Molecular and Cellular Biochemistry **243:** 87–95, 2003.

Transcriptional regulation of Na^+/H^+ exchanger expression in the intact mouse

Carmen V. Rieder and Larry Fliegel
Department of Biochemistry, CIHR Membrane Protein Group, Faculty of Medicine, University of Alberta, Edmonton, Alberta, Canada

Received 15 March 2002; accepted 7 August 2002

Abstract

We examined regulation of the Na^+/H^+ exchanger (NHE1 isoform) in the developing mouse. We generated transgenic mice with the Na^+/H^+ exchanger promoter directing expression of the β-Galactosidase reporter. We found that expression of the Na^+/H^+ exchanger was maximum in the heart and liver of 12-day-old embryonic mice. Similar results were found in mice using the green fluorescent protein reporter driven by the Na^+/H^+ exchanger promoter. Detailed examination of the myocardium revealed that the GFP reporter protein was expressed in the cytoplasm of cardiomyocyte cells. We examined NHE1 protein expression in transgenic mice lacking the transcription factors AP-2α or the transcription factor COUP-TF1. Eighteen-day-old AP-2α heterozygote mice show no large changes in NHE1 expression in heart, lung, liver, kidney and brain. In contrast, 18-day-old embryos from AP-2α null mice showed a large increase in Na^+/H^+ exchanger protein expression in the brain. NHE1 protein levels in COUP-TF1 knockout embryos did not differ from wild type embryos. The results suggest that AP-2α and COUP-TF1 are not critical to NHE1 expression in the late stage embryo and that other related transcription factors may function in regulation of the Na^+/H^+ exchanger. (Mol Cell Biochem **243**: 87–95, 2003)

Key words: AP-2, COUP-TF, differentiation, neonatal development, pH regulation

Introduction

The Na^+/H^+ exchanger is a plasma membrane protein responsible for regulating intracellular pH in eukaryotic cells. It removes one intracellular proton in exchange for an extracellular Na^+ when intracellular pH declines. Several isoforms of the protein exist. The NHE1 isoform was the first discovered and is ubiquitous in mammalian cells. It is the only plasma membrane isoform present in the myocardium in significant quantities [1]. The NHE1 isoform of the Na^+/H^+ exchanger is important in a variety of cellular processes related to pH regulation. For example, NHE1 participates in calcium overload in the heart during ischemia and reperfusion. The Na^+/H^+ exchanger removes protons that accumulate during ischemia and the resultant Na^+ is thought to reverse or reduce the activity of the Na^+/Ca^{2+} exchanger. The consequent calcium overload is implicated in many effects detrimental to the myocardium [2, 3]. More recently the myocardial Na^+/H^+ exchanger has been shown to contribute to hypertrophy of the heart and blockage of the Na^+/H^+ exchanger prevents hypertrophy [4, 5].

In other tissues, the Na^+/H^+ exchanger is important in pH regulation, but also plays a role in growth and development. Expression of the Na^+/H^+ exchanger is increased during differentiation of HL-60 (human leukemic) cells [6, 7]. We have demonstrated that there is a transient increase in the level of NHE1 transcription during retinoic acid induced differentiation of P19 (embryonal carcinoma) cells and this is accompanied by an increase in activity of the protein that is necessary for cell differentiation [8, 9]. A number of other studies have suggested that the Na^+/H^+ exchanger causes a transient rise in intracellular pH that is important for growth and differentiation in some cell types [10–13]. Deletion of the Na^+/H^+ exchanger in mice causes neurological defects and greatly

Address for offprints: L. Fliegel, Department of Biochemistry, Faculty of Medicine, University of Alberta, 347 Medical Science Building, Edmonton, Alberta T6G 2H7, Canada (E-mail: lfliegel@ualberta.ca)

reduces their growth and viability [14]. This suggests that the Na^+/H^+ exchanger plays an important, though not essential role in cell growth and differentiation.

In earlier studies, we have examined the regulation of expression of the Na^+/H^+ exchanger in the myocardium and other tissues. We [15] and others [16] have shown that the expression of the Na^+/H^+ exchanger is increased in the myocardium during ischemia and reperfusion. We cloned and examined the characteristics of the mouse Na^+/H^+ exchanger promoter (NHE1 isoform). The mouse NHE1 promoter/enhancer region possesses a number of putative regulatory elements including two CAT boxes, an SP-1 site, a cyclic AMP response element binding site (CREB) and an AP-2 site [17]. Deletion of all these sites except the AP-2 consensus sequence reduces the transcription activity of a reporter gene by 30–70%, depending on cell type [9, 17]. Gel mobility shift analysis showed that the transcription factor AP-2 can bind to this region, and DNase I footprinting analysis showed that this region is protected by nuclear extracts from NIH 3T3 cells. In addition, transfection of AP-2 deficient cells with an AP-2α expression plasmid results in increased activity of the NHE1 promoter [17]. In the myocardium, the transcription factor AP-2 regulates NHE1 promoter activity; however this effect is less in the heart compared to other tissues [18]. It is of note that the AP-2 site of the mouse promoter is involved in the regulation of the Na^+/H^+ exchanger during differentiation of embryonal carcinoma cells [9].

More recently, we have shown that more distal sites of the NHE1 promoter are also involved in regulation of the Na^+/H^+ exchanger gene expression. Mitogenic activation of cells increases Na^+/H^+ exchanger transcription in some cell types and involves a distal site on the NHE1 mouse promoter [19]. We discovered that COUP-TF I and II (originally discovered as chicken ovalbumin upstream promoter-transcription factors) are involved in regulation of Na^+/H^+ exchanger expression in this region of the promoter. COUP-TFI and II are orphan receptors that are implicated in regulation of embryonic development and neuronal cell fate determination. We showed that the Na^+/H^+ exchanger is a novel target for COUP-TF I and II regulation. The COUP-TF proteins were found to bind to a region between –841/–800 nt of the mouse NHE1 promoter. *In vivo* expression of COUP-TF isoforms in NIH3T3 or CV1 cells transactivated the promoter from this element and from the entire NHE1 promoter [20].

While we have shown that both the transcription factors AP-2 and COUP-TF I and II are important in Na^+/H^+ exchanger transcription in cultured cells [9, 15, 20], their role in regulation of Na^+/H^+ exchanger expression in intact animals has not been examined. In this study, we examined regulation of Na^+/H^+ exchanger expression in the myocardium and other tissues. We also examined the specific effects of ablation of the transcription factors AP-2α and COUP-TF1 on Na^+/H^+ exchanger expression in intact mice. The results are the first demonstration of the effect of transcription factor deletion on NHE1 expression in intact animals.

Materials and methods

Transgenic mice

We examined regulation of the NHE1 promoter *in utero* in the myocardium by constructing transgenic mice with the β-Galactosidase (β-Gal) enzyme under the control of the NHE1 promoter. The promoter-reporter construct contained a 3.8-kb fragment of the mouse NHE1 promoter and β-Gal enzyme placed after the transcription start site.

The β-Gal reporter construct was generated as follows. A 3.8-kb portion of the mouse NHE1 promoter was obtained from the plasmid pXP1–5.0 [21]. A HindIII to SalI digestion was used to obtain a 3.8-kb NHE1 promoter fragment, which was cloned into the corresponding sites in the vector, pSP73 (Promega) to make pHS-SP as described earlier [22]. XhoI and SalI digestions removed a 4.6-kb fragment containing the β-Gal gene from pGal-Basic (CLONTECH). Next, the β-Gal gene was ligated into pHS-SP at the SalI site, thereby placing the β-Gal gene 16 basepairs downstream of the transcription start site, and producing the desired plasmid, pHS-SP (β-Gal). Restriction mapping and DNA sequencing confirmed that pHS-SP(β-Gal) was correctly constructed.

Before constructing the transgenic mice, XhoI and SalI restriction enzyme digests extracted the 8221-bp NHE1 promoter and β-Gal reporter gene from pHS-SP(β-Gal). This linearized DNA fragment was injected into the pronucleus of preimplantation embryos (obtained from the oviducts of pregnant FVB/N females less than 20 h after fertilization). Once injected the fertilized eggs were transferred into the oviduct of a 1/2-day-old postcoitum pseudopregnant FVB/N female. Mating with vasectomized males was used to generate the pseudopregnant females. (All transgenic procedures were performed by Dr. Peter Dickie of the Transgenic Facility, University of Alberta Health Sciences Laboratory Animal Services, Edmonton, Alberta, Canada). All procedures on animals conformed to the Canadian Council on Animal Care regulations.

Tail biopsies of embryos or ear notches of neonates were used to obtain genomic DNA from putative transgenic mice. Mice harboring the transgene were identified by polymerase chain reaction using 5′ and 3′ primers in the coding region of the reporter. Specifically, the primers were as follows:

β-Gal Forward #1: 5′-CCA TGT CGT TTA CTT TGA CCA ACA A-3′

β-Gal Reverse #6: 5′-CGG CCT CAG GAA GAT CGC ACT CC-3′

Two β-Gal-expressing mouse lines were maintained. Selective breeding generated homozygotes, which was desirable for increased transgene expression, ease in future breeding and subsequent analysis of litters.

X-Gal staining of β-Galactosidase reporter embryos

Mouse embryos were dissected from the uterus at embryonic day 12 and immediately placed in an ice-cold phosphate buffer (P-buffer) containing 13.8 g/l NaH_2PO_4 and 14.2 g/l Na_2HPO_4. Next, embryos were transferred to individual wells of a 12-well plate and fixed. Fixation was done for 1 h, at room temperature, with agitation, in Fix buffer (0.2% gluteraldehyde, 5 mM EGTA pH 7.3, and 20 mM $MgCl_2$ in P-buffer). Next, embryos were rinsed 3 times, for 30 min each, in Wash buffer (100 mM sodium phosphate pH 7.3, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40). Embryos were stained for 36–48 h at 37°C in staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mg/ml X-Gal in Wash buffer). Once sufficient staining was evident, embryos were post-fixed overnight in 10% formalin, at 4°C with agitation. At this point, embryos were washed twice with water for 30 min each, and then placed in 70% ethanol for storage at 4°C.

Prior to photographing stained whole embryos, tissues were cleared with methyl salicylate. Embryos were initially dehydrated by replacing the 70% ethanol storage solution with 95% ethanol, and incubating for 30 min, at room temperature, with agitation. Embryos were then washed twice, for 30 min each, with 100% ethanol. Finally, embryos were transferred to 100% methyl salicylate in glass tubes, and incubated at room temperature, with agitation, for 10–15 min until the tissue cleared sufficiently. Embryos were photographed using a Leica MonoZoom 7 light microscope equipped with a Sanyo Hi-Resolution Color CCD camera.

To see the staining of internal organs more clearly, some whole stained embryos were cut in half using a cryostat. The embryos were frozen in cryomatrix as described earlier [22]. Sections of 20 μm thicknesses were cut and discarded until about half of the embryo remained. The embryos were washed with water to remove any residual cryomatrix material, dehydrated with ethanol, and then cleared with methyl salicylate as described above. The bisected embryos were photographed using a Leica MonoZoom 7 light microscope equipped with a Sanyo Hi-Resolution Color CCD camera. The given results are representative of 4–5 individual embryos for both controls and β-Gal-positive lines.

Preparation and microscopy of GFP-positive embryos

For some experiments, we used transgenic mice that contained an identical promoter construct to the β-Gal-positive lines except that the β-Gal reporter was replaced with a green fluorescent protein (GFP) reporter. Construction of the mice and examination of the GFP positive embryos was as described earlier [22]. Images of sagittal cryostat sections were obtained using a 10× or 100× objective on a Zeiss confocal microscope with fluorescein isothiocyanate filters. Identical confocal settings were used to collect images of controls and transgenic embryos and images were reconstructed using Adobe Photoshop.

NHE1 Western blot analysis

Preparation of total protein from tissues

Organs were harvested from 18-day-old mouse embryos and immediately frozen in liquid nitrogen. Crude microsome preparations were prepared essentially as described earlier [22]. To obtain sufficient protein, organs from several littermates were pooled. Pellets containing the membrane fractions were resuspended in buffer containing 1M NaCl, 100 mM Tris pH 7.4, 0.1 mM PMSF, 0.1 mM Benzamidine, 37.5 μM ALLN (calpain I inhibitor) and a proteinase inhibitor cocktail [23] with the addition of 1% SDS to aid in solubilization. Protein was quantified using the Bio-Rad D_C Protein Assay kit.

NHE1 immunoblots

Expression of the NHE1 protein was examined using an anti-NHE1 monoclonal antibody purchased from CHEMICON International as described earlier [22]. Briefly, crude membrane fractions containing 60–100 μg total protein were run on 10% polyacrylamide gels, followed by transfer to nitrocellulose membranes. Equal transfer of proteins was ensured by Ponceau S staining the nitrocellulose membranes. After immunoblotting with anti-NHE1 antibody, the Amersham Enhanced Chemiluminescence reaction was used to visualize immunoreactivity. An internal sample of 1 μg of GST protein was added to each total protein sample to use as a control for protein loading and efficiency of transfer. The immunoblots were stripped and probed for the GST protein after probing for NHE1.

AP-2 and COUP-TF1 knock-out mice

Mice with a disruption of the AP-2α gene were obtained from Dr. T. Williams (Yale University, New Haven, CT, USA) and have been described earlier [24]. Tissues from chicken ovalbumin upstream promoter transcription factor I (COUP-TFI) knockout mice were generously provided by Dr. M.J. Tsai (Department of Cell Biology, Baylor College of Medicine, Houston, TX, USA).

Maintenance and transfection of cell lines

NIH3T3 cells were grown on coverslips as described earlier [17]. Cells were transfected with 4 μg of either NHE1-GFP reporter, no reporter (mock transfected) or NHE1-β-Gal as described earlier [17]. To examine GFP fluorescence, coverslips were washed with PBS, fixed with 4% paraformaldehyde and photomicrographs were taken using an Olympus BX50 fluorescent microscope (Olympus, Japan) equipped with a SPOT digital camera (Diagnostics Instruments). β-Gal activity was measured using the Galacto-Light Plus Chemiluminescent Reporter Assay (Tropix). β-Gal activity of the control was set to one and other values given are relative to the control.

Results

In this study we examined regulation of Na^+/H^+ exchanger expression using two different promoter-reporter DNA constructs in transgenic mice. The use of two different promoter-reporter constructs allowed us to verify that the results we observed were not due to an artifact of the reporter system. Figure 1A is a schematic diagram illustrating the β-Gal NHE1 promoter reporter gene construct used to generate the transgenic mice described in this study. Figure 1B illustrates our previously made construct [22] which has the same fragment of the mouse NHE1 promoter driving expression of the GFP. Figure 2 confirms that the NHE1 promoter directs expression of the reporter plasmids. Figure 2A illustrates that the GFP protein is produced under the direction of the NHE1 promoter in NIH3T3 cells. Mock transfected cells showed virtually no fluorescence while the GFP transfected cells showed marked expression of this protein. To test the β-Gal reporter construct

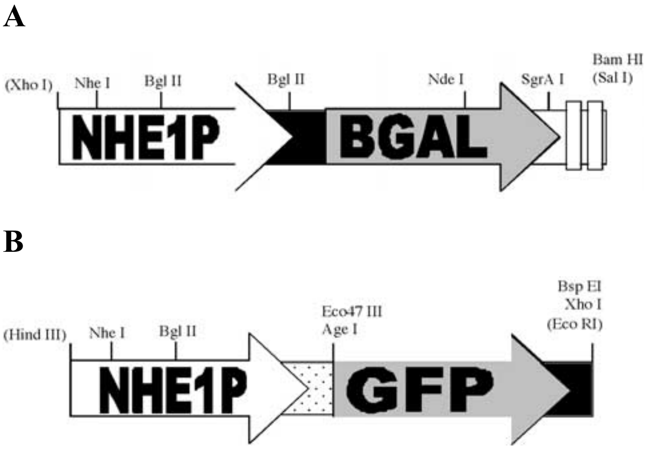
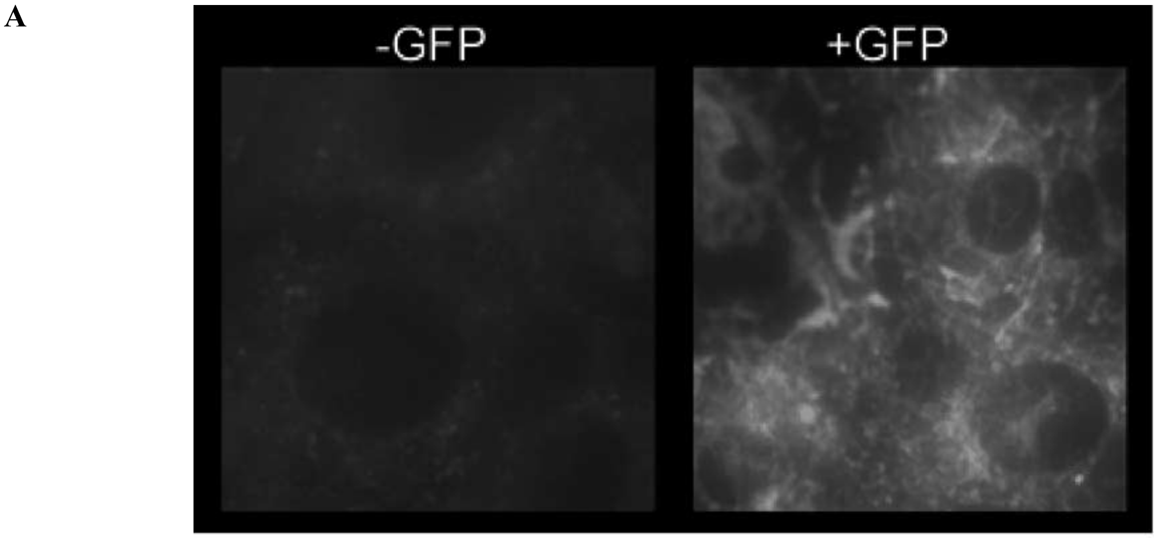


Fig. 1. Schematic diagram of the NHE1 promoter-reporter gene DNA constructs used. (A) NHE1 promoter-β-Galactosidase reporter gene construct. (B) NHE1 promoter – GFP reporter gene construct.



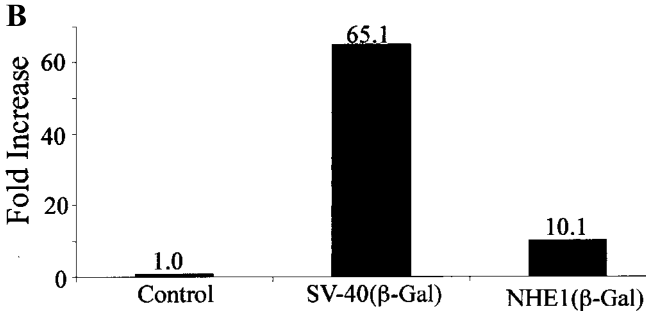


Fig. 2. Expression of the NHE1 promoter-reporter gene constructs in transfected NIH3T3 cells. (A) Expression of the GFP reporter in NIH3T3 cells. Cells were transiently transfected with 4 μg of GFP reporter (+GFP) and grown on coverslips as described in Materials and methods. Photomicrographs were taken using an Olympus BX50 fluorescent microscope with a SPOT digital camera. (B) Expression of the β-Gal reporter in NIH3T3 cells. Cells were transfected with 24 μg of NHE1-β-Gal reporter, 14 μg of SV-40-β-Gal reporter or no DNA (Control). β-Galactosidase activity was then determined and the control was set to a value of 1. (Results are shown in grayscale.)

we measured activity of the β-Gal enzyme in transfected cells. Cells transfected with the NHE1 promoter directing expression of β-Gal showed a 10-fold greater enzyme activity compared to background. By comparison, cells transfected with β-Gal under control of the strong viral SV-40 promoter, showed a much higher level of expression. These results were similar to those we demonstrated earlier which suggested that relative to the viral promoters, the NHE1 promoter directs a much lower level of transcription [17].

Having demonstrated the efficacy of the NHE1 promoter constructs, we examined the production of the appropriate reporters in transgenic mice harbouring the NHE1 promoter reporter plasmids. We have earlier suggested that the expression from the Na^+/H^+ exchanger promoter was maximal in the heart and liver of 12-day-old embryos [22]. We thus examined Na^+/H^+ exchanger transcription levels in intact embryos of this age from transgenic mice that contained the β-Gal reporter gene driven by the Na^+/H^+ exchanger promoter. Figure 3 shows 12-

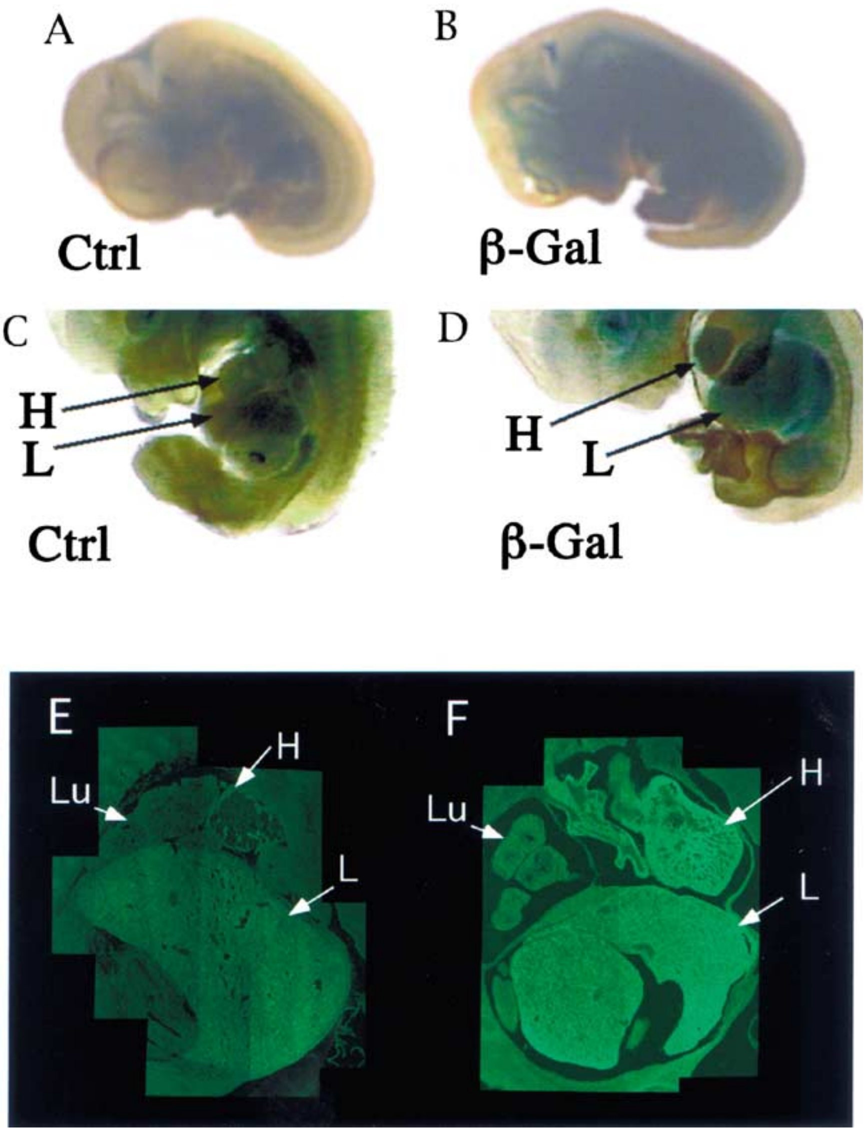


Fig. 3. Activation of the NHE1 promoter in 12-day-old embryos. (A–D) Activation of the NHE1 promoter in β-Gal-positive embryos. (A and B) Whole embryos stained with X-Gal. Embryos were harvested, stained and cleared with methyl salicylate. Photomicrographs were with a Leica MonoZoom 7 light microscope equipped with a Sanyo Hi-Resolution Color CCD camera. A – control embryo; B – β-Gal-positive transgenic. (C and D) Cross-section of X-Gal stained embryos. C – control; D – β-Gal-positive transgenic. Whole X-Gal stained embryos were frozen in cryomatrix, cut in half using a cryostat and cleared with methyl salicylate. Photomicrographs were obtained as with (A) and (B). H – heart; L – liver. (E and F) Activation of the NHE1 promoter in GFP-positive embryos. Representative sections through the heart, liver and lung of control (E) and GFP-positive (F) mice showing relative fluorescence. H – heart; L – liver; Lu – lung.

day-old embryos stained with X-Gal to visualize the β-Gal reporter. Figures 3A and 3B are control and β-Gal transgenic whole embryos (respectively). There was clearly more staining of the β-Gal transgenic embryo in comparison to the control, though it was difficult to discern individual organs in the whole embryos. To examine the individual organs that were stained in more detail, we made cross sections of 12-day-old β-Gal-positive and age-matched control embryos. In the cross-sectional views there was significant blue staining in the heart and liver of the β-Gal-positive animal compared to the control (Figs 3C and 3D). These results showed that the heart and liver transcribe relatively high levels of the Na^+/H^+ exchanger at this age in embryogenesis. Two independently made lines of transgenic β-Gal-positive mice gave similar results, with the heart and liver of the positive transgenics displaying more β-Gal activity compared to the control.

To confirm these results, we repeated the experiment using intact embryos from transgenic mice with the Na^+/H^+ exchanger promoter driving expression of the GFP reporter. The results are given in Figs 3E and 3F. The figure shows an enlargement of the heart, liver and lung of control (Fig. 3E) and GFP-positive (Fig. 3F) transgenic animals. The results are typical of over 5 determinations from two different transgenic mouse lines. GFP fluorescence was significantly greater in the heart and liver compared to control animals. The fluorescence of the lung was not elevated over background. Thus, it appears that NHE1 transcription is activated in both the heart and liver at embryonic day 12, while it remains below the level of detection in the lung and other tissues (not shown). The results with the GFP reporter are similar to those we have observed earlier [22]. We noted that the liver usually contained higher staining than the myocardium in both the β-Gal-positive mice and the GFP reporter system. In the β-Gal-positive mice we also sometimes noted significant staining of the eye region, however this was not demonstrated in the GFP reporter system (not shown) suggesting it might be an artifact of the reporter system being used.

To examine the cells expressing the GFP protein in more detail we examined a 100× magnification of the heart wall (myocardium). We chose this region because it was found to fluoresce brightly at lower magnifications. The photomicrographs (Fig. 4) demonstrate that the GFP protein is expressed in the cytosol as expected. Control tissue showed a greatly reduced level of fluorescence. It was noted that a subset of the cells had a higher level of transgene expression. Based on their numbers, morphology and location, the GFP-expressing cells were cardiomyocytes, indicating that the Na^+/H^+ exchanger promoter was active in cardiomyocytes at this age.

To further understand the regulation of the Na^+/H^+ exchanger expression in the developing mouse, we examined expression of the Na^+/H^+ exchanger protein in tissues from 18-day-old embryos that lacked the AP-2α transcription factor (homozygote), contained only one copy of the AP-2α gene (heterozygotes) or had both copies of the AP-2α gene (homozygote, wild type). Figure 5A shows a Western blot of heterozygote and wild type heart, lung, liver, kidney and brain from 18-day-old embryos. In the heterozygotes, Na^+/H^+ exchanger protein abundance is unaltered by the AP-2α gene disruption (Fig. 5A). In contrast, brain tissue from an 18-day-old embryo homozygous for the AP-2α knockout shows a surprisingly large (7–8-fold) increase in Na^+/H^+ exchanger expression. The homozygous AP-2α knockout embryo is severely deformed [24] thus only the brain could be readily identified for this analysis. For this experiment homozygote and wild type samples contained tissues from 5 animals. Protein levels were normalized using Ponceau S staining and immunoblotting of internal protein standards after probing for the Na^+/H^+ exchanger.

The COUP-TF transcription factors have been shown to be important in Na^+/H^+ exchanger expression in cultured cells [20]. To examine the role that the COUP-TF transcription factors could have in expression of the Na^+/H^+ exchanger, we used a similar approach to the AP-2α analysis described above. Figure 5B shows the abundance of NHE1 protein in the same tissues from wild type and COUP-TF1 knock out mice at embryonic day 18. In all tissues studied, no major changes in NHE1 protein expression occurred and there was no evidence of major decreases in NHE1 expression in these tissues. Slight variations in the molecular weight of NHE1 were likely caused by different degrees of glycosylation which has been reported earlier [25]

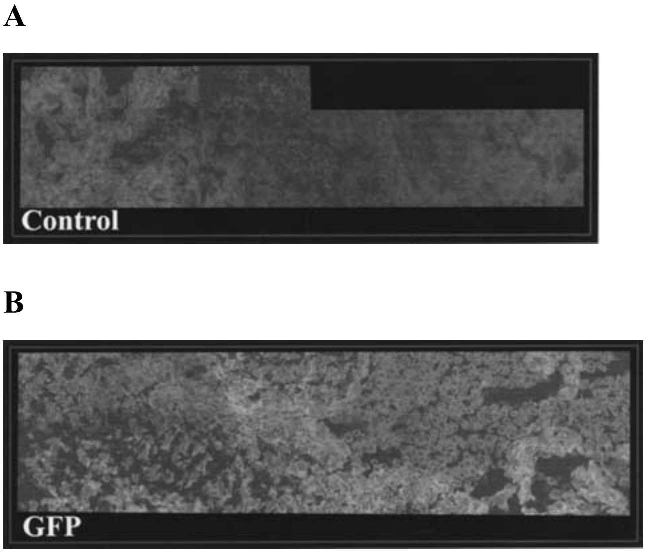


Fig. 4. Activation of the NHE1 promoter in 12-day-old embryo hearts visualized at high magnification. Images were obtained using a 100× Plan-Apochromat objective on a Zeiss LSM 510 confocal microscope. Images were assembled using Adobe Photoshop to illustrate a larger region of the myocardium. Control sections were from a wild type 12-day-old embryo and sections labeled GFP were from a GFP-positive transgenic mouse. (Shown in grayscale.)

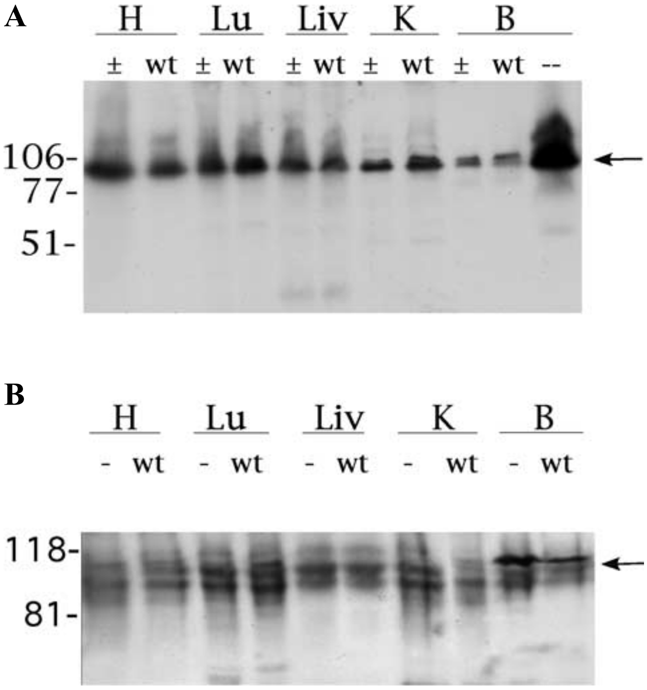


Fig. 5. Analysis of NHE1 protein expression in mice lacking the transcription factors AP-2α or COUP-TFI. (A) Western blot of NHE1 protein expression in the heart (H), lung (Lu), liver (Liv), kidney (K) and brain (B) of wild type (wt), heterozygous AP-2α knockouts (+/–) and homozygous AP-2α knockout (--) in 18-day-old embryos. (B) Western blot of NHE1 protein expression in 18-day-old embryos from wild type (wt) and homozygous COUP-TFI knockout mice (–). Arrows indicate the position of the 105–110 kDa NHE1 protein.

Discussion

Because of the critical role the Na^+/H^+ exchanger plays in development, differentiation and heart disease, regulation of the Na^+/H^+ exchanger expression in the myocardium and other tissues is of great importance. In this study, we used a number of transgenic approaches to study the regulation of the Na^+/H^+ exchanger in the intact mouse. In one approach, we made a transgenic mouse with a 3.8 kb fragment of the Na^+/H^+ exchanger promoter driving expression of the β-Gal reporter. This sensitive system was necessary to detect Na^+/H^+ exchanger transcription in the intact animal because the NHE1 promoter is relatively weak, as demonstrated in this (Fig. 2) and our earlier study [17]. When we examined the levels of promoter activity in whole mouse embryos, we found that there was significant Na^+/H^+ exchanger transcription in the heart and lung of 12-day-old embryos. This occurred with the β-Gal reporter and with the GFP reporter system. We have earlier demonstrated a similar result with the GFP reporter system [22], and the present results confirm this finding using a novel β-Galactosidase reporter system.

To confirm that the transcription of the Na^+/H^+ exchanger was occurring in cardiomyocytes, we examined sections of the heart wall at higher magnification. The sections (Fig. 4) confirmed that the GFP protein was found in the cytosol and that expression was occurring in cardiomyocyte cells. From these results it was clear that the early expression of the Na^+/H^+ exchanger gene in the 12-day-old embryonic heart was occurring predominantly in the developing cardiomyocyte.

Previous studies have characterized the NHE1 promoter and have identified binding sites for numerous transcription factors including AP-2 [17] and COUP-TF [20]. Dyck *et al.* [17] demonstrated the importance of the AP-2 site for NHE1 transcription. In these studies, constructs containing truncated versions of the NHE1 promoter were coupled to the luciferase reporter gene and transfected into human hepatoma and mouse fibroblast cell lines. It was found that deletion of the promoter upstream of the AP-2 site resulted in a 25% reduction in reporter activity however removal of the same upstream region plus the AP-2 site caused a 6-fold decrease in NHE1 transcription. Similarly, the AP-2 binding site is important for NHE1 expression in P19 cells [9], and rat neonatal myocytes [19]. Because of the apparent importance of this transcription factor in intact cells, we examined the effect of deletion of this gene on the amount of NHE1 protein in mice. In embryonic day 18 mice, no difference in Na^+/H^+ exchanger protein expression was detected in heart, lung, liver, kidney and brain from animals heterozygous for the AP-2α deletion (Fig. 5A). Interestingly, a marked increase in NHE1 protein expression was demonstrated in the brain of an 18-day-old embryo homozygous for the AP-2 disruption (Fig. 5A). Due to the severe deformity of the homozygous AP-2 knockouts, and the fact that these animals die at birth, no other organs could be clearly distinguished and it was not possible to obtain tissues from older animals for analysis. Regardless, taken together, these results provide some insight into the role of AP-2 in NHE1 transcription. Since NHE1 protein levels were similar in wild type and heterozygote at the two ages examined, it may be the transcription factor AP-2 activates expression of the NHE1 gene much earlier in embryonic development. Thus, by embryonic day 18, one copy of the AP-2 gene was sufficient to activate Na^+/H^+ exchanger expression to produce an appropriate amount of protein. It is of note that the heterozygote mice are relatively normal in growth and appearance [26] and exhibit only some decreases in fertility and other minor physical problems. It may be that the level of expression of AP-2 protein from the heterozygote may be similar to the wild type and therefore Na^+/H^+ exchanger expression is not affected.

An alternative explanation for the results with the AP-2 heterozygote mice is that another member of the AP-2 transcription factor family may be responsible for activation of the NHE1 gene. To date, three proteins have been placed in this family: AP-2α, AP-2β, and AP-2γ. Although they recognise a common DNA binding sequence, members differ somewhat in their expression patterns [26]. It may be that when one member is knocked-out, other proteins from that family are overexpressed. This may explain the abundance of Na^+/H^+ exchanger protein observed in the brain of the 18-day-old homozygote knockout mouse (Fig. 5A). In several cases complete knockout or inactivation of one member of a gene family has resulted in compensatory increase in other members of a gene family [27, 28]. If there is a compensatory increase by other members of the AP-2 family that are important in NHE1 expression, this would explain the increase we saw in the brain of these mice. In this regard it is of interest that in gel mobility shift assays, purified AP-2α protein produces a smaller shift than that seen with proteins derived from nuclear extracts [21]. Thus, it is plausible that NHE1 expression may be regulated by a different member of the AP-2 transcription factor in tandem or in combination with other transcription factors that differ from the AP-2α isoform disrupted in mice used in the present study.

We also examined NHE1 protein levels in various organs from 18-day-old embryos of a COUP-TF1 knockout line. Our earlier results have shown COUP-TFs are important in Na^+/H^+ exchanger expression [20]. Figure 5B shows that there is no difference in the quantity of NHE1 protein in organs from animals lacking COUP-TF1 compared to wild type embryos. Analogous to the discussion of the AP-2α knock-out results above, it may be that COUP-TF1 acts on the NHE1 promoter at a different stage of development or that another member of the chicken ovalbumin upstream promoter family of transcription factors is responsible for activation of the NHE1 promoter. In our earlier study [20], we found that COUP-TFII was more effective in enhancing Na^+/H^+ exchanger expression than COUP-TFI. This may mean that it is more significant in regulating Na^+/H^+ exchanger transcription in intact animals than COUP-TF1. Since deletion of COUP-TFII results in death of embryos *in utero*, it was not possible to examine the effect of COUP-TFII knockouts on Na^+/H^+ exchanger expression.

Our study has shown that expression of the NHE1 isoform of the Na^+/H^+ exchanger is high *in utero* at embryonic day 12 in the heart and liver of the developing mouse fetus. Results with the β-Gal reporter confirm those with the GFP reporter system. We demonstrate that the transcription of the Na^+/H^+ exchanger is occurring in cardiomyocytes of the intact myocardium. Deletion of the transcription factors AP-2α and COUP-TFI did not affect Na^+/H^+ exchanger expression in the embryonic tissues examined. Further experiments are necessary to understand regulation of expression in the intact embryo.

Acknowledgements

We are grateful for the gift of AP-2α transcription factor disruption mice from Dr. T. Williams (Yale University, New Haven, CT, USA) and for the embryos from COUP-TF1 deletion mice from Dr. M.J. Tsai (Department of Cell Biology, Baylor College of Medicine, Houston, TX, USA). Supported by funding from the CIHR and Heart and Stroke Foundation of Canada. LF is a Scientist of the Alberta Heritage Foundation for Medical Research. CVR was supported by a studentship from the Alberta Heritage Foundation for Medical Research.

References

1. Fliegel L: Regulation of myocardial Na^+/H^+ exchanger activity. Basic Res Cardiol 96: 301–305, 2001
2. Karmazyn M: Therapeutic potential of Na-H exchange inhibitors for the treatment of heart failure. Expert Opin Invest Drugs 10: 835–843, 2001
3. Karmazyn M, Sostaric JV, Gan XT: The myocardial Na^+/H^+ exchanger: A potential therapeutic target for the prevention of myocardial ischaemic and reperfusion injury and attenuation of postinfarction heart failure. Drugs 61: 375–389, 2001
4. Kusumoto K, Haist JV, Karmazyn M: Na(+)/H(+) exchange inhibition reduces hypertrophy and heart failure after myocardial infarction in rats. Am J Physiol Heart Circ Physiol 280: H738–H745, 2001
5. Yoshida H Karmazyn M: Na(+)/H(+) exchange inhibition attenuates hypertrophy and heart failure in 1-wk postinfarction rat myocardium. Am J Physiol 278: H300–H3004, 2000
6. Rao GN, Sardet C, Pouyssegur J, Berk BC: Na^+/H^+ antiporter gene expression increases during retinoic acid-induced granulocytic differentiation of HL60 cells. J Cell Physiol 151: 361–366, 1992
7. Ladoux A, Miglierina R, Krawice I, Cragoe EJ, Abita JP, Frelin C: Single-cell analysis of the intracellular pH and its regulation during the monocytic differentiation of U937 human leukemic cells. Eur J Biochem 175: 455–460, 1988
8. Wang H, Singh D, Fliegel L: The Na^+/H^+ antiporter potentiates growth and retinoic-acid induced differentiation of P19 embryonal carcinoma cells. J Biol Chem 272: 26545–26549, 1997
9. Dyck JRB, Fliegel L: Specific activation of the Na^+/H^+ exchanger during neuronal differentiation of embryonal carcinoma cells. J Biol Chem 270: 10420–10427, 1995
10. Grinstein S, Rotin D, Mason MJ: Na^+/H^+ exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation. Biochim Biophys Acta 988: 73–97, 1989
11. Lane M, Baltz JM, Bavister DD: Regulation of intracellular pH in hamster preimplantation embryos by the sodium hydrogen (Na^+/H^+) antiporter. Biol Reprod 59: 1483–1490, 1998
12. Lagarde AE, Pouyssegur JM: The Na:H antiport in cancer. Cancer Biochem Biophys 9: 1–14, 1986
13. Rich IV, Brackamm. I, Worthington-White D, Dewey MJ: Activation of the sodium/hydrogen exchanger via the firbronectin-integrin pathway results in hemtopoietic stimulation. J Cell Physiol 177: 109–122, 1998
14. Bell SM, Schreiner CM, Schultheis PJ, Miller ML, Evans RL, Vorhees CV, Shull GE, Scott WJ: Targeted disruption of the murine Nhe1 locus induces ataxia, growth retardation, and seizures. Am J Physiol 276: C788–C795, 1999
15. Dyck JRB, Maddaford T, Pierce GN, Fliegel L: Induction of expression of the sodium–hydrogen exchanger in rat myocardium. Cardiovasc Res 29: 203–208, 1995
16. Gan XT, Chakrabarti S, Karmazyn M: Modulation of Na^+/H^+ exchange isoform 1 mRNA expression in isolated rat hearts. Am J Physiol 277: H993–H998, 1999

17. Dyck JRB, Silva NLCL, Fliegel L: Activation of the Na$^+$/H$^+$ exchanger gene by the transcription factor AP-2. J Biol Chem 270: 1375–1381, 1995
18. Yang W, Dyck JRB, Wang H, Fliegel L: Regulation of the NHE-1 promoter in the mammalian myocardium. Am J Physiol 270: H259–H266, 1996
19. Besson P, Fernandez-Rachubinski F, Yang W, Fliegel L: Regulation of Na/H exchanger gene expression: Mitogenic stimulation increases NHE1 promoter activity. Am J Physiol 274: C831–C839, 1998
20. Fernandez-Rachubinski F, Fliegel L: COUP-TF I and COUP-TFII regulate expression of the NHE through a nuclear hormone responsive element with enhancer activity. Eur J Biochem 268: 620–634, 2001
21. Yang W, Dyck JRB, Fliegel L: Regulation of NHE1 expression in L6 muscle cells. Biochim Biophys Acta 1306: 107–113, 1996
22. Rieder CV, Fliegel L: Developmental regulation of the Na$^+$/H$^+$ exchanger expression in the fetal and neonatal mouse. Am J Physiol 283: H598–H605, 2002
23. Silva NLCL, Haworth RS, Singh D, Fliegel L: The carboxyl-terminal region of the Na/H exchanger interacts with mammalian heat shock protein. Biochemistry 34: 10412–10420, 1995
24. Zhang J, Hagopian-Donaldson S, Serbedzija G, Elsemore J, Plehn-Dujowich D, McMahon AP, Flavell RA, Williams T: Neural tube, skeletal and body wall defects in mice lacking transcription factor AP-2. Nature 381: 238–241, 1996
25. Murtazina BR, Booth BJ, Bullis BL, Singh DN, Fliegel L: Functional analysis of polar amino acid residues in membrane associated regions of the NHE1 isoform of the Na$^+$/H$^+$ exchanger. Eur J Biochem 268: 1–13, 2001
26. Talbot D, Lorgin J, Schorle H: Spatiotemporal expression pattern of keratins in skin of AP-2A-deficient mice. J Invest Dermatol 113: 816–820, 1999
27. Somigliana E, Vigano P, Filardo P, Candiani M, Vignali M, Panina-Bordignon P: Use of knockout transgenic mice in the study of endometriosis: Insights from mice lacking beta(2)-microglobulin and interleukin-12p40. Fertil Steril 75: 203–206, 2001
28. Wang XJ, Greenhalgh DA, Donehower LA, Roop DR: Cooperation between Ha-ras and fos or transforming growth factor alpha overcomes a paradoxic tumor-inhibitory effect of p53 loss in transgenic mouse epidermis. Mol Carcinogen 29: 67–75, 2000