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COUP-TFI and COUP-TFII regulate expression of the NHE through a nuclear hormone responsive element with enhancer activity

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The chicken ovalbumin upstream promoter-transcription factors (COUP-TFs) are orphan receptors involved in regulation of embryonic development and neuronal cell fate determination. We identified a target of COUP-TF involved in cell proliferation and cell differentiation. Using reporter assays, footprint analysis, and electrophoretic mobility shift assays, we showed that a nuclear hormone-responsive element located at −841/−800 nt of the mouse Na^+/H^+ exchanger (NHE) promoter binds COUP-TF with enhancer activity. Mutation at −829/−824 nt (and secondarily at −837/−833) prevents COUP binding and activation of the NHE promoter. *In vivo* expression of COUP isoforms in NIH 3T3 or CV1 cells transactivates from the nuclear hormone-responsive element and from the entire NHE1 promoter. Transactivation is greater for COUP-TFII, is increased for either COUP isoform by the presence of high serum concentrations, and is greatly reduced by mutations preventing COUP binding. *In vivo* COUP expression in NIH 3T3 cells results in increased synthesis of NHE. Expression of COUP-TFII induced by either retinoic acid or dimethyl sulfoxide in differentiating P19 cells increases NHE expression. The results show that COUP-TF regulates expression of the NHE and provide a mechanism that may be important in physiological and pathological situations linked to its upregulation.

Keywords: chicken ovalbumin upstream promoter (COUP); NHE; regulatory element; serum; transcriptional regulation.

The Na^+/H^+ exchanger (NHE) is a mammalian plasma-membrane protein that exchanges an intracellular H^+ for an extracellular Na^+. Several isoforms of the protein have been identified and designated NHE1–NHE6. The isoform NHE1 is present in all mammalian cells [1], is involved in pH regulation [2] and control of cell volume [3], and is activated by growth factors [4]. The NHE is known to influence cell growth and differentiation and has recently been shown to be critical to neuronal cell growth and differentiation [5–7].

The mechanisms involved in long-term regulation of the Na^+/H^+ antiporter have only recently started to be studied. Considering its important role in cell growth and differentiation, it is not surprising that its expression is regulated during development and differentiation [5,8]. Several putative proximally acting elements are involved in regulation of basal NHE1 expression including AP-1, AP-2 and C/EBP [9–13]. Recently we showed that a more distal region of the mouse *NHE1* promoter is significant in moderating NHE expression during cell growth and proliferation [14]. We found that a variety of mitogenic factors activated the *NHE1* promoter. Promoter-deletion analysis and electrophoretic mobility-shift assays (EMSAs) showed that a region −1085 to −800 nt from the start site was involved in mediating these effects.

In this communication, we define the elements involved in this regulation of NHE expression. We identify an enhancer element at −841 to −800 nt that binds chicken ovalbumin upstream promoter transcription factor I and II (COUP-TFI and COUP-TFII) and regulates NHE expression [15]. COUP-TFI and COUP-TFII regulate NHE expression from this distal region of the promoter and not within other regions of the *NHE1* gene. We demonstrate that COUP-TF has a biological role in transactivation of the *NHE1* promoter and regulates *NHE1* mRNA expression in both NIH 3T3 and P19 cells. The results provide a mechanism by which COUP-TF may influence cell growth and differentiation, through the NHE.

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Abbreviations: COUP-TF, chicken ovalbumin upstream promoter transcription factor; EMSA, electrophoretic mobility-shift assay; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; NHE, Na^+/H^+ exchanger; NHR, nuclear hormone receptor; TK, thymidine kinase.

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MATERIALS AND METHODS

Materials

DNA-modifying enzymes were from New England Nuclear, Mississauga, Ontario, Canada, Boehringer-Mannheim (Laval, Quebec, Canada) and Bethesda Research Laboratories (Gaithersburg, MD, USA). pGEM, pSP and pGL plasmids were from Promega, Madison, WI, USA. pTK 81 and pTK 40-CAT were gifts from R. Rachubinski, University of Alberta and L. Belanger, University Laval, Quebec, respectively. [α-^{32}P]dCTP and [^{3}H]thymidine were purchased from ICN Biomedicals (Ottawa, Ontario, Canada). All other chemicals were of analytical or molecular biology grade and were purchased from Fisher Scientific (Ottawa, Ontario, Canada), Sigma (St Louis, MO, USA) or BDH (Toronto, Ontario, Canada). Vectors for

in vitro and mammalian expression of transcription factors have been described: human COUP-TFI, a version truncated in its first 51 amino acids (t-COUP-TFI), rat COUP-TFII, human RXRα, human rat PPARα, rat HNF4α, rat LXRα, rat RZRα [16,17], RARα and RARβ, [18], human mCAR [19], human GRα [20] and mouse CREB [21].

Cell culture, transfection, reporter assays, measurement of *NHE1* mRNA levels, and immunological assays

CV1 cells were obtained from M. Nemer of the Clinical Research Institute of Montreal, Canada. NIH 3T3 and CV1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg penicillin G/streptomycin·mL^{-1}. P19 cells were maintained as described previously [22]. Treatment of P19 cells to promote differentiation with retinoic acid (1 μM) or Me_2SO (1%) was as described previously [22,23]. Cells were transiently transfected with purified plasmids (Qiagen, Chatworth, CA, USA) at 50% confluence using $CaPO_4$ precipitation as described previously [9,14]. For transient transfections of cells, 5 μg reporter plasmid was used unless otherwise noted. After transfection, cells were incubated in medium containing 0.5% or 10% fetal bovine serum or charcoal-filtered delipidated bovine serum without phenol red (Sigma). At 36 h after transfection, cells were harvested and cell lysates assayed for protein concentration, luciferase and β-galactosidase activities as described previosuly [14]. The expression vectors for transcription factors (0.25–2.5 μg per plate) were added concomitantly to the promoter–reporter vectors (2.5 μg per plate). Total RNA was extracted with Trizol reagent (Gibco–BRL) from mammalian cells transfected with the expression vectors pSG5 or pCOUP-TFII-SG5 (10 μg per T75 flask). After DNAse treatment, phenol/chloroform extraction, and reprecipitation, polyadenylated RNA was isolated using oligo(dT) magnetic beads (Dynal, Oslo, Norway) according to the manufacturer's instructions. For comparison of mRNA expression, mRNA was reverse transcribed into cDNA using immobilized oligo(dT) as a primer (0.25 μg mRNA·100 μL^{-1} beads in a 50-μL reaction volume). Competitive PCR amplification of the NHE1 message as well as of a control cDNA [glyceraldehyde-3-phosphate dehydrogenase (G3PDH)] was as described previously [14]. When antibody to COUP-TFII was used, Western blots and immunoprecipitation with Protein A Dynabeads coupled to IgG were according to the manufacturer's instructions (Santa Cruz, San Diego, CA, USA and Dynal, Oslo, Norway).

Construction of plasmids

Synthetic oligonucleotides of the sequence 5′-CAAAGTG-AGTTCTAGACCAGGC-3′ (MP286PCR) and 5′-GCATT-CTAGTTGTGGTTTGTC-C′ (5′pXP-1) were used to amplify nucleotides −1085 to −800 of the *NHE1* promoter plus a small fragment of the vector pXP-1 as described previously [14]. The −1085 to −800-nt fragment was digested with the *Pst*I to remove sites from pXP-1 immediately upstream of the *NHE1* sequence. This modified product was then subcloned into the *Pst*I–*Eco*RV sites of pGEM5ZF+ (Promega Corp) to create −1085/−800pGEM5ZF+.

For analysis of reporter activities, the nucleotides −1085 to −800 from −1085/−800 nt GEM5ZF+ were transferred via pTZ19R (Pharmacia, Baie d'Urfe, Quebec, Canada) (*Pst*I–*Sph*I) into PTK81-*luc* [*Bam*HI(*Bam*)–*Hin*dIII] to create the plasmid −1085/−800PTK81-*luc*. PTK81-*luc* is a luciferase reporter plasmid containing the minimal promoter nucleotides −81 to +52 of thymidine kinase (TK) [16]. The blunt −1085/−800-nt sequences were also inserted upstream of a construct of the minimal endogenous NHE1 mouse promoter (Blunt *Bam* site/−92+24NHE1-*luc*); this procedure created −1085/−800 −92/+24 NHE1-pXP1-*luc*. The minimal *NHE1* promoter construct was previously referred to as pXP-AP2 [9]. The −1085/−800 element was also inserted as a single or multiple tandem copies (two and four elements) into the reporter plasmid pGL3-SV40 MP-*luc* (*Xho*I–*Sac*I), a luciferase reporter under the control of the simian virus 40 minimal promoter (SV40MP). To make this construct, linked copies of the −1085/−800 element were prepared from −1085/−800 pTK81-*luc* (*Sal* I) and shuttled via pBS-SK$^+$ (*Sal* I) into pGL3-SV40MP-*luc* (*Xho*I–*Sac*I).

A series of vectors were engineered using various fragments of the −1085/−800-nt element fused to the plasmid pGL-SV40MP-*luc*. These vectors were: pGL −1058/−857-SV40MP-*luc*, pGL −858/−800-SV40MP-*luc*, pGL −1085/−916-SV40MP-*luc*, pGL −918/−824-SV40MP-*luc*, pGL −1085/−924-SV40, pGL −923/−800-SV40 and pGL −841/−800-SV40MP-*luc*. Numbers preceding SV40MP indicate the region of the promoter inserted. The abbreviation FI after a restriction enzyme indicates that a particular restriction site was filled in using standard molecular-biological techniques for the purpose of blunt-end ligation. The starting material to subclone the subregions was *Pst*I–*Sac*II fragments of the −1085/−800 element in plasmid −1085/−800pGEM5ZF+ or *Hin*dIII fragments of the same element inserted into pBS-SK+. −1058/−857 *Mse*I and −858/−800 *Mse*I–*Sac*II, derived from these initial vectors, were shuttled via pGEM5F+ (*Nde*I and *Nde*I–*Sac*II), into pGL-SV40MP-*luc* (*Sac*I–*Nhe*I and *Sac*I–*Xho*I). −1085/−916 *Hin*dIII–*Dde*I(FI), −1085/−924 *Hin*dIII–*Eco*57I(FI), −923/−800 *Eco*571(FI)–*Hin*dIII and −841/−800 *Asp*I(FI)–*Hin*dIII were shuttled via pBS-SK+ (*Hin*dIII–*Eco*RV) into pGL-SV40MP-*luc* (*Kpn*I–*Bgl* II for the first two fragments, *Sac*I–*Xho*I for the last two). −918/−822 *Dde*I(FI) was shuttled via pBS-SK+ (*Eco*RV) into pGL-SB40MP-*luc* (*Sac*I–*Xho*I).

Additional vectors derived from wild-type and mutant oligonucleotides spanning the −841/−800-nt *NHE1* region were engineered as follows. Double-stranded oligonucleotides with either B 5′ ends (upper strand) or *Bgl* II 5′ end (lower strand) were subcloned into pSP73 (*Bam*). After determination of orientation, *Bam*–*Bgl* II inserts with one and four copies in tandem were subcloned into pXP1 −92/+24 NHE1 (*Bam*) or pTK −40/+51 *Luc*, a TK-derived plasmid engineered from pGL3 and pTK −40/+51 CAT. For the latter purpose, pSP73 inserts were shuttled via pSKII+ (*Bam*) into pTK −40/+51 *Luc* (*Nhe*I–*Xho*I). Additional constructs included the −1085/+24-nt region of the mouse *NHE1* gene in pXP1 [14]. The −1085/−800-nt region of the enhancer was also inserted as a single copy upstream of the TK promoter (*Kpn*I–*Xho*I) and into pXP1 −92/+24 NHE1 (*Bam*).

Standard and positive control reporter plasmids for transfection experiments were pSV40-βGAL (Promega) and pGL3-SV40 promoter/enhancer-*luc* (Promega). Negative control plasmids were the vectors used for subcloning pXP1 [24] and pGL3-*luc* (Promega). In addition, the −1085 to −800-nt *NHE1* sequences were also inserted into the *Xho*I–*Sac*I sites of the basic pGL3, yielding the plasmid pGL −1085/−800-*luc*. The approach used was identical with that described for the insertion of these sequences into pGL-SV40 MP-*luc*. Standards and positive control plasmids for transfection experiments were pSV40-βGal and pGL3-SV40 promoter/enhancer-*luc*. Transfection experiments were repeated at least eight times and results shown are mean ± SD.

Footprint analysis

Nuclear extracts from mouse liver and NIH 3T3 cells were prepared as described [25]. The probes used for footprinting were labeled by filling in with [α-^{32}P]dCTP and the Klenow fragment of *Escherichia coli* DNA polymerase I at the *Xba* or *Hin*dIII sites depending on the instance. DNA probes (50 000 c.p.m. per reaction) were incubated for 15 min at room temperature with or without 50 μg of nuclear extract from mouse liver or NIH 3T3 cells, in the presence of 5 μg poly(dI-dC) and BSA (25 μg). DNAse I digestion was carried at various concentrations (3–0.01 U·100 μL^{-1}), as indicated in the legend to Fig. 3, for 2 min in the absence of nuclear extracts or for 3 min in their presence. Other conditions were as described previously [26].

DNA-binding assays

Nuclear extracts were prepared from NIH 3T3, P19 or CV1 cells or mouse liver as described previously [26,27]. Wild-type synthetic oligonucleotides (with added *Bam* or *Bgl* II 5′ ends) or mutants of the −841/800-nt NHE1 region were used after annealing and labeling with Klenow and [α-^{32}P]dCTP (see Fig. 7 for detailed nucleotide sequences). DNA-binding reactions were at room temperature, with 5 μg NIH 3T3 nuclear extracts in binding buffer (5% glycerol, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, 20 mM Tris/HCl, pH 7.0) and in the presence of 1 μg poly(dI-dC), 0.1 μg carrier DNA (salmon sperm DNA) and 5 μg BSA. Then 0.5 ng α-^{32}P-labeled oligonucleotides were mixed for 20 min at room temperature with or without unlabeled competitors (tested at molar fold excesses ranging from 10 to 100). The competitor oligonucleotides were previously characterized elements including site A of the human antithrombin III promoter (ATIII-A) [26], the peroxisome proliferator responsive element of the rat hydratase dehydrogenase gene [16], the mouse cellular retinol-binding protein 1 responsive element (CRBP1-RE) [28], or binding sites for HNF4α, COUP-TF, βRARE, C/EBP, HNF3, TRα (palindromic element) [26,29] and GR-1 [20]. Binding sites of other factors (CREB, AP1, AP2, NFKB) were obtained commercially (Santa Cruz). For supershift assays, 1 μL antiserum or 0.5 μg IgG fraction was added for 30 min after probe addition (unless otherwise indicated). Conditions for electrophoresis and autoradiography were as described previously [14]. *In vitro* transcription-translation assays were with a rabbit reticulocyte lysate system (Promega). The efficiency of the reaction was judged by SDS/PAGE of aliquots translated concomitantly with L-[^{35}S]methionine. EMSAs used 3 μL programmed or unprogrammed lysate.

RESULTS

The −1085/−800-nt serum response element is a modular enhancer

When single or multiple copies of the −1085/−800 element were inserted into reporter plasmids, a progressive increase in luciferase expression was observed (Fig. 1). For example, when one, two, or four tandem copies of the −1085/−800 NHE1 element were inserted upstream of the SV40 minimal promoter there was a 2.8-fold, 9.5-fold and

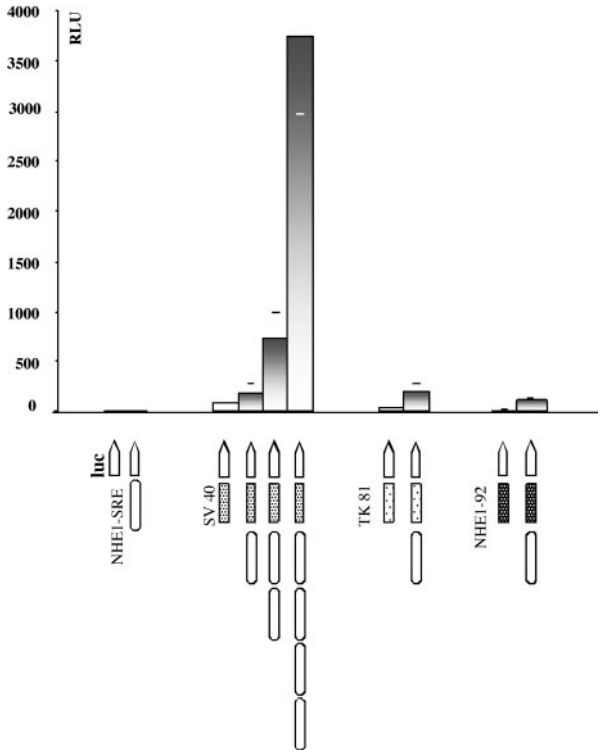


Fig. 1. Enhancer activity of the −1085/−800 element of the NHE promoter. NIH 3T3 cells were transiently transfected in 10% serum with luciferase reporter plasmids containing no promoter, or the minimal promoters of SV40, TK and the NHE (nt −92/+24). One, two or four copies of the −1085/−800 *NHE1* element were inserted upstream of the minimal promoters in a head to tail orientation where indicated. The plasmids used in order of appearance in the figure were pGL3, pGL −1085/−800, pGL3-SV40MP and pGL −1085/−800-SV40MP, pGL −1085–800 (× 2) -SV40MP, pGL −1085/−800 (× 4) -SV40MP, pTK81, pTK81 −1085/−800, pXP −92/+24 and pXP −1085/−800 NHE1 −92/+24. Abbreviations/illustrations are: Luc, luciferase; NHE1–92, plasmid pXP-NHE1 −92/+24 containing nucleotides −92/+24 of the NHE promoter; NHE1-SRE, refers to one copy of the −1085/−800 sequence of the mouse *NHE* promoter; TK 81, the minimal TK promoter; RLU, relative light unit; SV40, the minimal SV40 promoter. Results are mean ± SD from at least eight experiments.

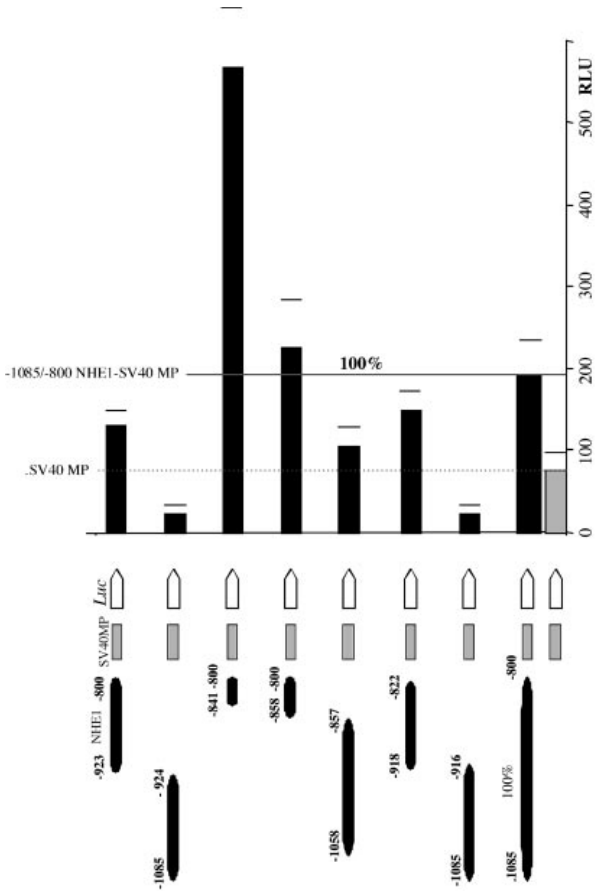


Fig. 2. Activity of subfragments within the −1085/−800-nt region of the *NHE* promoter. NIH 3T3 cells were transiently transfected in 10% serum with the plasmids; pGL −923/−800-SV40MP, pGL −1085/−924-SV40MP, pGL −841/−800-SV40MP, pGL −858/−800 -SV40MP, pGL −1058/−857-SV40MP, pGL −918/−822-SV40MP, pGL −1085/−916-SV40MP, pGL −1085/−800-SV40MP and pGL-SV40MP. The abbreviations are: *Luc*, luciferase; RLU, relative light unit; SV40MP, the minimal SV40 promoter. The dashed line indicates the activity of pGL-SV40MP and that of −1085/−800pGL-SV40MP was set at 100% and is indicated by the solid line. Results are mean ± SD from at least eight experiments.

49-fold increase in luciferase activity, respectively. With the minimal element of the TK promoter, a single copy of the *NHE1* element resulted in a sixfold increase in luciferase activity. With the minimal element of the *NHE1* promoter, a single copy of the *NHE1* element resulted in a 12-fold increase in luciferase activity. The increases occurred whether the DNA was in the normal (Fig. 1) or reverse orientation (not shown) upstream of the minimal promoters. Similar trends were observed in medium depleted of or rich in serum. These results suggest that the −1085/−800 sequence contains an enhancer element able to function in both homologous and heterologous promoter systems. Importantly, no luciferase activity was detected when the −1085/−800 *NHE1* sequence was inserted in the basal plasmid system (pGL3). This confirms that there was no read-through activity within the plasmid sequences and that the −1085/−800 *NHE1* sequence lacks promoter activity.

We designed subsequent experiments to delineate more clearly active regions within the −1085/−800-nt element. Figure 2 shows the transcriptional activity associated with different subfragments of the −1085/−800 element, after their insertion into pGL-SV40MP. The selection of these subfragments was based on the availability of restriction sites. Subfragment activity is compared with the activity of the whole −1085/−800-nt element (pGL-−1085/−800-SV40MP), which is assigned a value of 100%. The basal luciferase activity of the SV40 minimal promoter (pGL-SV40MP) is also indicated. These results indicate that the −1085/−800-nt region is a composite element with several modules, some able to increase activity of the SV40 minimal promoter and others able to decrease it. Footprinting analyses, which examined both strands of the −1085/−800-nt element, identified several areas of protection (Fig. 3). This is in keeping with our previous observation that proteins from NIH 3T3 nuclear extracts bind to several discrete subregions encompassed by the −1085/−800-nt serum responsive element [14]. Figure 3 illustrates the protection afforded by nuclear extracts from mouse liver and NIH 3T3 cells using probes for the 3′ end of the −1085/−800-nt region; specifically the DNA probes were at nt −923/−860 (Fig. 3A,B) and nt −859/−800 (Fig. 3C,D). The pattern of protection by nuclear extracts from mouse liver was almost identical with that by nuclear extracts from NIH 3T3 cells and is summarized in Fig. 4. The protection showed only slight differences in boundary areas for the two types of nuclear extract (Fig. 3).

The region of the promoter with the strongest enhancer activity was found in the most 3′ portion of the *NHE1* −1085/−800-nt region, in the −841/−800-nt element (Fig. 2). Specifically, the activity of this element was sixfold higher than the activity of the entire region, making it a good candidate for further characterization. In footprint analysis, we found that only one area was strongly protected at this location. For the upper DNA strand, the boundaries of protection were −830 to −808 (area slightly protected, as seen in Fig. 3C) and −803 to −800 nt (Fig. 3D). For the lower strand they were −838 to −814 nt (Fig. 3D) and −809 to −800 nt (Fig. 3D).

The −1085/−924-nt and −1085/−916-nt regions decreased the minimal activity of the SV40 promoter (Fig. 2). In contrast, SV40 promoter activity was restored and even slightly increased with the −1058/−857-nt region. This suggests that the extreme 5′ end of the NHE1 region, between nucleotides −1085 and −1058, contains a negative element. In DNA footprinting experiments, we found that the −1085/−916-nt region has six main areas of protection (summarized in Fig. 4). These regions include sequence homologies to binding sites for GF I, GATA, E47, LMO, XFD, Cdx a, Sox, SRY, HNF3, HFH, C/EBP, and Oct 1 families of transcription factors [30]. Some of these putative transcription factors have been localized to negative elements, acting alone and through heterodimerization, and others are known as true repressors [30].

The region from −923 to −839 nt includes three areas that were protected in the footprint experiments (Figs 3 and 4). Database analysis of this region reveals sequence homologies with a variety of binding sites for transcription factors, including the XFD, CdxA, SRY, AML1, HFH, and AP1 families [30]. Overall, our

footprinting experiments indicate that additional experimentation, including mapping, internal deletions, and testing of separate elements in reporter assays, will probably identify other *cis*-acting components within the −1085/−840-nt area.

Identification of transcription factors binding to the −841/−800-nt element

Figure 5 illustrates the results of EMSAs on the −841/−800-nt element, with nuclear extracts from NIH 3T3

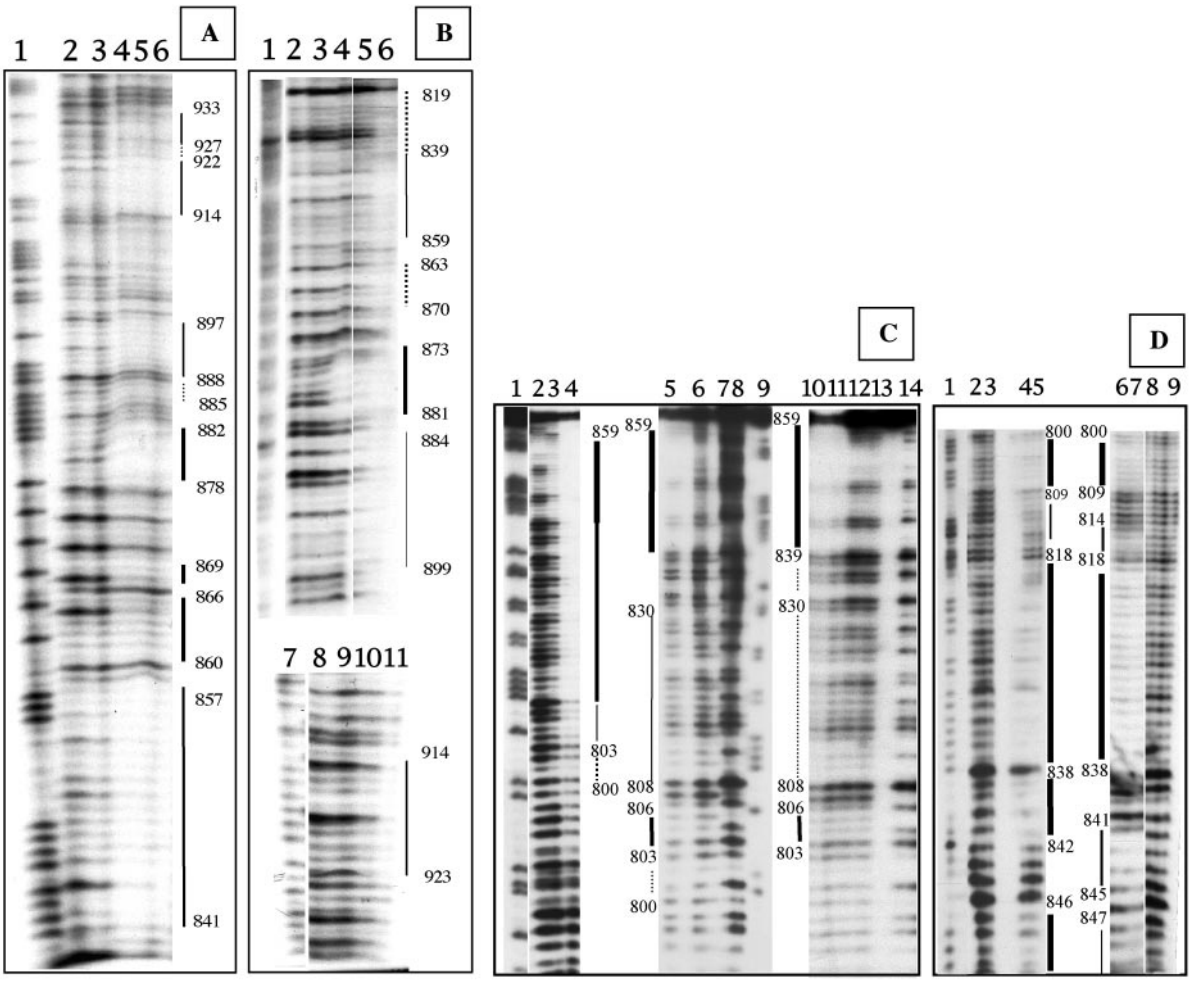


Fig. 3. Footprint analysis of the −923/−800-nt *NHE1* promoter region. A *Pst* I–*Hin*dIII* insert containing the −923/−800-nt *NHE1* sequences in pBS-SK allowed footprinting of the upper DNA strand of the −923/−800-nt *NHE1* sequences. A *Xba*I*–*Kpn*I insert of the same plasmid was used to label the lower DNA strand of the −923/−800-nt region. A *Sac*I–*Hin*dIII* insert containing the −858/−800-nt *NHE1* sequences in pTZ-19R allowed footprinting of the upper strand of the −858/−800-nt region. To label the lower strand of the same region, an *Nsi*I–*Apa*I insert containing the −858/−800-nt region in pGEM-5ZF + was subcloned into the *Pst* I-*Apa*I sites of pBS-SK$^+$. From this plasmid, a *Xba*I*–*Apa*I insert was excised and labeled. (A) −923/−805-nt region (upper strand). Lane 1, G + A sequencing ladder obtained with the *Pst* I–*Hin*dIII* −923/−800-nt probe. Lanes 2–3, patterns of protection obtained with the probe alone incubated with 0.03 and 0.01 U DNAse I, respectively. Lanes 4–6, patterns of protection obtained with nuclear extracts from NIH 3T3 cells incubated with 0.3, 0.1, 0.03 U DNAse I, respectively. Lines adjacent to the footprint indicate areas of protection, with thicker lines indicating greater protection. (B) −923/−805-nt region (lower strand). Lanes 1 and 7, G + A sequencing ladders obtained with the *Xba*I*–*Kpn*I −923/−800-nt probe. Lanes 2, 3, 8 and 9, digests obtained with the probe alone at 0.03 and 0.01 U DNAse I, respectively. Lanes 4–6, patterns of protection obtained with nuclear extracts from NIH 3T3 cells incubated with 0.3, 0.1 and 0.03 U DNAse I, respectively. Lanes 10 and 11, patterns of protection obtained with nuclear extracts from NIH 3T3 cells incubated with 0.3 and 0.03 U DNAse I, respectively. (C) − 858/−800-nt NHE1 region (upper strand). Lanes 1 and 9, G + A sequencing ladders obtained with the *Sac*I–*Hin*dIII* −858/−800-nt probe. Lanes 2–6, patterns of protection obtained with nuclear extracts from mouse liver with DNA incubated with 1, 0.1, 0.01, 0.03, and 0.3 U DNAse I, respectively. Lanes 7 and 8, DNA digestion pattern obtained with the DNA probe alone incubated with 0.03 and 0.01 U DNAse I, respectively. Lanes 10–13, patterns of protection obtained with nuclear extracts from NIH 3T3 cells, with DNA incubated with 0.03, 0.1, 0.3 and 1 U DNAse I, respectively. Lanes 9, 14, digests obtained with the probe alone incubated with 0.01 U DNAse I. (D) −858/−800-nt region (lower strand). Lane1, G + A sequencing ladder obtained with the *Xba*I*–*Kpn*I −858/−800-nt probe. Lanes 2 and 3, and lanes 8 and 9, DNA digestion pattern obtained with the probe alone incubated with 0.03 and 0.01 U DNAse I, respectively. Lanes 4 and 5, patterns of protection obtained with nuclear extracts from NIH 3T3 cells incubated with 0.1 and 0.03 U DNAse I, respectively. Lanes 6 and 7, patterns of protection obtained with nuclear extracts from mouse liver incubated with 0.1 and 0.03 U DNAse I, respectively.

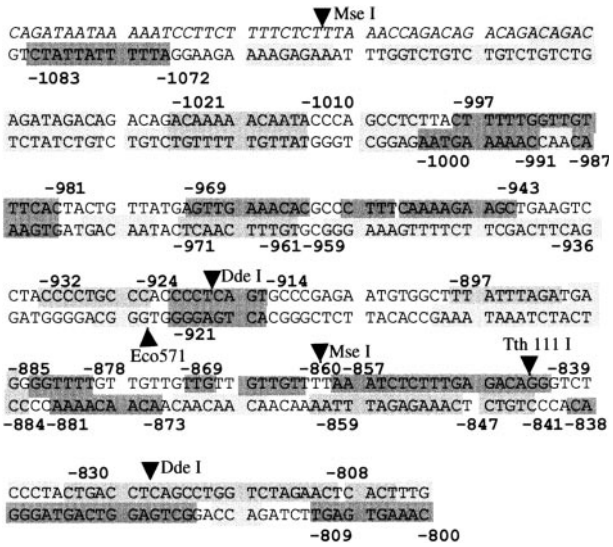


Fig. 4. Nucleotide sequence of the enhancer region of the mouse NHE gene indicating regions of DNA that were protected in footprinting experiments. Shaded areas indicate areas protected. Darker shading indicates greater protection. Numbering refers to the beginning and end of the shaded areas. The arrowheads indicate locations of restriction sites used to study reporter activity of the −1085/−800 subfragments. The summary of the data comes from Fig. 3 and from other footprint analysis not shown.

cells. A number of oligonucleotide-responsive elements, which are known to bind nuclear hormone receptors, competed either totally or partially with the −841/−800-nt region. Lanes 6, 8 and 14, containing no competing oligonucleotides, show two shifted bands. Competition is considered partial when the lower band of the shift is still present after addition of competing oligonucleotide. The following oligonucleotides competed with the −841/−800-nt region: HD-PPRE (lane 2), AT III-A (lane 3), βRARE (lane 5), CRBP1-RE (data not shown), binding sites for GR (lane 1), TR palindrome (lane 4), COUP-TF (lane 11), and HNF4 (lane 12). Some transcription-factor-binding sites other than nuclear receptor responsive elements also competed, for example AP1 and CREB (both competed partially, lanes 10 and 13, respectively). An NFKB element (lane 9) showed partial competition with both species of the shift. A number of subfragments of the −1085/−800-nt element and of the −1085/+24-nt *NHE1* promoter were also tested as competitors. Interestingly, the binding of nuclear extract proteins to the −841/−800-nt probe was partially reduced by a −814/−398-nt fragment of the *NHE1* promoter (immediately downstream of the region studied here; lane 16). In contrast, no competition was observed with other subfragments of the −1085/−800-nt element, such as the −1085/−916-nt fragment and the −918/−859-nt fragment (lane 15).

Figure 6 shows the nucleotide sequence of the −841/−800-nt element. Several putative ACTGGA repeats [perfect and imperfect half-site-binding motifs for nuclear hormone receptors (NHRs)] are illustrated. They represent regions of the −841/−800-nt element with direct and palindromic repeats, in a direct or inverted orientation. To characterize NHR binding to the native −841/−800-nt element, we performed EMSA and supershift experiments with nuclear extracts from NIH 3T3 cells, and with NHR translated *in vitro* (Fig. 7). The band shift generated using NIH 3T3 extracts reacted with antibodies against COUP, suggesting that endogenous COUP isoforms are present in this cell line (Fig. 7A,C). In addition, two isoforms translated *in vitro*, human COUP-TFI and rat COUP-TFII, bound directly to the −841/−800-nt element (Fig. 7B,D,E). For supershift experiments, we selected two antibodies known to cross-react with mouse COUP-TF isoforms. These were rabbit polyclonal IgG against human COUP-TFI and a different rabbit polyclonal against rat COUP-TFII (Fig. 7A,C) [16,17]. Both antibodies recognized the shift band generated by lysates programmed with COUP-TF isoforms (Fig. 7A,C). In EMSA, they decreased the amount of the COUP-TF shift species generated, and this was occasionally associated with the appearance of a supershifted band, either at the well location or at the top of the gel. The specificity of the shifts was confirmed by the addition of a control element known to bind these two isoforms of COUP-TF (Fig. 7A).

To identify the half-site motifs of the −841/−800-nt element involved in NHR binding, the putative ACTGGA repeats (Fig. 6) were mutated and tested for binding and competition by EMSA. The mutants selected were M1–M6 which contained variants or deletion muants of the DRA (−837/−833 nt), DRB (−829/−824 nt) and DRC (−818/−813 nt) motifs as indicated in Fig. 6. In direct EMSA, we found that the mutation of DRB (M2) reduced binding of COUP-TFI and COUP-TFII to the −841/−800-nt element (Fig. 7B). As expected, M6 (mutation of DRA and DRB) did not bind COUP (Fig. 7B). In contrast, M1 and M3, with mutations in DRA and DRC, respectively, were still able to bind COUP-TFI and COUP-TFII, although some reduction in the binding to M1 was evident. The two deletion mutants (M4, with intact DRA and DRB, and M5, with intact 3′ end of DRA and DRB, DRC) were both able to bind COUP-TFI and COUP-TFII (Fig. 7B). Together, these results suggest that DRA and DRB play, respectively, a minor and a major role in binding COUP-TFI and COUP-TFII.

Next, we tested the ability of mutants M1 to M6 to compete with COUP-TFI binding to the native −841/−800-nt element and to the mutated elements M1 and M3. The results are summarized in Table 1. Competition for

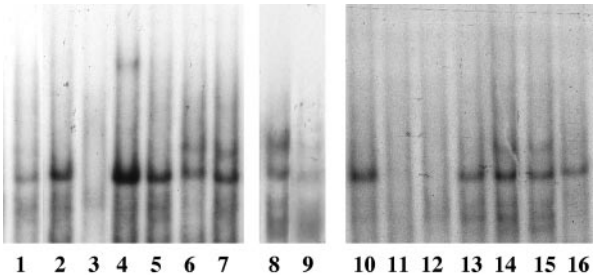


Fig. 5. EMSA of the −841/−800 element of the mouse *NHE* promoter in the presence or absence of various DNA competitors. Lanes 6, 8, and 14, NIH 3T3 nuclear extracts without competitor. Lane 7, polylinker from pBS-SK+ (*Sac*I–*Kpn*I insert). Lanes 1–5, 9–13, 15–16 contain a 50-fold molar excess of oligonucleotides for GR, HD-PPRE, ATIII-A, TRE (palindrome), βRARE, NFκB, CREB, HNF4, COUP-TF, AP1, NHE1 −1085/−916 nt, and NHE1 −814/−398 nt.

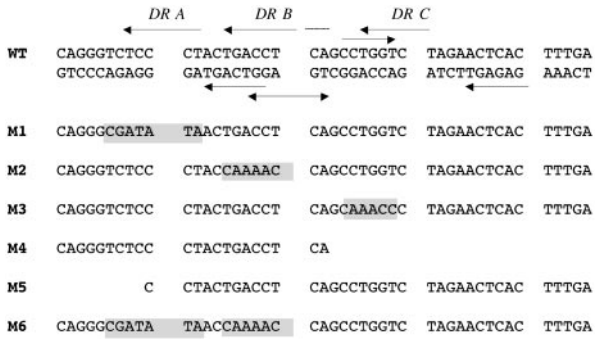


Fig. 6. Nucleotide sequence of the wild-type and mutant −841/−800-nt element and putative nuclear receptor half-binding sites. The double-stranded sequence of nucleotides −843 to −799 is illustrated. Arrows indicate several putative direct and palindromic repeats in a normal or inverted orientation. DR A, B and C indicate the three specific regions selected for mutation studies. M1–M6 are mutants of this region used as competitors in EMSA. Shaded areas indicate regions mutated.

COUP-TF binding to the native element (WT) is exhibited mostly by M1 and M3, and to a lesser degree by M4 followed by M5. In contrast, M2 is almost entirely unable to compete. These results again suggest that DRB plays a major role in COUP-TF binding to the native element. Nevertheless, as before, we found that COUP-TF binding was not exclusive to the DRB half-site, as neither M4 nor M5 was able to totally compete with binding. These results were further supported by competition with COUP-TF binding to radiolabeled M1 and M3 (Table 1). COUP-TFI binding to M3 (with a DRC mutation) was partly prevented by M2 (with a DRB mutation). This could only have occurred via competition with DRA. Finally, addition of M6 (which has DRA and DRB mutations) did not prevent binding of COUP-TFI by the wild-type element (Table 1), suggesting that DRC and nucleotides 3′ to it are not involved in interaction with this factor.

In further experiments, we investigated whether COUP-TFI and COUP-TFII interact at their binding site on the −841/−800-nt element. To detect potential dimerization, we used a truncated form of COUP (t-COUP-TFI) that can easily be differentiated from intact proteins in EMSA.

Table 1. EMSA of the control and mutant −841/−800 element of the mouse NHE promoter in the presence or absence of the DNA competitors M1–M6 (illustrated in Fig. 7). COUP-TFI was expressed in a coupled reticulocyte lysate system, and EMSA gels were quantified using a BioRad phosphorimager.

		Percentage competition	
Labeled DNA element	Competitor	100-fold excess	10-fold excess
Wild-type	M1	89	67
	M2	77	2
	M3	96	73
	M4	87	46
	M5	70	33
	M6	0	0
M1	M2	7	–
	M3	91	–
M3	M1	73	–
	M2	43	–

t-COUP-TFI has intact DNA-binding regions [17]. Figure 7D,E illustrates *in vitro* interactions of COUP-TF bound to control and mutated −841/−800-nt elements. Figure 7D demonstrates that a homodimer forms between COUP-TFI bound to the −841/−800-nt element and COUP-TFI bound to the mutants M1 and M3. Intact COUP-TFI gave a larger species than truncated COUP-TFI, and, as expected, a mixture of intact and truncated COUP-TF1 gave a species with an intermediate mobility. Heterodimers between COUP-TFI (truncated form) and COUP-TFII were also generated, using the same experimental approach (Fig. 7E).

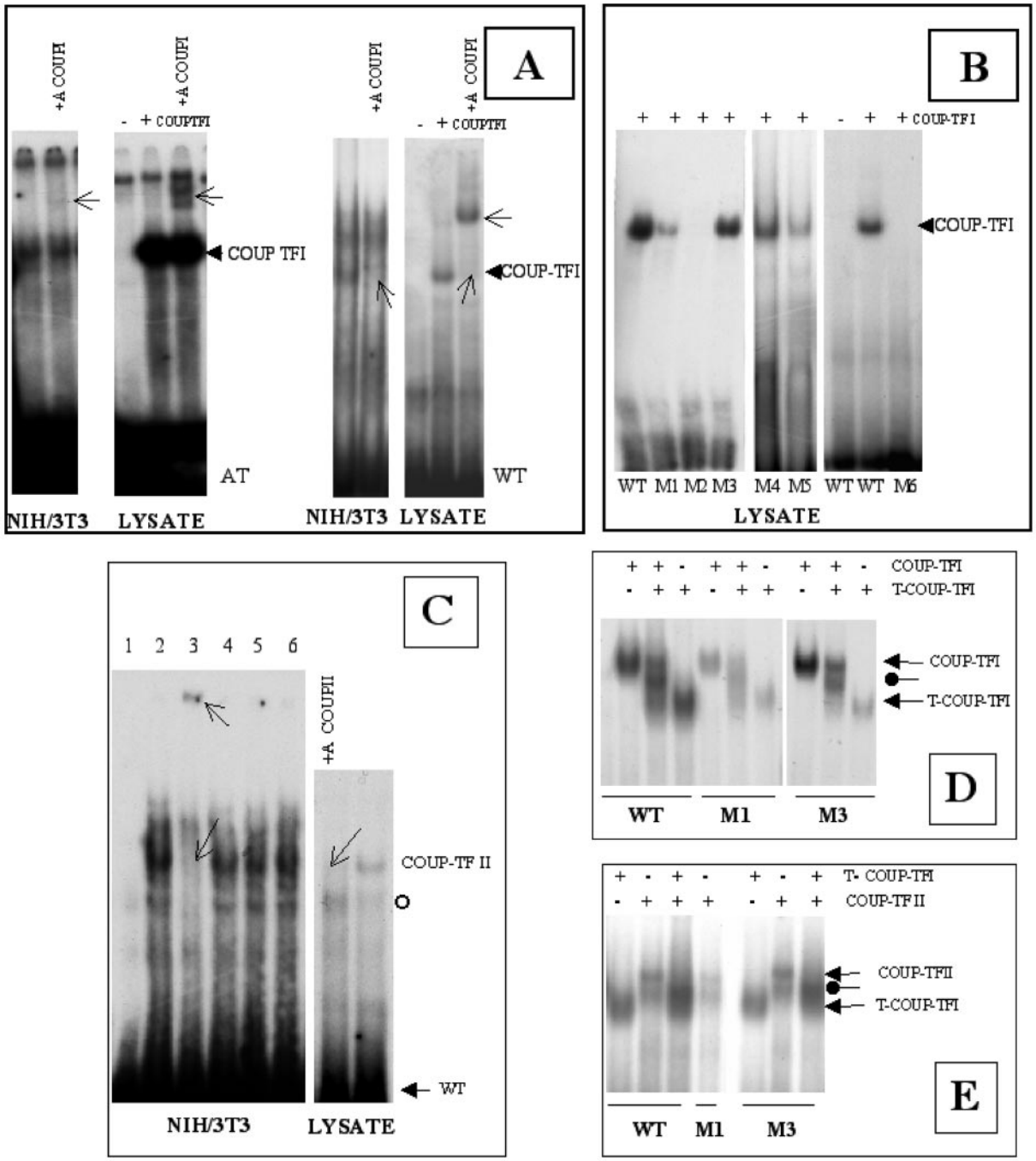
Transcriptional activity of the −841/−800-nt element

Figure 8 illustrates the transcriptional activity of wild-type and mutant, single and multiple [4] copies of the −841/−800-nt element in NIH 3T3 cells. Both single and multiple (four tandem) copies of the wild-type element increased luciferase expression driven by the *NHE1* minimal promoter (pXP −92/+24NHE1-*luc*). The activity

Fig. 7. EMSA of *in vitro* binding of COUP-TFI, COUP-TFII on the wild-type −841/−800-nt element and mutants M1–M6. (A) COUP-TFI binding and reactivity of COUP-TFI antibodies with NIH 3T3 nuclear extracts and in reticulocyte lysates programmed (+) or unprogrammed by COUP-TFI. DNA for mobility shifts was a control probe (AT, antithrombin promoter-element A) or the −841/−800-nt element of the NHE promoter (WT). COUP antibody was added where indicated at the top of the gels. Arrows and arrowheads indicate COUP-reactive species and supershifted proteins. A decrease in COUP-I-reactive species was always observed after antibody addition whereas supershifts were only occasionally seen in the upper part of the gel and/or at the well position, especially in NIH 3T3 extracts. (B) Binding of COUP-TFI expressed in reticulocyte lysates to wild-type (WT) and mutated (M1–M6) −841/−800-nt elements. (C) Reactivity of antibodies for NHRs in supershift assays of the −841/−800-nt element of the *NHE*. Left, experiments with NIH 3T3 extracts (NIH/3T3); right, experiments with COUP-TFII protein produced in reticulocyte lysates (LYSATE). All lanes contained the labeled −841/−800-nt DNA element with NIH 3T3 cell extracts where indicated. Left, lane 1, no extract; lanes 2–6 contain antibodies against HNF4α (2), COUP-TFII (3), PPARγ (+ α,β) (4), RXRα (5) and RARα (+ β,γ) (6). Right, *in vitro* translated COUP-TFII. + A COUPII indicates addition of antibody to COUP-TFII. Open circle denotes non-specific band. For experiments with both NIH 3T3 extracts and *in vitro* translated COUP-TFII, COUP-TFII antibody reactivity in NIH 3T3 extracts results in a decrease in the reactive species and occasionally an additional band at the well site as indicated by the arrows. (D) Receptor interactions on the −841/−800-nt element of COUP-TFI and t-COUP-TFI (truncated COUP-TFI). *In vitro* translated COUP-TFI and truncated COUP-TFI were incubated either separately or together with the −841/−800-nt element and the mutant forms M1 and M3 where indicated. The closed circle indicates the presence of a dimer. (E) Similar to (D) except with t-COUP-TFI and full-length COUP-TFII.

of the M2 mutant was greatly reduced. The activity of M1 (single and multiple copies) and M3 (multiple copies only) was slightly reduced in this plasmid system. Similar results were obtained with these mutants in preliminary experiments with the TK minimal promoter (pTK −40/+51, not shown).

Figure 9 illustrates the dose-dependent effects of cotransfection of COUP-TF isoforms with tandem copies of the −841/−800-nt element. The experiments were conducted in the presence of 0.5% and 10% fetal bovine serum. As with the experiments presented in Fig. 8, the minimal promoter of *NHE1* (−92/+24 nt) (Fig. 9A,B) and a truncated version of the TK promoter (−40/+51 nt) (Fig. 9C) were used in luciferase systems. In the absence of additional elements, COUP-TF isoforms did not modify the basal luciferase activity driven by the two promoters. When four tandem copies of the −841/−800-nt element were placed in front of the *NHE1* minimal promoter, luciferase activity in 10% serum (without stimulation by exogenous COUP-TF) was approximately double that in 0.5% serum. The effects of COUP-TF isoforms on activity are given relative to the appropriate matched controls with or without serum. Cotransfection with vectors for COUP-TF isoforms increased the luciferase activities



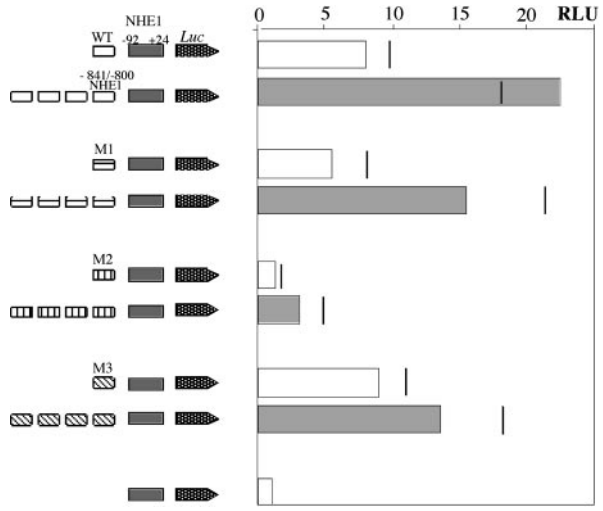


Fig. 8. Transcriptional activity of wild-type and mutant, single and multiple copies (four) of the −841/−800-nt element in NIH 3T3 cells. Cells were transiently transfected with plasmids containing a minimal promoter plus single or multiple (four) copies of the wild-type and mutant −841/−800-nt element. Cells were transfected and luciferase activity measured as described in the Materials and methods. The abbreviations are: *Luc*, luciferase; RLU, relative light unit. The *NHE1* minimal promoter–reporter system was used containing nucleotides −92/+24. Each plate was transfected with 2.5 μg reporter plasmid. Results are the mean ± SD from at least eight experiments.

described above, at both low and high concentrations of serum, and in both NIH 3T3 and CV1 cells. At each 'dose' of COUP-TF tested, a larger increase in luciferase activity was obtained in 10% serum. The maximal stimulation of promoter activity was mediated by cotransfection with the COUP-TFII vector, with 10% serum. The COUP-TFI vector showed the same pattern of activation, but with a lesser magnitude. We obtained similar results when COUP-TFI and COUP-TFII were expressed using the plasmid pRC/CMV instead of pSG5 (data not shown). Finally, in both cell lines, cotransfection of the COUP-TF vectors increased the basal luciferase activity of plasmids constructed with M1 (instead of the wild type −841/−800-nt element). This increase was smaller than that seen with the wild-type element. In contrast, when plasmids were constructed with the mutated element M2, barely detectable luciferase activities were seen, either with or without the addition of vectors for the COUP-TF isoforms.

Mechanisms of the effects of COUP-TF isoforms on endogenous NHE1 expression

We next investigated the effects of COUP-TF isoforms on the transcriptional activity of the serum responsive region at −1085/−800 nt and of the −1085/+24-nt *NHE1* endogenous promoter. The effects of the COUP-TF isoforms were compared with those seen in the −841/−800-nt NHR-responsive element. The −1085/−800-nt element was inserted upstream of the NHE1 minimal promoter or upstream of the TK minimal promoter (Table 2). Overexpression of either COUP-TF isoform was performed as described above for Fig. 9. COUP-TF transactivated the three elements tested, including the entire −1085/+24-nt *NHE1* promoter. Another series of experiments were carried out to examine if the binding activity of COUP-TF was restricted to the distal end of the *NHE1* promoter. We examined binding of translated COUP-TF by NHE1 promoter fragments located at −1085/−842 (*Hin*dIII–*Tth*111I), −814/−398 nt (*Xba*I–*Acc*I); −396/−91 nt (*Acc*I–*Bsg*I) and −92/+24 nt (*Bam*–*Hin*dIII of pXP-AP2). None of these NHE1 promoter fragments were able to bind directly COUP-TF isoforms translated *in vitro* (data not shown).

To investigate further the biological effects of COUP-TF on NHE1 expression, we selected two experimental approaches. The first system used transient COUP-TF overexpression in NIH 3T3 cells as described above for Table 2. Nuclear extracts and mRNA samples were collected 72 h after addition of the control plasmid pSG5 or pCOUP-TFII-SG5. The ability of COUP-TF to bind to the −841/−800 nt was examined after its overexpression in NIH 3T3 cells (Fig. 10A, panel I). To ensure satisfactory transfection, we simultaneously transfected some of the cells with the −841/−800-nt luciferase reporter and confirmed the expected effects on transcriptional activity, as described for Fig. 8. For EMSA with NIH 3T3 nuclear extracts, we used only 1 μg nuclear proteins, as this produces little mobility shift from endogenous COUP-TF in the absence of COUP-TF overexpression. We found that the mobility shifts generated with the COUP produced from the expression plasmids migrated at the same position as endogenous COUP. For both COUP-TF isoforms, we also found that transfection increased quantities of the shifted species that react with COUP-TF antibodies (Fig. 10A, panel I). This suggests that the exogenously added COUP-TF, expressed *in vivo,* binds to the *NHR* element. The addition of COUP-TF antibodies reduced quantities of the shift species. These results also confirmed that COUP overexpression occurred in these cells. In addition, Western blots of nuclear extracts (10 μg per lane) also confirmed that COUP was overexpressed in NIH 3T3 cells (Fig. 10A, panel II).

To examine the effects of COUP overexpression on mRNA levels of the *NHE*, we used a competitive PCR assay we have described previously [14]. After isolation of mRNA, reverse transcription was performed, followed by competitive PCR of cDNA anchored to magnetic beads. Figure 10A, panel III, illustrates the amplification of the *NHE1* cDNA region over exons 8–9 (180-bp product, lanes 2–5) and of the larger *NHE1* 404-bp competitor template. Simultaneously, a *G3PDH* cDNA and control templates were amplified, giving a product of 396 nt from the reverse-transcribed cDNA (lanes 7–10) and a 241-nt product with the G3PDH competitor plasmid (lane 6). As illustrated in Fig. 10, transfection of COUP-TFII into NIH 3T3 cells resulted in a large increase in *NHE1*-derived PCR products generated from the corresponding cDNA library. In contrast, no major differences were seen in G3PDH-derived PCR products between the two libraries. The result was typical of three independent experiments performed with different RNA preparations.

The second intact cell system used to examine the endogenous induction of COUP-TFII expression was P19 embryonal carcinoma cells treated with either retinoic acid (1 μM) or Me_2SO (1%) [22,23]. RNA samples and nuclear

Table 2. Effect of COUP-TFI and COUP-TFII on transcriptional activity of the endogenous *NHE1* promoter (−1085/+24 nt) and the −1085/−800 nt *NHE1* enhancer placed upstream of the *NHE1* or *TK* minimal promoters. Luciferase values are relative to each reporter transfected with pSG5 (100%) for serum regimens of 0.5% or 10%. A 1.25 μg sample of each expression vector was added per NIH 3T3 plate.

	Expression vector			
	pCOUP-TFI-SG5		pCOUP-TFII-SG5	
Luciferase reporter	0.5% serum	10% serum	0.5% serum	10% serum
−1085/−800-nt NHE1MP-*luc*	615	970	980	1310
−1085/+24-nt NHE1-*luc*	363	504	673	1894
−1085/−800-nt TKMP-*luc*	398	585	746	1001

extracts were collected before and 72 h after treatment of the cells with retinoic acid or Me_2SO. Nuclear proteins prepared after retinoic acid treatment were able to bind the −841/−800-nt element and reacted with COUP-TFII antibody (Fig. 10B, panel I). The increase in synthesis of endogenous COUP-TFII after treatment with retinoic acid or Me_2SO was confirmed by Western blotting of the nuclear extracts (Fig. 10B, panel II). Before retinoic acid or Me_2SO treatment, and in 10 μg extract, no COUP-immunoreactive band was detected. However, a low-intensity band could be

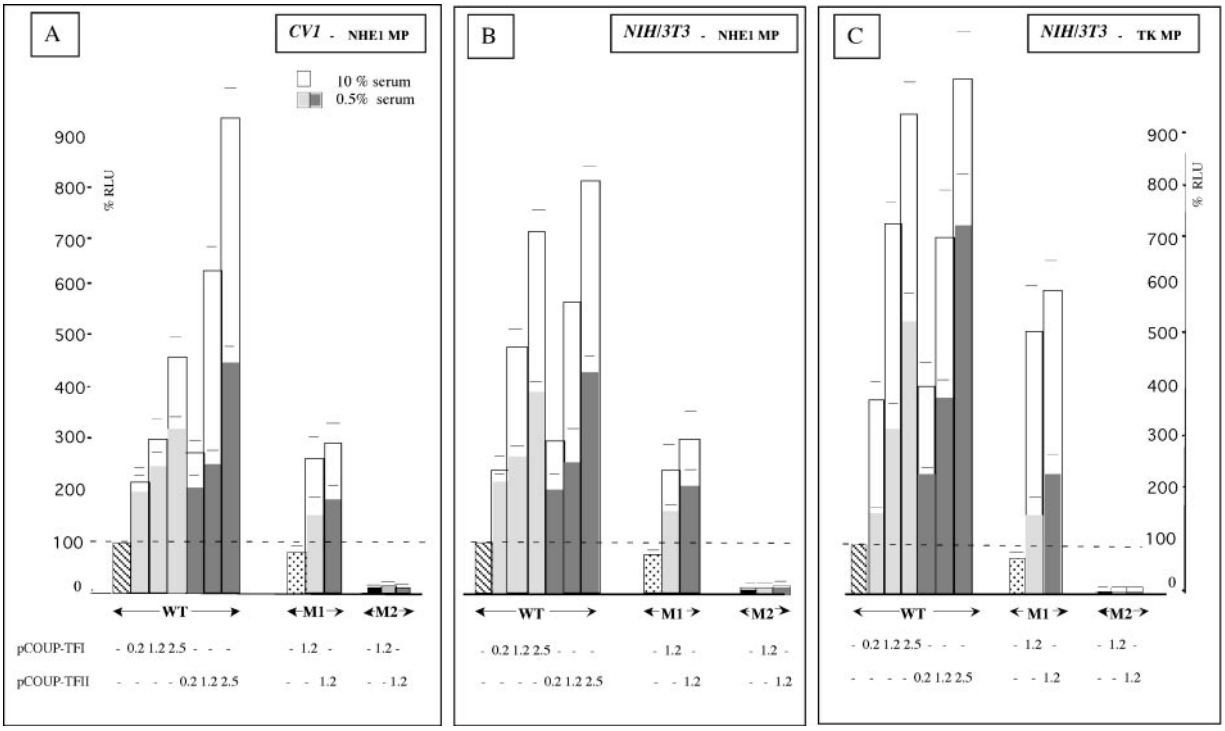


Fig. 9. Effect of COUP-TFI, COUP-TFII on reporter activity of the −841/−800-nt element in the presence of 0.5% or 10% fetal bovine serum. The wild-type −841/−800-nt element was used or mutant M1 and M2 with the mutations indicated in Fig. 6. (A) Expression vectors for COUP-TF isoforms were cotransfected in CV1 cells with the NHE1 reporter plasmid which included four tandem copies of the −841/−800-nt wild-type element upstream of the *NHE1* minimal promoter or of the mutants M1 or M2. Each plate was transfected with 2.5 μg reporter plasmid in the presence of 0.25, 1.25 or 2.5 μg expression vector for the various nuclear receptors. Control values of −841/−800-nt wild-type element without addition of COUP plasmids were set at 100% and are indicated by the hatched bar. For comparison with other values, a dotted line extends the 100% value. In 0.5% and 10% serum the relative luciferase activity was 13.2 ± 3.8 and 26.9 ± 4.4 (mean ± SD), respectively. The effects of the receptors are expressed as a percentage of the reporter activity in low serum (0.5%, shaded) or normal (10%, unshaded) serum. Effects of pCOUP-TF isoforms on activity are given relative to the appropriate matched control with or without serum. Spotted and black bars indicate luciferase values of M1 and M2 mutant, respectively, relative to wild-type controls without addition of COUP-containing plasmids. (B) Transfection and reporters were the same as in (A) but with NIH 3T3 cells. In 0.5% and 10% serum, the wild-type relative luciferase activity was 8.9 ± 1.7 and 22.8 ± 4.7 (mean ± SD), respectively. (C) Transfection and reporters were the same as in (A) but with NIH 3T3 cells and with the reporters including the four tandem copies of the −841/−800-nt element upstream of the *TK* minimal promoter instead of the *NHE1* minimal promoter. In 0.5% and 10% serum, the relative luciferase activity was 9.04 ± 3.1 and 15.5 ± 5.4 (mean ± SD), respectively. In all cases a constant amount of expression vector was maintained by cotransfection of the empty expression vector pSG5. Baseline values for all experiments used for normalization in the absence of nuclear receptors were measured in the presence of 2.5 μg pSG5 to normalize for the amount of vector transfected. Results presented are mean ± SD from at least eight experiments.

seen for extract at concentrations of 20 μg and above, suggesting that low amounts of this factor are synthesized in P19 cells. We also examined mRNA levels after retinoic acid and Me_2SO treatments using competitive PCR as described in Fig. 10A. Competitive conditions were applied to estimate semiquantitatively differences in mRNA expression. Figure 10B, panel III illustrates the products obtained after coamplification of cDNA aliquots and various dilutions of the competitor plasmids. An increase in products derived from the *NHE1* mRNA message (180-nt product) was obtained in P19 cells treated with retinoic acid (lanes 5–8 compared with lanes 1–4 from the control library) or Me_2SO (not shown). These experiments were repeated three times and the results were highly reproducible. No marked difference in mRNA expression was seen for G3PDH, as shown by the 396-nt product in lanes 1–4 and 5–8. Finally, following the experimental protocol used for isolation of mRNA and nuclear protein, untreated,

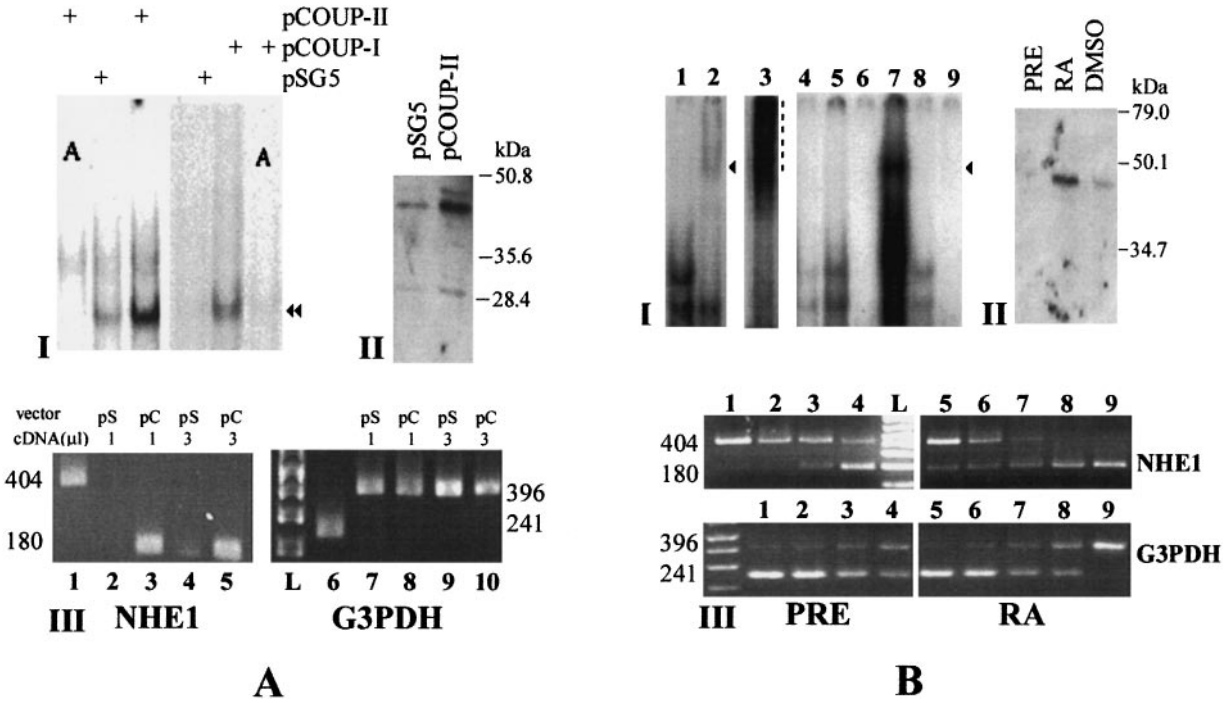


Fig. 10. Effects of COUP on NHE1 expression in NIH 3T3 cells (A) and P19 cells (B). A-I, EMSA following *in vivo* overexpression of COUP-TFI and COUP-TFII. Nuclear extracts were prepared from cells transfected (12.5 μg/T75 flask) with pSG5, pCOUP-TFI-SG5, or pCOUP-TFII-SG5. A 1 μg sample of each type of nuclear extract was incubated with the wild-type −841/−800-nt element. 'A' indicates addition of COUP antibody. The double arrowhead indicates the location of COUP-reactive species in the mobility shift. A-II, Western blots using COUP-TFII antibody and 10 μg nuclear extract/lane. Cells were transfected with pSG5 or pCOUP-TFII-SG5. The position and sizes of the protein markers are indicated on the right. A-III, PCR products in 2% agarose gels after amplification of G3PDH and *NHE1* cDNA (left and right panels, respectively). Lanes 1 (404-bp NHE1 product) and 6 (241-bp G3PDH product) are positive controls from the amplified competitive templates. In contrast, no products were generated after amplification of the reaction mixture without primers or without template. Similarly, no products were amplified from the mRNAs before reverse transcription. The products generated from the amplified, reverse-transcribed mRNA are shown in lanes 2–5 (*NHE1*, 180 bp) and 7–10 (G3PDH, 396 bp). Either 1 or 3 μL cDNA beads were amplified from the pSG5 (pS) or the pCOUP-TFII (pC) libraries. Each lane contains 10% of the total PCR products/reaction. Lane L, 1-kb Plus DNA ladder (Gibco–BRL). The results are typical of three independent experiments. B-I, EMSA of P19 nuclear extracts from cells treated with retinoic acid. Lane 1, nuclear proteins binding to the −841/−800-nt element. Lane 2, reaction as in lane 1 but with COUP-TFII antibody. The single arrowhead indicates COUP-immunoreactive species. Lanes 3–9 illustrate EMSA with the −841/−800-nt element after immunoprecipitation with a specific antibody (against COUP-TFII, lanes 3, 4, 6, 7 and 9) or a non-specific antibody (lanes 5 and 8). In lane 3, retinoic acid-treated nuclear extracts were specifically bound to the beads, washed and eluted. The eluate was then tested in EMSA with the wild-type *NHR* element. The predominant COUP species seen in this lane (3) are COUP-TF supershifts (dashed line to the right). Lanes 4–6 illustrate wild-type binding by the residual nuclear fractions not bound by either specific or non-specific antibodies (lanes 4 and 5, respectively). Lane 6 is without nuclear extract. Lanes 7–9 illustrate wild-type binding of the total reaction mixture containing nuclear extracts plus immunobeads coupled to the specific (lane 7) or the non-specific (lane 8) antibody. Lane 9 is without extract. B-II, Western blots of nuclear extracts with COUP-TFII antibody. The extracts (10 μg/lane) were prepared before and after treatment with retinoic acid or Me_2SO. B-III, PCR products in 2% agarose gels after competitive PCR amplification of *G3PDH* and *NHE1* cDNA for P19 cells treated or not with retinoic acid. The negative controls described for A-III for NIH 3T3 cells also tested negative for P19 cells. The competitive templates amplified for control (lanes 1–4) and retinoic acid-treated cells (lanes 5–9) were added as 10-fold serial decreasing dilutions (lanes 1–8, *NHE1* 404-nt and *G3PDH* 241-nt products). The initial amount of competitor added in lanes 1 and 5 for *G3PDH* and *NHE1* were 10 ng and 500 pg, respectively. The PCR products from 1 μL of the cDNA before and after treatment with retinoic acid, amplified concomitantly, are illustrated in lanes 1–4 and 5–8, respectively (180 bp for *NHE1* and 396 bp for *G3PDH*). The products shown in lane 9 were generated by the amplification of 1-μL library aliquots amplified without the competitive templates. Each *NHE1* and *G3PDH* lane contains 40% and 10% of the total PCR products/reaction, respectively.

retinoic acid-treated or Me_2SO-treated P19 cells were cotransfected with pSG5, pCOUP-TFI-SG5 or pCOUP-TFII-SG5 together with the 4 × (−841/−800 nt) NHE1-MP reporter. Luciferase expression generated from the *NHR* element was increased an average of 30-fold after retinoic acid treatment (average of three experiments) and an average of eightfold after Me_2SO addition. In addition, in transient transfection experiments of the P19 cells, overexpression of exogenous COUP-TFI or COUP-TFII also activated *NHE* promoter activity from the *NHR* element 2.5-fold and sixfold, respectively. Further transactivation was obtained in the presence of retinoic acid, 200-fold for pCOUP-I-SG5 and 600-fold for pCOUP-II-SG5. In the presence of Me_2SO, 15-fold and 28-fold further transactivation was obtained for COUP-TFI and COUP-TFII, respectively.

DISCUSSION

COUP-TF is mostly known as a negative transcriptional regulator of RXR-mediated hormone-signaling pathways. However, transactivation by COUP-TF has also been widely reported [15–17,31,32]. COUP-TF has been implicated in the regulation of several important biological processes such as embryonic development and neuronal cell differentiation [33]. In our study, we clearly identified a NHR COUP responsive element from nt −841 to −800 of the *NHE* promoter. The two isoforms we tested, COUP-TFI and COUP-TFII, formed homodimers and heterodimers on the −841/−800-nt *NHE* element (Fig. 7).

COUP-TF homodimers are known to accommodate various direct and palindromic repeats of various arrangements of AGGTCA [15,34]. Binding and competition experiments with mutants of the −841/−800-nt element localized the COUP-TF-binding region to two putative direct repeats DRA and DRB, from −837 to −824 nt (Figs 6 and 7; Table 1). The DRA–DRB area contains several possible NHR half-sites, including a DR separated by 2 bp (DR2) at −837 to −824 nt, and an inverted palindrome (Ipal) at −837 to −822 nt (IPal 4, Fig. 6) [15,29,35]. COUP-TF isoforms may also bind as a homodimer on one DNA half-site [36,37]. In this case, the DNA consensus for COUP-TF binding is TAGGTCA [38] and this is similar to (but not identical with) the TGAGGTCA motif in DRB. Our results clearly showed that this motif, DRB, plays a major role in binding COUP-TF. The transcriptional activity of this element *in vivo* was greatly reduced by mutations (M2, Fig. 8). This mutation was also associated with suppression of COUP-TF transactivation (Fig. 9). The related DRA area, with its imperfect NHR half-site consensus, also appears to partly influence binding *in vitro*, although not as much as DRB. When the DRA was mutated (M1), transcriptional activity was present *in vivo* even if somewhat reduced. Furthermore, exogenous COUP-TF was transactivated from the M1 element, in NIH 3T3 and CV1 cells (Fig. 9), although this was lower than that seen for the wild-type element, suggesting a more minor role for DRA.

It is likely that additional NHR transcription factors and mediators interact at the −841/−800-nt site and modulate COUP-TF activity. Of the NHRs we tested, no interactions were detected with RXR and RAR. No RXR binding was seen, either directly or after heterodimerization, with PPAR, RAR, RXR, LXR, RZR, or mCAR. Similarly, no heterodimers were formed between COUP-TFI/II and RXRα, RARα or TRα (data not shown). UR and NGF1β, two orphan receptors upregulated by cell activation and often present on COUP-TF-responsive elements [20], did not bind to the NHE1 site. Also, the presence of a putative Pal3 element, TGGAGTcggACCAGA, at −827 to −813 nt, suggests possible interactions with members of the family of classic steroid receptors. We tested antibodies for GR and ERE in EMSA but detected no immunoreactivity. From competition studies, translated CREB was suggested as a possible regulator (Fig. 5), but it also did not bind to the −841/−800-nt *NHE1* element (data not shown). Database homologies at the −841/−800 site suggest binding sites for v-Erb A, AP1, CREB, NK25, Lyf 1, MZF 1, delta E or c-Myb [30]. Characterization of additional transcriptional regulators at this site therefore warrants further investigation. However, it is clear that, of those tested, COUP-TF is one of the few transcription factors that can interact either directly or indirectly at this location.

To examine the specificity of the COUP-TF effects further, we determined whether COUP-TF isoforms could bind outside the −841/−800-nt element. We found that COUP-TF isoforms do not bind additional sites on the −1085/−800-nt region or sites more proximal to this region in the *NHE1* promoter. However, a −814/−398 *NHE1* fragment competed with NHR binding to the −841/−800-nt element (Fig. 5). Whether cross-talk occurs between these two regions of the *NHE1* gene and modulates activity of the *NHR* element is not known at the moment, but we cannot ignore the possible presence of additional modulatory mechanisms for COUP-TF activity on NHE1 expression. This region contains consensus sequences for CREB (−790), SP1 (−600) and two CAAT boxes (at −568, −508), and bridging of COUP with other DNA-bound transcription factors has accounted for COUP positive activation in several genes [39]. Nevertheless, the specificity of COUP-TF binding to the −841/−880 region suggests that this is a key site of COUP-TF regulation of NHE expression.

COUP-TF isoforms have been implicated in the regulation of several important biological processes such as embryonic development, neuronal cell differentiation, and heart development [33,40]. However, it has been difficult to define downstream targets of COUP-TF because COUP knockout mice show early lethality [15]. Therefore, the physiological role of COUP-TF isoforms and their role in regulation of NHE1 expression were investigated in two cell model systems in which COUP expression was induced. Transient overexpression of COUP-TF increased production of *NHE1* mRNA (Fig. 10A) in NIH 3T3 cells. The same result was found when retinoic acid or Me_2SO (Fig. 10B) induced endogenous COUP-TFII expression in P19 cells. These cells have been reported to contain very low levels of COUP-TFI and COUP-TFII when not treated with retinoic acid or Me_2SO [23]. Our results confirm this observation (Fig. 10B, panel II). Furthermore treatment of P19 cells with retinoic acid or Me_2SO resulted in increased transcriptional activity of the −841/−800-nt *NHR* element. Transient expression of pCOUP-TFI or pCOUP-TFII further enhanced activation from the *NHR* element. It is

important to again note that the −841/−800-nt element is not a retinoic acid-responsive element, because it does not bind RXR, RAR, or RAR–RXR heterodimers. Therefore, the stimulating effect of retinoic acid on the −841/−800-nt element in P19 cells must be of indirect origin. It is likely that the increase in COUP-TF endogenous expression is responsible for the observed transcriptional effects of retinoic acid on the NHE. We have previously shown that the activity of the *NHE1* promoter is increased during retinoic acid-induced differentiation of P19 cells [22]. Moreover, retinoic acid-induced differentiation of P19 cells is eliminated in a P19 cell line made deficient in the NHE, or by treatment of P19 cells with the Na^+/H^+ exchange inhibitor Hoe 694 [41]. Thus it is clear that the NHE plays a permissive role in differentiation of these cells. This may be the reason for the upregulation of its expression seen on treatment with retinoic acid. Our previous study [22] showed that the transcription factor AP-2 is important in increasing *NHE1* promoter activity. It is therefore clear from this [22] and other studies of ours [42] that the distal responsive element of the gene is not the only region significant in regulating NHE expression in P19 and other cell types. However, the correlation of COUP expression and effects on *NHE1* promoter activity in this study suggest that retinoic acid-induced P19 differentiation is linked to NHE1 upregulation through COUP *in vivo*. Further experiments are therefore necessary to confirm that COUP plays an important role in NHE1 expression in neuronal cells *in vivo*. It is, however, interesting to note that NHE knockouts, although not lethal, are also associated with severe neurological defects [6].

The effects we observed on transcription were greatly enhanced by the presence of 10% serum. This result agrees with our previously study in which we showed that mitogenic activation of cells activated the *NHE* promoter through this region of the gene [14]. These results suggest that we may have defined an important mechanism by which COUP-TF regulates NHE expression and thereby influences cell growth and differentiation. However, it should be noted that enhancer activity of the −845/−800-nt element, as well as transactivation by COUP-TF, were also observed in 0.5% serum and charcoal-filtered delipidated serum. This occurred even though lower luciferase activity was observed compared with that in 10% serum (Fig. 9). This effect was seen in NIH 3T3 cells and also in CV1 cells, a cell line commonly used for COUP-TF-over-expression studies [15]. Therefore, the potential of COUP-TF for transactivation cannot only be attributed to the presence of serum in the medium, although, in this setting, COUP activity was markedly enhanced (Fig. 9; Table 2). An identical observation has been reported in studies of COUP transactivation of RZR/ROR responsive elements [37].

The involvement of COUP-TF isoforms in the control of cell proliferation is well documented, although downstream targets have not been well characterized [43,44]. It also well known that the NHE is regulated in response to a variety of external stimuli and is also involved in regulation of cell proliferation in many cell types [7,45,46]. Evidence from our laboratory suggests that NHE expression and promoter activity (−1085/+24 nt) are increased during NIH 3T3 cell proliferation and serum stimulation [14]. Therefore, our results provide a mechanism by which COUP-TF could account for increased NHE expression and activity during cell growth and differentiation [14,22,41,46]. Growth stimulation and upregulation of NHE expression has also been implicated in various pathological situations, such as heart ischemia and hypertrophy [47]. In addition, the involvement of COUP-TFII in angiogenesis and vascular remodeling during early embryonic life [15] as well as the re-activation of 'fetal programs' of cell activation leading to cardiac hypertrophy make the study of *NHE1* expression via the −845/−800-nt COUP-responsive element an attractive candidate for further study.

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REFERENCES

1. Fliegel, L. & Frohlich, O. (1993) The Na^+/H^+ exchanger: an update on structure, regulation and cardiac physiology. *Biochem. J.* **296**, 273–285.
2. Boron, W.F. & Boulpaep, E.L. (1983) Intracellular pH regulation in the renal proximal tubule of the salamander. *J. Gen. Physiol.* **81**, 29–52.
3. Grinstein, S., Clarke, C.A. & Rothstein, A. (1983) Activation of Na^+/H^+ exchange in lymphocytes by osmotically induced volume changes and by cytoplasmic acidification. *J. Gen. Physiol.* **82**, 619–638.
4. Pouyssegur, J. (1985) The growth factor-activatable Na^+/H^+ exchange system: a genetic approach. *Trends Biochem. Sci.* **10**, 453–455.
5. Wang, J., Singh, D. & Fliegel, L. (1997) The Na^+/H^+ exchanger potentiates growth and retinoic acid induced differentiation of P19 embryonal carcinoma cells. *J. Biol. Chem.* **272**, 26545–26549.
6. Cox, G.A., Lutz, C.M., Yang, C.-L., Biemesderfer, D., Bronson, R.T., Fu, A., Aronson, P.S., Noebels, J.L. & Frankel, W.N. (1997) Sodium/hydrogen exchanger gene defect in slow-wave epilepsy mice. *Cell* **91**, 139–148.
7. Grinstein, S., Rotin, D. & Mason, M.J. (1989) Na^+/H^+ exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation. *Biochim. Biophys. Acta* **988**, 73–97.
8. Rao, G.N., Sardet, C., Pouyssegur, J. & Berk, B.C. (1992) Na^+/H^+ antiporter gene expression increases during retinoic acid-induced granulocytic differentiation of HL60 cells. *J. Cell. Physiol.* **151**, 361–366.
9. Dyck, J.R.B., Silva, N.L.C.L. & Fliegel, L. (1995) Activation of the Na^+/H^+ exchanger gene by the transcription factor AP-2. *J. Biol. Chem.* **270**, 1375–1381.

10. Miller, R.T., Counillon, L., Pages, G., Lifton, R.P., Sardet, C. & Pouyssegur, J. (1991) Structure of the 5′-flanking regulatory region and gene for the human growth factor-activatable Na^+/H^+ exchanger NHE-1. *J. Biol. Chem.* **266**, 10813–10819.
11. Horie, S., Moe, O., Yamaji, Y., Cano, A., Miller, R.T. & Alpern, R.J. (1992) Role of protein kinase C and transcription factor AP-1 in the acid-induced increase in Na/H antiporter activity. *Proc. Natl Acad. Sci. USA* **89**, 5236–5240.
12. Kolyada, A.Y., Johns, C.A. & Madias, N.E. (1995) Role of C/EBP proteins in hepatic and vascular smooth muscle transcription of human NHE1 gene. *Am. J. Physiol.* **269**, C1408–C1416.
13. Kolyada, A.Y., Lebedeva, T.V., Johns, C.A. & Madias, N.E. (1994) Proximal regulatory elements and nuclear activities required for transcription of the human Na/H exchanger (NHE-1) gene. *Biochim. Biophys. Acta* **1217**, 54–64.
14. Besson, P., Fernandez-Rachubinski, F., Yang, W. & Fliegel, L. (1998) Regulation of Na/H exchanger gene expression: Mitogenic stimulation increases NHE1 promoter activity. *Am. J. Physiol.* **274**, C831–C839.
15. Tsai, S.Y. & Tsai, M.J. (1997) Chick ovalbumin upstream promoter-transcription factors (COUP-TFs): coming of age. *Endocr. Rev.* **18**, 229–240.
16. Zhang, B., Marcus, S.L., Sajjadi, F.G., Alvares, K., Reddy, J.K., Subramani, S., Rachubinski, R.A. & Capone, J.P. (1992) Identification of a peroxisome proliferator-responsive element upstream of the gene encoding rat peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase. *Proc. Natl Acad. Sci. USA* **89**, 7541–7545.
17. Marcus, S.L., Winrow, C.J., Capone, J.P. & Rachubinski, R.A. (1996) A $p56^{lck}$ ligand serves as a coactivator of an orphan nuclear hormone receptor. *J. Biol. Chem.* **271**, 27197–27200.
18. Umesono, K., Murakami, K.K., Thompson, C.C. & Evans, R.M. (1991) Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. *Cell* **65**, 1255–1266.
19. Choi, H.S., Chung, M., Tzameli, I., Lee, Y.K., Seol, W. & Moore, D.D. (1997) Differential transactivation by two isoforms of the orphan nuclear hormone receptor CAR. *J. Biol. Chem.* **272**, 23565–23571.
20. Bernier, D., Thomassin, H., Allard, D., Guertin, M., Hamel, D., Blaquiere, M., Beauchemin, M., LaRue, H., Estable-Puig, M. & Belanger, L. (1993) Functional analysis of developmentally regulated chromatin-hypersensitive domains carrying the alpha 1-fetoprotein gene promoter and the albumin/alpha 1-fetoprotein intergenic enhancer. *Mol. Cell. Biol.* **13**, 1619–1633.
21. Roesler, W.J., Graham, J.G., Kolen, R., Klemm, D.J. & McFie, P.J. (1995) The cAMP response element binding protein synergizes with other transcription factors to mediate cAMP responsiveness. *J. Biol. Chem.* **270**, 8225–8232.
22. Dyck, J.R.B. & Fliegel, L. (1995) Specific activation of the Na^+/H^+ exchanger during neuronal differentiation of embryonal carcinoma cells. *J. Biol. Chem.* **270**, 10420–10427.
23. Jonk, L.J.C., de Jonge, M.E.J., Pals, C.E.G.M., Wissink, S., Vervaart, J.M.A., Schoorlemmer, J. & Kruijer, W. (1994) Cloning and expression during development of three murine members of the COUP family of nuclear orphan receptors. *Mech. Dev.* **47**, 81–97.
24. Nordeen, S.K. (1988) Luciferase reporter gene vectors for analysis of promoters and enhancers. *Biotechniques* **6**, 454–457.
25. Gorski, K., Carneiro, M. & Schibler, U. (1986) Tissue-specific *in vitro* transcription from the mouse albumin promoter. *Cell* **47**, 767–776.
26. Fernandez-Rachubinski, F.A., Weiner, J.H. & Blajchman, M.A. (1996) Regions flanking exon 1 regulate constitutive expression of the human antithrombin gene. *J. Biol. Chem.* **271**, 29502–29512.
27. Graves, B.J., Johnson, P.F. & McKnight, S.L. (1986) Homologous recognition of a promoter domain common to the MSV LTR and the HSV tk gene. *Cell* **44**, 565–576.
28. Vista-Picard, N.L., Hobbs, P.D., Pfahl, M., Dawson, M.I. & Pfahl, M. (1996) The receptor–DNA complex determines the retinoid response: a mechanism for the diversification of the ligand signal. *Mol. Cell. Biol.* **16**, 4137–4146.
29. Tran, P., Zhang, X.K., Salbert, G., Hermann, T., Lehmann, J.M. & Pfahl, M. (1992) COUP orphan receptors are negative regulators of retinoic acid response pathways. *Mol. Cell. Biol.* **12**, 4666–4676.
30. Quandt, K., Frech, K., Karas, H., Wingender, E. & Werner, T. (1995) MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res.* **23**, 4878–4884.
31. Hall, R.K. & Sladek, F.M. (1995) The orphan receptors COUP-TF and NHF-4 serve as accessory factors required for induction of phosphoenolpyruvate carboxykinase gene transcription by glucocorticoids. *Proc. Natl Acad. Sci. USA* **92**, 412–416.
32. Power, S.C. & Cereghini, S. (1996) Positive regulation of the vHNF1 promoter by the orphan receptors COUP-TF1/Ear3 and COUP-TFII/Arp1. *Mol. Cell. Biol.* **16**, 778–791.
33. Ktistaki, E. & Talianidis, I. (1997) Chicken ovalbumin upstream promoter transcription factors acts as auxiliary cofactors for hepatocyte nuclear factor 4 and enhance hepatic gene expression. *Mol. Cell. Biol.* **17**, 2790–2797.
34. Munoz, A. & Bernal, J. (1997) Biological activities of thyroid hormone receptors. *Eur. J. Endocrinol.* **137**, 433–445.
35. Cooney, A.J., Tsai, S.Y., O'Malley, B.W. & Tsai, M.-J. (1992) Chicken ovalbumin upstream promoter transcription factor (COUP-TF) dimers bind to different GGTCA response elements, allowing COUP-TF to repress hormonal induction of the Vitamin D3, Thyroid hormone, and retinoic acid receptors. *Mol. Cell. Biol.* **12**, 4153–4163.
36. Klinge, C.M., Silver, B.F., Driscoll, M.D., Sathya, G., Bambara, R.A. & Hilf, R. (1997) Chicken ovalbumin upstream promoter-transcription factor interacts with estrogen receptor, binds to estrogen response elements and half-sites, and inhibits estrogen-induced gene expression. *J. Biol. Chem.* **272**, 31465–31474.
37. Schrader, M., Danielsson, C., Wiesenberg, I. & Carlberg, C. (1996) Identification of natural monomeric response elements of the nuclear receptor RZR/ROR. They also bind COUP-TF homodimers. *J. Biol. Chem.* **271**, 19732–19736.
38. Schrader, M., Becker-Andre, M. & Carlberg, C. (1994) Thyroid hormone receptor functions as monomeric ligand-induced transcription factor on octameric half-sites. Consequences also for dimerization. *J. Biol. Chem.* **269**, 6444–6449.
39. Pipaon, C., Tsai, S.Y. & Tsai, M.J. (1999) COUP-TF upregulates NGFI-A gene expression through an Sp1 binding site. *Mol. Cell. Biol.* **19**, 2734–2745.
40. Pereira, F.A., Qiu, Y., Zhou, G., Tsai, M.-J. & Tsai, S.Y. (1999) The orphan nuclear receptor COUP-TFII is required for angiogenesis and heart development. *Genes Dev.* **13**, 1037–1049.
41. Wang, H., Singh, D. & Fliegel, L. (1997) The Na^+/H^+ antiporter potentiates growth and retinoic-acid induced differentiation of P19 embryonal carcinoma cells. *J. Biol. Chem.* **272**, 26545–26549.
42. Yang, W., Wang, H. & Fliegel, L. (1996) Regulation of Na^+/H^+ exchanger gene expression. Role of a novel poly(dA:dT) element in regulation of the NHE1 promoter. *J. Biol. Chem.* **271**, 20444–20449.
43. Qiu, Y., Krishnan, V., Pereira, F.A., Tsai, S.Y. & Tsai, M.J. (1996) Chicken ovalbumin upstream promoter-transcription factors and their regulation. *J. Steroid Biochem. Mol. Biol.* **56**, 81–85.
44. Kerber, B., Fellert, S. & Hoch, M. (1998) Seven-up, the *Drosophila* homolog of the COUP-TF orphan receptors, controls cell proliferation in the insect kidney. *Genes Dev.* **12**, 1781–1786.
45. Elsing, C., Reichem., J., Marti, U. & Renner, E.L. (1994) Hepatocellular Na^+/H^+ exchange is activated at transcriptional and posttranslational levels in rat biliary cirrhosis. *Gastroenterology* **107**, 468–478.

46. Rao, G.N., Sardet, C., Pouyssegur, J. & Berk, B.C. (1990) Differential regulation of Na^+/H^+ antiporter gene expression in vascular smooth muscle cells by hypertrophic and hyperplastic stimuli. *J. Biol. Chem.* **265**, 19393–19396.

47. Karmazyn, M., Gan, T., Humphreys, R.A., Yoshida, H. & Kusumoto, K. (1999) The myocardial Na^+-H^+ exchange. Structure, regulation, and its role in heart disease. *Circ. Res.* **85**, 777–786.