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Developmental regulation of Na^+/H^+ exchanger expression in fetal and neonatal mice

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Rieder, Carmen V., and Larry Fliegel. Developmental regulation of Na^+/H^+ exchanger expression in fetal and neonatal mice. *Am J Physiol Heart Circ Physiol* 283: H273–H283, 2002. First published February 28, 2002; 10.1152/ajpheart.00042.2002.—We examined the hypothesis that Na^+/H^+ exchanger expression is regulated during fetal and neonatal development and differentiation. To examine transcriptional regulation of the NHE1 isoform of the Na^+/H^+ exchanger, transgenic mice were created that contained the mouse NHE1 promoter driving expression of green fluorescent protein. The level of NHE1 transcription varied between tissues and with the stage of embryonic development. The highest expression was in the heart and liver of 12- to 15-day-old mice, and this declined with age. To examine Na^+/H^+ exchanger protein levels, we immunoblotted mouse tissues from 18-day-old embryos, neonates, and adults. Protein levels increased after embryonic *day 18* and peaked at 14 days of age in the heart, lung, liver, kidney, and brain. The greatest rise in NHE1 protein expression occurred in the heart, whereas the smallest increase was in the brain. The results suggest that Na^+/H^+ exchanger transcription and protein levels are controlled in a tissue-specific and time-dependent manner during development.

differentiation; neonatal development; pH regulation

MAMMALIAN GROWTH AND DEVELOPMENT from conception to adulthood is a complex process involving specific regulation of a large number of proteins. During this process, a variety of cell types undergo growth and differentiation and form specific cell lineages and tissues. Variations in intracellular pH (pH_i) have long been suggested to be important in these processes (20, 32). A variety of evidence has also suggested that the Na^+/H^+ exchanger is important in growth and differentiation of many cell types and is responsible for the elevation of pH_i in these cells (for a review, see Ref. 20). Furthermore, the Na^+/H^+ exchanger generates a permissive pH_i that is critical for the development of mitogenic responses elicited by growth factors (31, 40). Also, deletion of the Na^+/H^+ exchanger in mice causes neurological defects, and the growth and viability of these mice is greatly reduced; therefore, the Na^+/H^+ exchanger plays an important role in cell growth and differentiation (3).

The NHE1 isoform of the Na^+/H^+ exchanger is ubiquitous throughout mammalian tissue. Other isoforms have more specific tissue and cell localization with more specialized functions and distributions in these tissues (16, 38, 41). A number of reports have suggested that expression of the NHE1 isoform of the Na^+/H^+ exchanger is increased in cell growth and differentiation, possibly allowing it to facilitate these processes. Studies have shown that, during differentiation of human leukemic cells (HL-60), there is an 18-fold increase in NHE1 transcription and a 7-fold increase in protein levels (30, 38, 39). More recently, our laboratory (13, 47) reported a transient increase in the level of NHE1 transcription during retinoic acid-induced differentiation of P19 cells and a corresponding rise in NHE1 activity, which was necessary for cell differentiation. Similarly, we showed that NHE1 mRNA levels increase when L6 cells differentiate from myoblast to myotubes. Also, the NHE1 promoter was activated during L6 muscle cell differentiation (50) and with mitogenic activation of cells (4). These data suggest that the Na^+/H^+ antiporter plays an important role in the differentiation of some cells; however, this phenomenon has not been shown to occur universally in all cell types (46).

In the myocardium, regulation of the Na^+/H^+ exchanger levels is of special importance. The Na^+/H^+ exchanger plays a key role in damage that occurs to the myocardium during injury by ischemia and reperfusion. The exchanger removes excess protons that result from ischemia. This produces an increase in intracellular sodium that causes an increase in intracellular calcium either by reversing the activity of the Na^+/Ca^{2+} exchanger or reducing its activity. The excess intracellular calcium causes a variety of detrimental effects in the myocardium (for reviews, see Refs. 27 and 28). The Na^+/H^+ exchanger has also recently been shown to play an important role in myocardial hypertrophy. Recent results have shown that blockage of Na^+/H^+ exchanger activity can prevent myocardial hypertrophy (29, 51). We (12) and others (17) have also

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shown that the level of Na^+/H^+ exchanger message is variable in the heart and is induced by acidosis and ischemia.

Developmental regulation of the Na^+/H^+ exchanger has not been well studied. Some reports have demonstrated that the amount of NHE1 message is greater in the newborn heart compared with the adult (6, 23). However, there has not been a systematic evaluation of NHE1 expression in the heart and in various tissues during the early developmental process. In addition, the level of protein (as opposed to mRNA levels) has not been surveyed throughout development in the heart and other tissues. In this study, we investigated the regulation of NHE1 transcription and protein levels during mouse development. We found that NHE1 is subject to both age-dependent and tissue-specific developmental regulation.

EXPERIMENTAL PROCEDURES

Construction of Transgenic Mice with Reporter Genes

Reporter gene transgenic mice were generated to examine the transcriptional activity of the NHE1 gene during mouse development. Specifically, these animals expressed green fluorescent protein (GFP) under the control of the NHE1 promoter. The approach used was similar to that reported earlier by several authors (26, 35–37, 52). A promoter-reporter plasmid was constructed for this purpose, and three independent mouse cells lines were made for analysis. The promoter-reporter construct contained a 3.8-kb fragment of the mouse NHE1 promoter and the GFP gene placed after the transcription start site.

The 3.8-kb portion of the mouse NHE1 promoter was obtained from pXP1–5.0 (48). pXP1–5.0 contains 5 kb of the mouse NHE1 gene originally isolated from a mouse lambda Gem-11 genomic DNA library. The plasmid was cut with *Hin*dIII and *Sal*I to obtain a 3.8-kb NHE1 promoter fragment, which was cloned into the corresponding sites in the vector pSP73 (Promega). The resulting plasmid was named pHS-SP.

To obtain the gene for the GFP reporter, pS65T-C1 (Clontech) was digested with *Bgl*II and *Bam*HI to remove 51 bp of the polylinker and was religated. Removal of a portion of the polylinker facilitated future cloning steps by eliminating certain restriction enzyme sites. The "linkerless" pS65T-C1 vector was digested with *Nhe*I and *Stu*I to produce a 1.9-kb fragment containing the GFP gene. This fragment was cloned into the *Xba*I-*Sma*I sites of pTZ18R (Pharmacia) to yield the construct named pTZ(GFP). Finally, the 3.8-kb portion of the mouse NHE1 promoter was extracted from the pHS-SP vector described above with *Hin*dIII and *Sal*I digestions and was placed in the corresponding sites of pTZ(GFP). The final construct contained the 3.8-kb mouse NHE1 promoter fragment (including the transcription start site, which was assigned as *base pair 0*) upstream of the GFP gene, which began at *base pair 31*. The correct construction of this plasmid [named pHS-TZ(GFP)] was confirmed by restriction mapping and DNA sequencing.

Before the transgenic mice were constructed, restriction enzyme digests extracted the NHE1 promoter and reporter gene from the plasmid [*Hin*dIII and *Eco*RI cut pHS-TZ(GFP), creating a 5,572-bp piece]. The linearized DNA fragments were injected into the pronucleus of preimplantation embryos (obtained from the oviducts of pregnant FVB/N females <20 h after fertilization). Once injected, fertilized eggs were transferred into the oviduct of a 0.5-day-old postcoitum pseudopregnant FVB/N female. Mating with vasectomized males generated the pseudopregnant females. (Dr. Peter Dickie of the Transgenic Facility, University of Alberta Health Sciences Laboratory Animal Services, Edmonton, Alberta, Canada, performed all transgenic procedures.) All procedures on animals were performed according to the Canadian Council on Animal Care regulations.

Genomic DNA was obtained from tail biopsies of embryos or ear notches of neonates from transgenic litters. Mice harboring the transgene were identified by PCR (24) using 5′ and 3′ primers in the reporter coding region. Specifically, the primers were as follows: GFP forward 5′-TGG TGA TGT TAA TGG GCA CAA-3′ and GFP reverse 5′-CAG CAC GTG TCT TGT AGT TCC CG-3′.

Of the three GFP transgenic lines generated, two were found to express the GFP protein strongly and are denoted as G16 and G34. Selective breeding generated homozygotes, which was desirable for increased transgene expression, ease in future breeding, and subsequent analysis of litters.

Preparation and Microscopy of GFP-positive Embryos

Mouse embryos were harvested at different ages, fixed in 2% paraformaldehyde for 60 min, and embedded in 30% sucrose at 4°C overnight. Next, embryos were washed with PBS, incubated in 50% Cryomatrix (Shandon)-PBS for 8 h at room temperature, and then placed at 4°C overnight in 100% Cryomatrix. The embryos were frozen in 2-methylbutane cooled by liquid nitrogen. Sagittal cryostat sections were cut to a thickness of 10 μm and mounted in GelTol mounting medium (Shandon). Images were obtained using a ×10 objective on a Zeiss confocal microscope with fluorescein isothiocyanate filters. Identical confocal settings were used to collect images of controls and transgenic embryos at each age. Images were reconstructed using Adobe Photoshop.

Quantification of GFP Fluorescence

Fluorescence intensity was quantified using NIH Image software. Briefly, the organ of interest was selected using the polygon tool, and a histogram of fluorescence intensity was generated. The fluorescence intensity of the organ was taken as the sum of all counts with a pixel intensity >240. Quantification was repeated for at least three similar sections from the same organ to account for potential differences in fluorescence with respect to the depth of the section. Similar methods of GFP fluorescence quantification have been previously reported (18, 34). Statistical analysis was done using Statview software, and results are expressed relative to controls of the same age.

NHE1 Western Blot Analysis

Preparation of total protein from tissues. Organs were harvested from mouse embryos (embryonic *day 18*), neonates (1, 2, and 14 days old), and adult mice (>8 wk old) and immediately frozen in liquid nitrogen. To obtain sufficient protein from embryos and neonates for analysis, organs from littermates of the same age were pooled. Therefore, embryonic *day 18* crude microsome preparations contained 6–10 organs and neonatal preparations contained 3–6 organs. It was not necessary to pool adult organs; therefore, each adult crude microsome preparation was derived from a single animal. Tissues were placed in a buffer containing 1 M NaCl, 100 mM Tris (pH 7.4), 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 37.5 μM ALLN (calpain I inhibitor), and a proteinase inhibitor cocktail (42) for homogeniza-

tion. Samples were homogenized at 4°C for 30 s, incubated on ice for 30 s, and then homogenized again for 30 s using an Omni International 2000 electric homogenizer. To obtain crude membrane fractions (which contained the NHE1 protein within cells), homogenates were subjected to a series of centrifugation steps (2). Initial centrifugation was for 10 min at 3,000 rpm. The pellet was discarded, and the supernatant was centrifuged at 10,000 rpm for 15 min. The resulting pellet was again discarded, and the supernatant was centrifuged at 30,000 rpm for 1 h to obtain a fraction enriched in crude microsomes. The pellet containing the membrane fraction was resuspended in the same buffer as described above with the addition of 1% SDS to aid in solubilization. Total protein was quantified using a Bio-Rad DC Protein Assay kit.

NHE1 immunoblots. Anti-NHE1 monoclonal antibody was purchased from Chemicon to quantify NHE1 protein in crude microsomes. This antibody was generated in mice using an immunogen that consisted of the entire COOH-terminal, hydrophilic domain of porcine NHE1 coupled to a maltose binding protein. Although porcine NHE1 was used as the immunogen to generate the antibody, NHE1 is highly conserved between mouse, porcine, and other mammalian species.

For NHE1 immunoblots, crude membrane fractions containing 60–100 μg total protein were run on 10% polyacrylamide gels, followed by transfer to nitrocellulose membranes. Membranes were stained with Ponceau S to confirm that all lanes were loaded equally. Next, membranes were incubated overnight at 4°C in 10% milk-Tris-buffered saline (TBS) and then washed three times for 5 min each in TBS at room temperature. Membranes were probed at 4°C overnight with anti-NHE1 monoclonal antibody (Chemicon) at a concentration of 1:2,000 in TBS. After three washes of 5 min each with TBS, membranes were incubated with 1:5,000 goat anti-mouse antibody in TBS at room temperature for 1 h. After three 5-min washes in TBS, the Amersham enhanced chemiluminescence reaction was used to visualize immunoreactivity. Blots were scanned and quantified using Image Gauge software (Bio-Rad) essentially as previously described (5, 21). This procedure has also been used previously for NHE1 and NHE3 isoforms of the Na^+/H^+ exchanger (41).

As noted above, all blots were stained with Ponceau S after transfer to ensure equivalent protein transfer in each lane. As an additional control for protein loading and efficiency of transfer, 1 μg glutathione-*S*-transferase (GST) protein was added to each total protein sample before the polyacrylamide gels were loaded. After NHE1 was probed for, the immunoblots were stripped and probed for the GST protein. All lanes included in the NHE1 immunoblot quantification analysis contained equivalent amounts of protein as revealed by Ponceau S staining and GST immunoblotting.

Statistical Analysis

The Mann-Whitney *U*-test was used to statistically evaluate data. A difference of $P < 0.05$ was considered significant.

RESULTS

Activation of the NHE1 Promoter During Embryonic Development

To determine the extent of NHE1 transcriptional activation during embryonic development, embryos were examined at 12, 15, and 18 days after conception. Figure 1*A* shows 12-day-old embryos from control, G16, and G34 mouse lines. Qualitative comparison of the GFP-positive pictures to those of the controls reveals increased fluorescence in the heart and liver, indicating an activation of NHE1 transcription at this age. Similarly, fluorescence of the heart and liver in 15-day-old embryos appears to be greater in G16 and G34 animals compared with controls (although to a lesser extent than in the 12-day-old embryos), as shown in Fig. 1*B*. Finally, Fig. 1*C* illustrates that the GFP reporter does not give an obvious fluorescent signal in any tissues by embryonic *day 18* compared with controls. Similar results were found for another independently made GFP transgenic mouse line (G34, data not shown).

Next, we undertook quantitative analysis of GFP fluorescence using NIH Image software as previously described (18, 34) and as in EXPERIMENTAL PROCEDURES (Fig. 2). Figure 2*A* is an enlargement of heart and liver sections from control, G16, and G34 lines. The fluorescence quantification of the heart, liver, and lung are depicted in Fig. 2, *A–C*, respectively. GFP fluorescence was found to be >5-fold greater in the heart compared with control animals (Fig. 2*B*), whereas the liver exhibited a 12-fold increase in reporter expression (Fig. 2*C*). The fluorescence of the lung was quantified as a negative control and did not show significant elevation over background levels (Fig. 2*D*). Thus it appears that NHE1 transcription is activated in both the heart and liver at embryonic *day 12*, whereas it remains below the level of detection in the lung and other tissues.

Figure 3 shows the same analysis of GFP fluorescence in 15-day-old embryos. Representative sections of heart and liver regions are given in Fig. 3*A*. GFP fluorescence was found to be about twofold greater in the 15-day-old hearts compared with controls (Fig. 3*B*). In the liver, the reporter signal was ~2.8-fold higher than the background (Fig. 3*C*). As with the 12-day-old embryos, the lung did not show significant fluorescence (Fig. 3*D*). Although NHE1 transcriptional activation could be detected in both the heart and liver of 15-day-old embryos, this activation was less than what was observed at embryonic *day 12*.

Subsequent quantitative analysis was performed exclusively on the hearts of mice at embryonic *day 18* and older. Hearts from 18-day-old embryos, 1-day-old neonates, 14-day-old neonates, and adults are illustrated in Fig. 4*A*. Values were normalized to that of the equal-aged controls, which were assigned a value of 1.0. Figure 4*B* shows that none of the GFP-positive hearts had significantly greater fluorescence than controls at these ages, suggesting that the level of NHE1 transcription had declined compared with younger embryos. Clearly, the level of cardiac NHE1 transcription was below the level of detection in the 18-day-old embryo, neonate, and adult.

NHE1 Protein Expression in the Late Embryo to the Adult

Immunoblotting was used to examine the level of NHE1 protein in the mouse tissues. We initially confirmed the ability of the commercial anti-NHE1 mono-

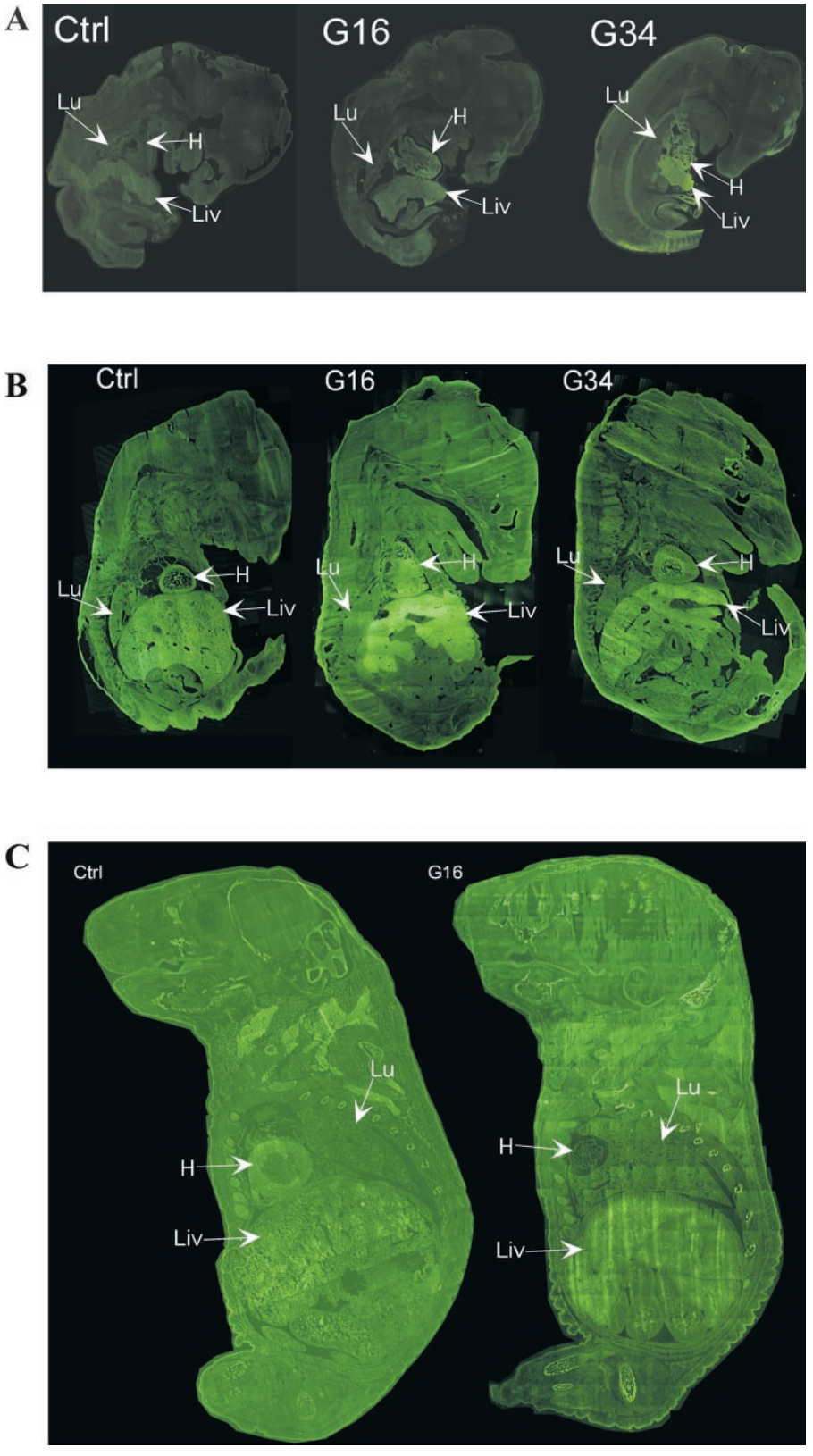


Fig. 1. Activation of the Na^+/H^+ exchanger isoform 1 (NHE1) promoter in 12- (*A*), 15- (*B*), and 18-day-old (*C*) embryos. Sagittal sections were prepared from mouse transgenic lines with the Na^+/H^+ exchanger promoter directing expression of the green fluorescent protein (GFP) reporter. Sections from control (Ctrl), G16, and G34 transgenic mouse lines were prepared at a thickness of 10 μm as described in EXPERIMENTAL PROCEDURES. Images were viewed with a confocal microscope for GFP fluorescence and were reconstructed from smaller fragments. H, heart; Liv, liver; Lu, lung.

clonal antibody to detect the bona fide NHE1 protein. A polyclonal antibody raised against the COOH-terminal 178 amino acids of the Na^+/H^+ exchanger (22, 42) was used to immunoprecipitate NHE1 from a rat heart extract. The immunoprecipitate was run on SDS-PAGE and probed with the commercial anti-NHE1 monoclonal antibody. The anti-NHE1 monoclonal antibody recognized a band of ~100 kDa, confirming that

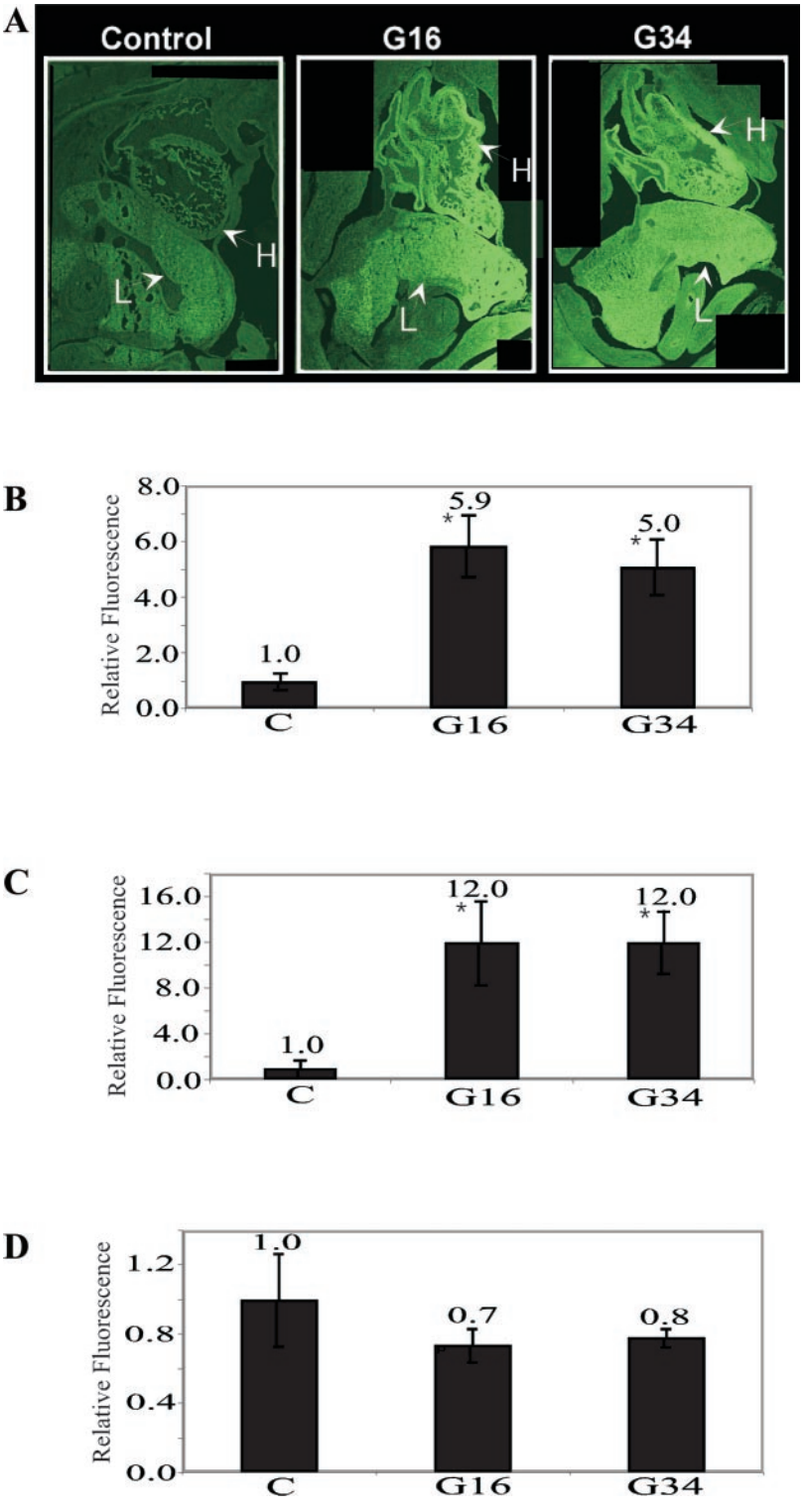


Fig. 2. NHE1 promoter activity in the heart, liver, and lung of 12-day-old embryos. *A*: enlargement of representative heart (H) and liver (L) sections of 12-day-old embryos from control (C), G16, and G34 lines. *B–D*: relative GFP fluorescence in the heart (*B*), liver (*C*), and lung (*D*), respectively. Fluorescence intensity was quantified using NIH Image software as described in EXPERIMENTAL PROCEDURES. Results are expressed relative to control fluorescent intensities and are means ± SE of 3–4 sections for each sample represented. *Fluorescence was significantly greater than controls ($P < 0.05$).

it recognizes the Na^+/H^+ exchanger protein (data not shown).

Western blotting was then used to examine NHE1 expression in the heart, lung, liver, kidney, and brain of older mice. These five organs were chosen because of the results with the transgenic reporter experiments and because it was possible to obtain reasonable quantities of these tissues from embryos and young neonates. The relative amount of Na^+/H^+ exchanger protein was compared from 18-day-old embryos, 1-day-old neonates, 2-day-old neonates, 14-day-old neonates, and adults. Representative Western blots are shown in Fig. 5 adjacent to graphs of the quantified results. In all tissues studied, the amount of NHE1 protein peaked in the 14-day-old neonates. Specifically, compared with 2-day-old neonates, the NHE1 protein is 6.1-, 1.8-, 2.5-,

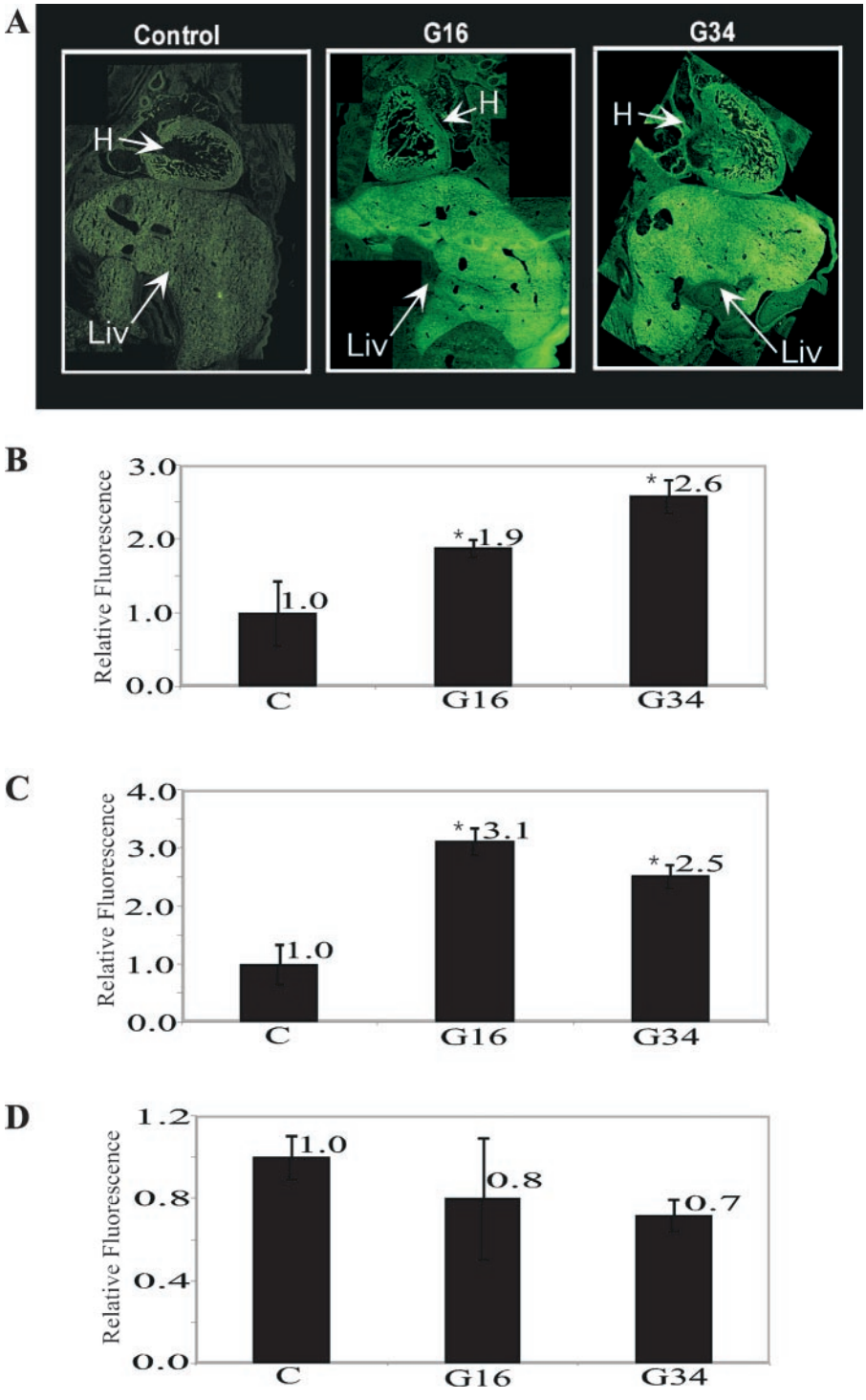
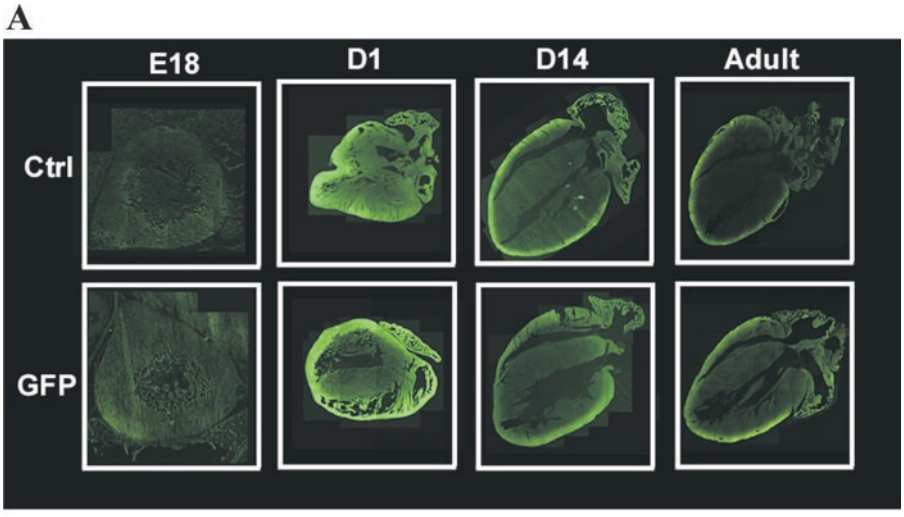


Fig. 3. NHE1 promoter activity in the heart, liver, and lung of 15-day-old embryos. *A*: representative heart and liver sections of 15-day-old embryos from control and Na^+/H^+ exchanger transgenic reporter G16 and G34 lines. *B–D*: relative GFP fluorescence in the heart (*B*), liver (*C*), and lung (*D*), respectively. Fluorescence intensity was quantified using NIH Image software as described in EXPERIMENTAL PROCEDURES. Results are expressed relative to control fluorescent intensities and are means ± SE of 3–4 sections for each sample represented. *Fluorescence was significantly greater than controls ($P < 0.05$).

2.8, and 1.3-fold more abundant in the heart, lung, liver, kidney, and brain of the 2-wk-old neonates, respectively.

It is noteworthy that in some tissues (lung and liver) the NHE1 immunoblot reveals a single band of ~100 kDa; however, in other tissues (heart, kidney, and brain), multiple bands are evident. The NHE1 protein is often found as a band of ~105–110 kDa plus an isoform ~10–15 kDa smaller. The smaller isoforms represent deglycosylated or partially glycosylated protein (22, 38, 48). We have previously shown that there are multiple carbohydrate moieties on the Na^+/H^+ exchanger that can affect the apparent molecular weight of the mature protein on the plasma membrane (22). In our analysis, all immunoreactive bands of ~90–110 kDa were included for quantification.

We also examined the relative amount of NHE1 protein present in the heart, lung, liver, kidney, and brain at several ages. Figure 6 shows representative Western blots for tissues from 18-day-old embryos,



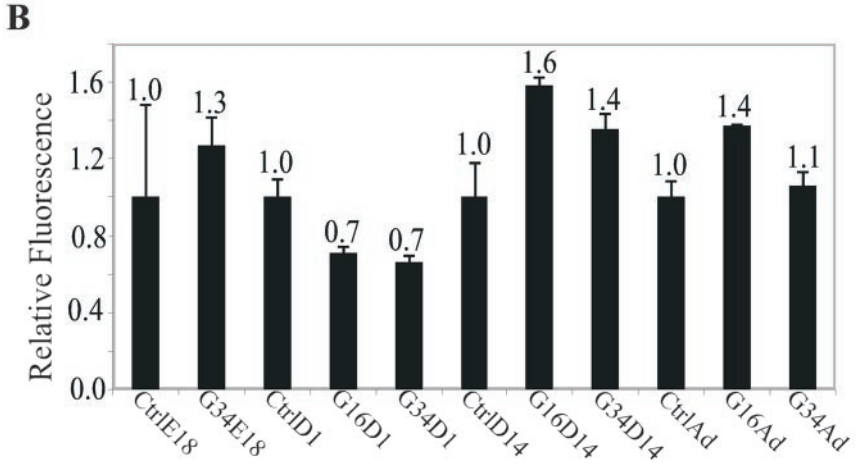


Fig. 4. NHE1 promoter activity in hearts of 18-day-old embryos, 1-day-old neonates, 14-day-old neonates, and adults from G16 and G34 mouse transgenic lines. *A*: representative heart sections from control and GFP-positive lines at the indicated ages. All ages are shown on a scale relative to the controls. *B*: relative GFP fluorescence in the heart. Fluorescence intensity was quantified using NIH Image software as described in EXPERIMENTAL PROCEDURES. Results are expressed relative to control fluorescent intensities. Results are expressed as means ± SE of 3–4 measurements for each sample shown. Fluorescence was not significantly greater than controls in any of the GFP-positive hearts at these ages ($P < 0.05$). E18, embryonic *day 18* hearts; D1 and D14, 1- and 14-day-old hearts, respectively; Ad, adult hearts.

1-day-old neonates, 14-day-old neonates, and adults. Quantified values are expressed relative to the amount of NHE1 measured in the liver at each age. At embryonic *day 18* and postnatal *day 1*, NHE1 protein is two- to threefold higher in the heart, lung, liver, and kidney compared with the brain. By 14 days of age, however, there is a change in relative NHE1 protein levels such that it is 5.3-, 4.1-, 3.9-, and 2.2-fold greater than the brain in the heart, lung, liver, and kidney, respectively. In the adult, the brain also expressed the least amount of NHE1 compared with other tissues.

DISCUSSION

In this study, we examined developmental and tissue-specific regulation of Na^+/H^+ exchanger expression in the embryo and in the newborn mouse. To investigate regulation of transcription during embryological development, we used transgenic mice expressing a GFP reporter coupled to the mouse Na^+/H^+ exchanger promoter. The 3.8-kb NHE1 promoter fragment contains all of the upstream elements that are important for expression in mammalian cells (50). This sensitive system was necessary to detect changes in the level of NHE1 transcription because the Na^+/H^+ exchanger promoter is a relatively weak promoter (14) and only a very low level of NHE1 protein is expressed in cells (45).

When we examined the relative levels of transcription in whole mouse embryos using GFP as a reporter, we found a time- and tissue-dependent regulation of NHE1 transcription. The Na^+/H^+ exchanger promoter activity was greatest in the heart and liver at embryonic *days 12* and *15*. In our system, only these two tissues consistently showed Na^+/H^+ exchanger expression above background levels. Clearly, the NHE1 promoter is more active in the heart and liver at these stages of fetal growth and development.

Surprisingly, we found that Na^+/H^+ exchanger transcription declined during embryonic development. At embryonic *day 15*, transcription in the heart and liver was less than at embryonic *day 12*. By embryonic *day 18*, the GFP reporter signal was not significantly greater than background levels. Therefore, it is apparent that the level of NHE1 transcription decreases with embryo age. From our previous experiments (see below) and from other studies that have examined mRNA levels, it is clear that transcription of the NHE1 promoter does not cease at embryonic *day 18*. Rather,

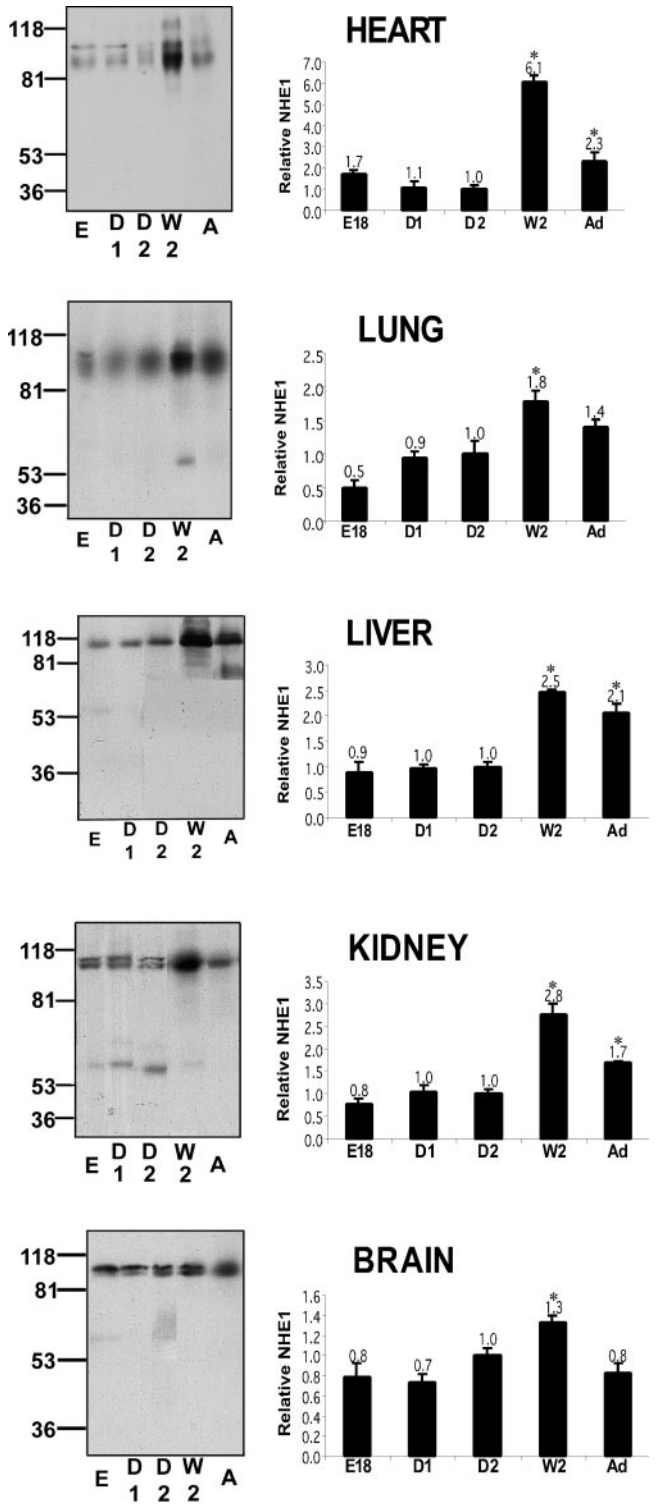


Fig. 5. NHE1 protein expression during development of heart, liver, lung, kidney, and brain from 18-day-old embryos (E) to adults (A or Ad). *Left*: representative Western blots of tissues collected at the indicated ages and probed with anti-NHE1 monoclonal antibody. *Right*: quantification of relative amounts of NHE1 protein. Blots were scanned and quantified using Image Gauge software (Bio-Rad). Quantification results were derived from immunoblotting 3–4 unique crude microsome fractions prepared by pooling organs of littermates as described in EXPERIMENTAL PROCEDURES. Results are expressed as means ± SE of the 3–4 organ pools measured relative to the amount of protein in the 2-day-old (D2) tissues. D1, *day 1*; W2, *day 14*.

it is apparent that our GFP reporter system was unable to detect the lower NHE1 transcriptional activity in these tissues. Similarly, it is likely that transcription of the Na^+/H^+ exchanger occurred in other tissues at younger ages but was below the level of detection.

We found a heightened level of Na^+/H^+ exchanger transcription in the myocardium at embryonic *days 12* and *15*. If this results in a concomitant increase in protein levels, it may be that there is a functional role of increased expression of the Na^+/H^+ exchanger in the heart during embryonic development. Recent reports have shown that hypertrophy of the myocardium results in elevation of Na^+/H^+ exchanger levels (7, 44). Also, blockage of the Na^+/H^+ exchanger attenuates hypertrophy in the myocardium (7, 51). Thus it is clear that the Na^+/H^+ exchanger plays a significant role in adult myocardial growth during these hypertrophic stimuli (7, 44, 51). We have previously noted that a number of stimuli that increase growth and development of vascular smooth muscle and the myocardium increase NHE1 message levels (16). This includes endothelin, α_1-adrenergic stimulation, serum, and blood pressure (for a review, see Ref. 16). In our study, we found that, with the rapid growth that occurs in the fetus, NHE1 transcription was elevated compared with other tissues. These results are consistent with the suggestion that stimuli that promote growth of the myocardium increase Na^+/H^+ exchanger expression. It may be that the stimuli that increase NHE1 expression during development are similar to those that activate expression in hypertrophy. It must be noted, however, that pathological hypertrophy is distinct from fetal cardiac growth. Further experiments are therefore necessary to examine the role of the Na^+/H^+ exchanger in fetal development.

The liver also showed a relatively high level of Na^+/H^+ exchanger transcription in the developing fetus compared with other tissues. The reason for the elevated expression in this tissue is not completely clear. However, a number of studies have suggested that activity of the Na^+/H^+ exchanger is critical for growth and viability of the human liver and related cell types. Recently, it has been shown that Na^+/H^+ exchanger inhibition impairs cell growth and viability of human and rat hepatocarcinoma cells (19). Also, in hepatic stellate cells, Na^+/H^+ exchange inhibition prevents cell proliferation (10), and Na^+/H^+ exchanger activation may play a critical role in growth of some hepatic tumors (43). Additionally, growth factors can activate the Na^+/H^+ exchanger in the liver and hepatic cell lines (43), which further emphasizes the importance of NHE1. Yet another study has also demonstrated that the Na^+/H^+ exchanger is activated during liver regeneration (9). Finally, a role for the Na^+/H^+ exchanger has been described in a cirrhosis model characterized by chronic stimulation of hepatocyte proliferation. In this instance, both the activity and mRNA levels of the Na^+/H^+ exchanger were greatly increased (15). Overall, it is apparent that the Na^+/H^+ exchanger plays a significant role in hepatocyte growth and pH maintenance.

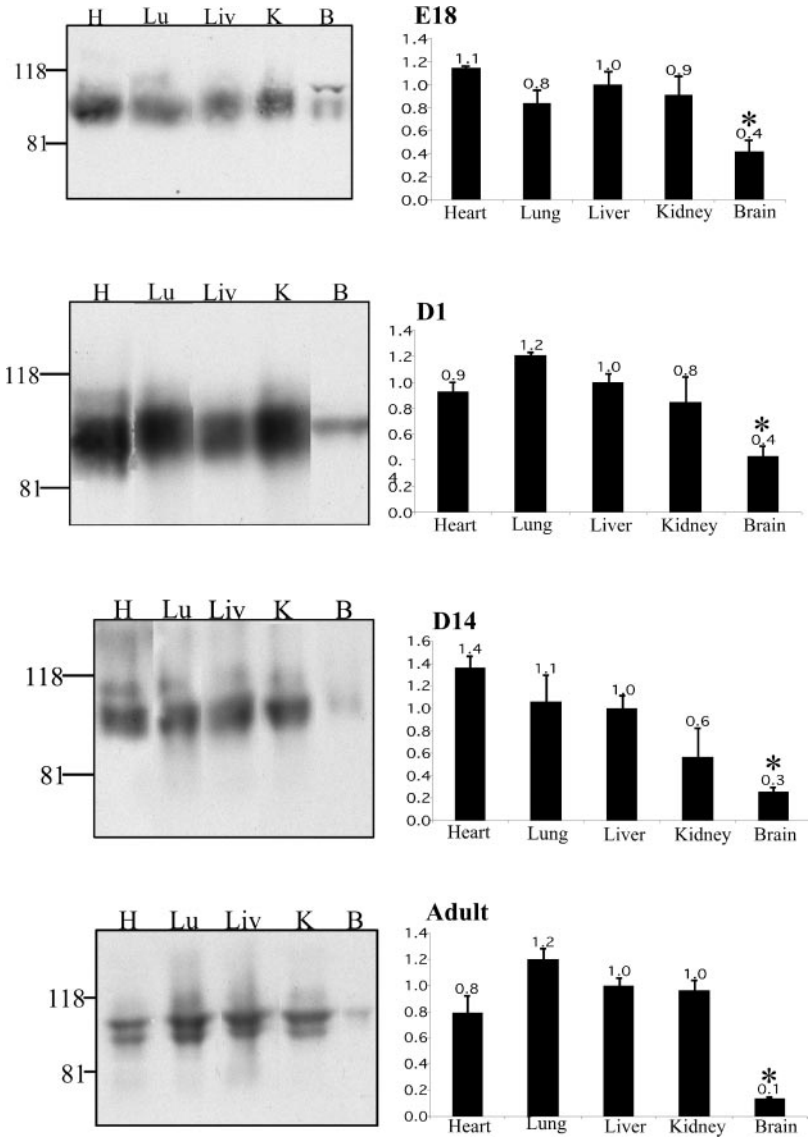


Fig. 6. Relative levels of Na^+/H^+ exchanger expression in heart, lung, liver, kidney (K), and brain (B) during development. The relative levels of Na^+/H^+ exchanger expression were compared between various tissues of mice at embryonic *day 18*, 1 day old, 14 days old, and adult. *Left*: representative Western blots of tissues collected at the indicated ages and probed with anti-NHE1 monoclonal antibody. *Right*: quantification of relative amounts of NHE1 protein. Blots were scanned and quantified using Image Gauge software. Quantification results were derived from immunoblotting 3–4 unique crude microsome fractions prepared by pooling organs of littermates as described in EXPERIMENTAL PROCEDURES. Results are expressed as means ± SE of the 3–4 organ pools measured relative to the amount of protein present in liver tissue at that age group.

Our study also examined developmental regulation of NHE1 protein levels in the heart, lung, liver, kidney, and brain of 18-day-old embryos to adults. As shown in Fig. 5, NHE1 protein levels peaked in 14-day-old tissues from the heart, lung, liver, kidney, and brain. The quantity of NHE1 protein remained high in the adult lung, liver, and kidney compared with the 14-day-old neonate but decreased in the adult heart to essentially the same amount found in the 18-day-old embryos. The decrease in Na^+/H^+ exchanger protein observed in the heart is consistent with an earlier study (23) that showed that sarcolemma NHE1 activity was reduced by two- to threefold in adult rats compared with 6-day-old neonatal rats. Chen et al. (6) also reported a 1.6-fold decrease for similar ages in rabbit myocardium. The reason for the increase in NHE1 protein at 2 wk of age is still uncertain. It may be relevant that at this age of mouse development the animals begin weaning. This process involves a switch from a high fat-low carbohydrate diet to a low fat-high carbohydrate diet (25). Concurrently, the activity for enzymes of fatty acid oxidation decreases, and there is an increase in activity of enzymes of carbohydrate metabolism (25). A recent publication (33) has shown that uncoupled glucose metabolism as an energy source results in significant increases in proton production in the myocardium. It is also well known that acidosis increases Na^+/H^+ exchanger expression (12, 17). Thus the shift in metabolism could result in increased proton production, which in turn causes an increase in Na^+/H^+ exchanger levels. An alternative explanation may be related to hormone levels. Thyroid hormone levels and corticosterone levels rise at this age in development (25). It has recently been shown that thyroid hormone increases NHE1 message levels in the myocardium (49). It is thus possible that changes in hormone levels or metabolism affect NHE1 protein expression. Further experiments are necessary to elucidate the precise mechanisms of regulation of the NHE1 protein in this period.

It is of note that two separate studies of NHE1 knockout mice have emphasized the importance of NHE1 in neonates. Bell et al. (3) generated NHE1 knockout mice that exhibited a normal phenotype at birth, but by 14 days of age, mutants were significantly smaller than controls. Also, the 14-day-old NHE1-null animals displayed an ataxic gait, and 68% of the mutants died between 16 and 29 days of age. Cox et al. (8) examined a different NHE1 knockout line and also reported the development of an ataxic gait and a smaller size in mutants 11–14 days of age. It is apparent that the precise role of NHE1 at this stage is complex, with multifaceted effects. Even so, the importance of NHE1 to the neonatal mouse is emphasized and is therefore consistent with the present study.

It is of interest that, although NHE1 protein levels peak at 14 days after birth (Fig. 5), we did not detect a significant, corresponding increase in NHE1 promoter activity at this age using the GFP reporter (Fig. 4). However, others have shown that NHE1 mRNA in rat ventricles (23) and rabbit hearts (6) is higher at 2 wk of age compared with the adult. There are two possible explanations for this apparent discrepancy. One possibility is that promoter activity is elevated, but this activity is still below the detection level of our system. Alternatively, elevated mRNA levels in the neonatal heart may result from changes in RNA stability and not alterations in transcription levels. Changes in the mRNA levels may then affect protein translation and resultant protein levels. Further experiments are necessary to clarify which of these alternatives is correct.

NHE1 protein levels were also compared in the heart, lung, liver, kidney, and brain from mice of the same age. The brain expressed the least NHE1 protein at all ages examined. Interestingly, the pattern of relative NHE1 expression changed significantly between neonatal *days 1* and *14*. More specifically, NHE1 was only twofold more abundant in the heart, lung, liver, and kidney compared with the brain at embryonic *day 18* and 1 day of age. However, NHE1 protein was four- to fivefold greater than that of the brain in the heart, lung, and liver of 14-day-old animals (Fig. 6). This increase further emphasizes the importance of NHE1 in certain tissues at 2 wk of age. It was surprising that Na^+/H^+ exchanger expression was relatively low in the brain. Recent studies have shown that deletion of NHE1 results in severe neurological defects in mice (8). We have also shown that inhibition or deletion of NHE1 function inhibits neuronal differentiation of P19 cells (47). Thus it is clear that the Na^+/H^+ exchanger plays an important role in this tissue. Although it is not clear why the protein is present in lower abundance in this tissue relative to others, it is noteworthy that a brain-specific isoform, NHE5, exists (1) and that it could complement activity of the NHE1 isoform. A recent study (11) has shown a similar pattern of NHE1 developmental expression in the rat brain.

Overall, our results have shown that expression of the Na^+/H^+ exchanger protein is regulated in both a time-dependent and tissue-specific manner. In utero, transcription of the Na^+/H^+ exchanger message is greatest in the heart and liver, with the highest levels observed at the youngest age examined (embryonic *day 12*). This elevated level of transcription may be related to differentiation or cell and organ growth. We also found that NHE1 protein expression peaked in several organs at 14 days after birth. NHE1 protein levels were lowest in the brain, and this low level became more pronounced with age. Further experiments are necessary to understand the regulation of NHE1 protein expression in the intact embryo.

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