attenuated there must be some other mechanism by which retinoic acid stimulation activates this gene without the process of differentiation and the resulting cascade of cellular events. At the present

time the mechanism by which this occurs is not known. It is possible that retinoic acid acts acutely to regulate the NHE1 gene by a mechanism such as phosphorylation of AP-2 and that differentiation is required for further activation. It has yet to be determined whether this is what occurs in retinoic acid stimulation of RAC65 cells.

We also found that activation of the NHE1 promoter is an early specific event in retinoic acid stimulation of neuronal differentiation of P19 cells. Retinoic acid treatment of P19 cells or RAC65 cells resulted in large increases in NHE1 activity after only 40 h but had no effect on NIH 3T3 cells. The effects on P19 cells occurred before marked changes in neuronal markers such as β-tubulin. The results obtained from the stable cell line P19A2 confirm this observation. The peak activation of the NHE1 promoter occurred within 48 h. This observation suggests that NHE1 promoter activation may be an early event leading to and important in differentiation.

The role of the Na^+/H^+ antiporter in cellular differentiation remains controversial. Earlier studies have shown that intracellular alkalinization accompanies differentiation (32). It has been suggested that there is a causal link between increased antiporter activity and subsequent differentiation (33, 34). Alternatively, the increased antiporter activity may be involved in protection of the cell from acidosis produced during respiratory burst activation (9). However, it has been suggested that in HL60 cells the activation and increased activity of the Na^+/H^+ exchanger is not a requirement of differentiation (35). The exact role of the protein and its increased expression in differentiation still has to be elucidated. It seems very unlikely that a mechanism of large increases in activity and expression of the protein evolved without significant physiological function. The loss of the absolute requirement for the Na^+/H^+ exchanger in HL60 cells may be the result of transformation of these cell lines (35). In addition, it could be due to differences between cells grown in culture as opposed to cells growing *in vivo*. It has been shown that amiloride analogs that specifically inhibit the Na^+/H^+ exchanger may be useful *in vivo* in inhibition of some types of tumor cell proliferation (36). The exact role of the increased expression of the Na^+/H^+ exchanger in cellular differentiation *in vivo* has yet to be defined.

In addition to activation of the gene, our results show that the activity of the Na^+/H^+ exchanger protein is increased in cells treated with retinoic acid. In undifferentiated P19 cells, the Na^+/H^+ exchanger protein has been shown to be constitutively activated and unaffected by extracellular stimuli (22). In addition, retinoic acid stimulation of HL60 cells does not appear to act by increasing activity of the Na^+/H^+ antiporter directly. Stimulation by retinoic acid resulted in decreased phosphorylation of the protein despite a rise in internal pH (37). Because differentiation may require either intracellular alkalinization or increased Na^+/H^+ exchange capacity, it may be that the mechanism to fulfill this requirement is increased Na^+/H^+ exchanger gene expression followed by increased protein expression and activity. The early activation of transcription of the gene followed by its decline after the differentiation process suggests that there is an important role in the process of differentiation itself. Future studies will examine the function of the Na^+/H^+ exchanger in this process.

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