Replacement of Nonmuscle Myosin II-B with II-A Rescues Brain but Not Cardiac Defects in Mice*

Received for publication, March 30, 2007, and in revised form, May 17, 2007. Published, JBC Papers in Press, May 22, 2007, DOI 10.1074/jbc.M702731200

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The purpose of these studies was to learn whether one isoform of nonmuscle myosin II, specifically nonmuscle myosin II-A, could functionally replace a second one, nonmuscle myosin II-B, in mice. To accomplish this, we used homologous recombination to ablate nonmuscle myosin heavy chain (NMHC) II-B by inserting cDNA encoding green fluorescent protein (GFP)-NMHC II-A into the first coding exon of the Myh10 gene, thereby placing GFP-NMHC II-A under control of the endogenous II-B promoter. Similar to B-/B− mice, most B+/B+ mice died late in embryonic development with structural cardiac defects and impaired cytokinesis of the cardiac myocytes. However, unlike B+/B− mice, 15 B+/B+ mice of 172 F2 generation mice survived embryonic lethality but developed a dilated cardiomyopathy as adults. Surprisingly none of the B+/B+ mice showed evidence for hydrocephalus that is always found in B−/B− mice. Rescue of this defect was due to proper localization and function of GFP-NMHC II-A in place of NMHC II-B in a cell-cell adhesion complex in the cells lining the spinal canal. Restoration of the integrity and adhesion of these cells prevents protrusion of the underlying cells into the spinal canal where they block circulation of the cerebral spinal fluid. However, abnormal migration of facial and pontine neurons found in NMHC II-B mutant and ablated mice persisted in B+/B+ mice. Thus, although NMHC II-A can substitute for NMHC II-B to maintain integrity of the spinal canal, NMHC II-B plays an isoform-specific role during cytokinesis in cardiac myocytes and in migration of the facial and pontine neurons.

Nonmuscle myosin II (NM-II), together with cardiac, skeletal, and smooth muscle myosins, belongs to the class II myosin family and is composed of one pair of heavy chains (230 kDa) and two pairs of light chains (20 and 17 kDa) (1, 2). To date, three isoforms of the NM-II heavy chain, termed NMHC II-A, NMHC II-B, and NMHC II-C, have been identified in vertebrates (3–6). The NMHCs are encoded by three different genes, Myh9, -10, and -14 (generating NMHC II-A, NMHC II-B, and NMHC II-C, respectively), which, in humans and mice, are located on different chromosomes (7–9). They have similar molecular structures in that each heavy chain contains an amino-terminal globular region that catalyzes ATP hydrolysis and binds to actin to generate force and an α-helical carboxyl-terminal coiled-coil rod region that is responsible for myosin filament assembly (1, 2). The primary structure of the three NMHC II isoforms is well conserved showing a 64–80% identity in amino acids. The three isoforms are widely expressed in most mammalian tissues and cells in a tissue-specific (6) and developmentally dependent manner (10).

The biological functions of the NM-II isoforms have been studied both in vitro and in vivo. In cultured cells, NM-II has been found to participate in such fundamental cellular processes as cytokinesis, cell migration, and maintenance of cell morphology (11–13). Although all three isoforms have been shown to have different kinetic properties with respect to their interaction with actin (14–17), they are capable of partially substituting for each other in experiments in cultured cells. A recent study showed that NMHC II-B small interfering RNA-induced multnucleation in COS-7 cells could be rescued by each of the three isoforms although not to the same extent (18). In contrast, the role of NMHC II-B in collagen fiber transport and cell migration differed from that of NMHC II-A in a number of vertebrate cultured cell lines (19–22).

In vivo studies showed that mice that have been ablated for NMHC II-B had major defects in their hearts and brains and died between embryonic day (E) 14.5 and birth (23, 24). The cardiac defects included a membranous ventricular septal defect (VSD), double outlet from the right ventricle (DORV) of the aorta and pulmonary artery, and a defect in cytokinesis resulting in a decreased number of cardiac myocytes. The brain abnormalities included a severe hydrocephalus starting at E12.5 and resulting in destruction of large amounts of brain tissue (25). A point mutation in the motor domain of NMHC II-B (R709C), coupled with decreased expression of the mutant myosin, led to the impaired migration of specific groups of neurons, including the cerebellar granule cells during brain development, accompanied by the cardiac defects outlined previously (24, 26). The hypomorphic mutant mice, which, unlike B+/B− mice, survived for up to 20 days after birth, had profound difficulties with their gait and balance and, similar to B+/B− mice, manifested a severe hydrocephalus (26). These
studies indicate the importance of NMHC II-B during murine cardiac and brain development. Because both the brain and heart (especially cardiac myocytes) are enriched in the expression of NMHC II-B relative to the other isoforms of NMHC II and because there is no obvious increase in the expression of NMHC II-A (and II-C) in cardiac myocytes and brain tissue in NMHC II-B ablated mice (see below), we are unable to distinguish whether the defects seen in B−/B− mice are due to the absence of the NMHC II-B isoform specifically or due to the absence of NM II in general. Similarly mice ablated for NMHC II-A die by E6.5 with a failure in the development of the visceral endoderm where only NMHC II-A, but not II-B, is expressed, again indicating that the defect may be due to the loss of NM II (27).

Another related question raised in this study is whether the expression of an NMHC II isoform not usually found in a particular cell type such as cardiac myocytes could cause an adverse effect on the heart. Thus, cardiac myocytes, unlike most cells including skeletal and smooth muscle cells, cease to express NMHC II-A at the protein level by E9.5. Moreover mutations in many sarcomeric and cytoskeletal proteins, such as cardiac actin, β-cardiac myosin heavy chain, and muscle LIM protein, in humans result in a dilated cardiomyopathy (28–30), a myocardial disorder characterized by cardiac dilatation and contractile dysfunction of the left and/or right ventricles (31). Here we present evidence for the first time that expression of NMHC II-A in adult cardiac myocytes results in a dilated cardiomyopathy.

Finally vertebrates differ from Drosophila and Dicyostelium in that they express three different NMHCs rather than one. Therefore, it becomes of interest to learn whether each isoform of NM II in vertebrates plays a unique role or whether they are interchangeable. In this study, we focused on whether NMHC II-A can substitute for NMHC II-B in vivo during mouse development. To answer the question of functional replacement, we used homologous recombination to ablate the message encoding NMHC II-B by inserting cDNA encoding human green fluorescent protein (GFP)-NMHC II-A into the first coding exon of the NMHC II-B gene. This ablates NMHC II-B and places GFP-NMHC II-A under control of the endogenous II-B promoter. We examined the mice generated following germ line transmission of the GFP-NMHC II-A allele (designated B+) with respect to the ability of NMHC II-A to replace NMHC II-B and rescue the defects in mouse brains and hearts in NM II-B ablated mice. We showed that NMHC II-A can rescue hydrocephalus by restoring the integrity of the cells lining the spinal canal but, in most cases, cannot rescue either the impaired migration of the facial and pontine neurons or the cardiac defects.

**EXPERIMENTAL PROCEDURES**

**Construction of the Targeting Vector**—A 5.8-kb genomic fragment containing exon 2 (351 bp), the first coding exon of the NMHC II-B gene, was cloned from a 129/Sv mouse genomic library (Stratagene, La Jolla, CA) for targeting vector construction as described previously (23). The GFP-tagged full-length coding region of human NMHC II-A with a poly(A) signal derived from SV40 was inserted into a BamHI site of exon 2 just after the initiating ATG to replace NMHC II-B. This was followed by insertion of a 1.8-kb neomycin resistance cassette in an intron upstream from exon 2 in a 3′→5′ direction for positive selection (see Fig. 1A). A diphtheria toxin cassette was used for negative selection (not shown). Homologous recombination between the SpeI sites results in disruption of the murine NMHC II-B gene by GFP-human NMHC II-A cDNA. During sequencing of the targeting vector, we found five nucleotide changes from the original report for human NMHC II-A that resulted in three conservative changes in amino acid sequence: K778R, an extra Glu residue at 1346, and I1626V. Studies from this laboratory and other investigators showed that these changes have no discernable effects on the properties of NM II-A (18, 21, 22, 32).

**Generation of Chimeric Mice and Germ Line Transmission of the Mutant Allele**—Linearized targeting vector was electroporated into embryonic stem (ES) cells, and clones were selected with G418. Genomic DNA from G418-resistant ES cell clones was extracted and digested with BamHI. The homologous recombinants were identified by an 8.9-kb band (versus a 6.7-kb band for the wild type) using Southern blot analysis with a 5′ external probe from a BamHI-SpeI fragment indicated in Fig. 1A (see Fig. 1B). Three heterozygous ES cell clones, which had undergone homologous recombination, were injected into C57BL/6 mouse blastocysts. The resulting chimeric mice were scored for germ line transmission by mating them with female C57BL/6 mice. The genotypes of the progeny were analyzed by Southern blot as described above. Only one of the ES cell clones was transmitted in the germ line. All procedures were conducted using an approved protocol in accordance with the NHLBI Animal Care and Use Committee.

**Immunoblot Analysis**—Lung, brain, and heart samples were snap frozen on dry ice and homogenized with Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 0.3 M NaCl, 1% (v/v) Nonidet P-40, 5 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and 1% by volume protease inhibitor mixture containing 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, E-66, bestatin, leupeptin, and aprotinin (Sigma) at 4 °C. After centrifugation at 10,000 × g for 10 min, the protein in the supernates was quantitated by Bio-Rad protein assay and analyzed by immunoblotting. Rabbit polyclonal antibodies raised against the carboxyl-terminal sequences of human NMHC II-A and II-B and mouse II-C were described previously (6, 33). Monoclonal anti-GFP antibody was purchased from BD Biosciences (Clone JL-8). Monoclonal anti-β-actin antibody was from Sigma-Aldrich. Immunoblots were visualized by SuperSignal West Pico (for brain and lung samples) and SuperSignal West Femto (for adult heart samples) Chemiluminescence Substrate (Pierce).

**Immunofluorescence Staining**—Frozen sections or paraffin-embedded sections were blocked with 4% normal goat serum in phosphate-buffered saline containing 3% bovine serum albumin for 30 min at room temperature and then incubated with the primary antibodies for 1 h at room temperature followed by incubation with Alexa Fluor 488- or 594-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:300; Molecular Probes, Eugene, OR) for 1 h at room temperature. The primary antibodies used in this study are as follows. Polyclonal antibodies
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against mouse NMHC II-A (1:500) and human NMHC II-B (1:5000) were characterized previously in our laboratory (33). Monoclonal antibody against sarcomeric α-actinin (1:1000; clone EA-53) was from Sigma-Aldrich, and monoclonal antibody against desmin (1:100; clone D33) was from Dako Cytomation (Carpenteria, CA). β-Catenin and N-cadherin were from Invitrogen (1:200). Because the GFP signal could not be visualized in paraffin-embedded tissue or in the adult heart, GFP antibody was used (1:500; Clontech). Alexa Fluor 594-conjugated wheat germ agglutinin (10 μg/ml) was from Invitrogen. Nuclei in some sections were counterstained with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). The images were collected using a Leica TCS-SP confocal microscope.

**Histological Analysis**—For histological analysis, fresh tissues were fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4), and 5–10-μm-thick paraffin-embedded sections were stained with hematoxylin and eosin (H&E). In some cases, the heart sections were stained with Masson’s trichrome to detect interstitial fibrosis.

**Flow Cytometric Detection of Cardiac Myocytes**—Hearts from E16.5 embryos were harvested, and the cells, including both cardiac and noncardiac cells, were isolated by digestion with 0.05% trypsin, EDTA at 37°C for 10 min. This digestion was repeated three times, removing the dissociated cells each time. The dissociated cells were filtered through a cell strainer with pore size of 70 μm (BD Biosciences Discovery Labware) and were fixed with 70% ethanol at −20°C overnight before flow cytometric analysis. An aliquot was removed to determine cell number. The fixed cells were then incubated with monoclonal antibody against sarcomeric α-actinin (1:800; clone EA-53, Sigma-Aldrich) for 45 min on ice. After three washes with phosphate-buffered saline, 0.1% bovine serum albumin, the cells were incubated with allopheocyanin-conjugated mouse IgG1 (1:100; BD Biosciences Pharmingen) for 30 min on ice followed by three washes with phosphate-buffered saline, 0.1% bovine serum albumin. One wild-type heart sample incubated with secondary antibody alone functioned as a negative control. Cells were analyzed using a BD FAC-Calibur system (BD Biosciences). Light scattered gates were set to exclude debris. The gates to distinguish positive and negative staining were established by staining with secondary antibody alone in a wild-type sample in each experiment.

**Echocardiographic Analysis**—Transthoracic echocardiograms were performed on both B-/+ and B**+/** mice. The investigators were blinded to genotypes. The echocardiograms were performed using an Acuson Sequoia 256c imaging system with a 15-MHz transducer. Left ventricular size, function, and wall motion were assessed using two-dimensional and m-mode echo modalities. All images were taken from the left ventricular short view at the papillary muscle level. All left ventricular measurements were performed from the m-mode. The measurements were performed off line using Prosvol Version 3.0 software. Three measurements were performed for each parameter and then averaged.

**Quantitative Reverse Transcription-PCR**—Heart samples were collected and frozen in liquid nitrogen. Total RNA from each heart sample was prepared using the RNasy kit (Qiagen, Valencia, CA), and reverse transcription was carried out using total RNA as described previously (18). Real time PCR was performed using a QuantiTect SYBR Green PCR kit (Qiagen) on a Chromo4 fluorescence detector (MJ Research, Inc., Waltham, MA) according to the manufacturer’s protocol. The PCR mixture contained 0.4 μM forward and reverse primers in a total volume of 50 μl. Amplification included one cycle of 95°C followed by 40 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s. A melting curve was used to identify a temperature where only the relevant amplified product, and not primer dimers, accounted for the SYBR Green-bound fluorescence. All the values were normalized to the mRNA expression levels of glyceraldehyde-3-phosphate dehydrogenase. The primer sequences used in this study are as follows: atrial natriuretic factor: forward 5′–3′ ACGCCAGCATGGCTCCTTCTCC, reverse 5′–3′ GCTGTTATCTTGAGTACCAGGAAG; brain natriuretic factor: forward 5′–3′ AAGCTGCTGGAGCTGATAGA, reverse 5′–3′ GTTACAGCCAAAAGACTGTA; α-cardiac myosin heavy chain: forward 5′–3′ GAATGGCAAGACGTTGACTGTGA, reverse 5′–3′ CCTCTTAGTGTTGACAGTCT; β-cardiac myosin heavy chain: forward 5′–3′ CCAGAAGAGAACTCCAT, reverse 5′–3′ ACTCTTGGTCTGATGGCA; glyceraldehyde-3-phosphate dehydrogenase: forward 5′–3′ ACCACGATCCATGCCATAC, reverse 5′–3′ TCCACACCCTGTGTCTGTA.

**RESULTS**

**Expression and Distribution of GFP-NMHC II-A in B**+/** Mice**—We disrupted the NMHC II-B gene with cDNA encoding NMHC II-A, thereby placing II-A under the control of the endogenous NMHC II-B promoter (see “Experimental Procedures” and Fig. 1, A and B). The absence of NMHC II-B was confirmed by immunoblot analysis of lung, heart, and brain tissues from 3-month-old homozygous mice, demonstrating that NMHC II-B expression was ablated from the B**+/** mice (Fig. 2A, top panel). Expression of GFP-NMHC II-A (GFP-II-A) was detected in all these tissues along with endogenous NMHC II-A using an antibody to NMHC II-A (Fig. 2A, second panel from top) and an antibody to GFP (Fig. 2A, middle panel). NMHC II-C expression was unchanged in these mice.

We also carried out experiments to quantify the expression of GFP-NMHC II-A in B**+/** mice by generating mice that express GFP-NMHC II-B as a fusion protein, again using homologous recombination. This also allowed us to monitor any potential adverse effects of GFP on the organs expressing a fusion protein of GFP-NMHC II. We generated these B**+/** mice by inserting GFP into the II-B gene in the first coding exon just after the translational start site. Importantly these mice express the same amounts of NM II-B as B-/+ mice. Using these mice and the B**+/** mice, we quantified the expression of GFP-NMHC II-B and GFP-NMHC II-A using an antibody to GFP. Quantitation of immunoblot analyses revealed that the expression of GFP-NMHC II-A in the hearts and lungs of the B**+/** mice was about 80% of the expression found for GFP-NMHC II-B in the B**+/** mice (see Fig. 2B) confirming that GFP-NM II-A was being expressed in B**+/** mice.

J. Bao and R. S. Adelstein, unpublished data.
mice at or near physiological levels of NM II-B expression in wild-type mice. These quantitative data also suggest that the abnormalities found in the cardiac myocytes and neuronal cells of the B\(^{**}\)/B\(^{**}\) mice are not due to excessive over- or underexpression of NMHC II-A (see below). Moreover there was no phenotypic difference between the B\(^{GFP}\)/B\(^{GFP}\) mice and B\(^{+}/B^{+}\) mice, showing that the fusion of GFP and myosin II was not toxic to mouse tissues.

Studies using immunofluorescence confocal microscopy of E16.5 paraffin-embedded heart sections of B\(^{**}\)/B\(^{**}\) mice confirmed the absence of NMHC II-B expression in the cardiac myocytes as well as the non-myocytes in the heart (Fig. 3G). The figure also shows the presence of GFP-NMHC II-A in both cell types of the B\(^{**}\)/B\(^{**}\) heart (Fig. 3F). Desmin, which is only present in the cardiac myocytes, was used to distinguish the myocytes from the non-myocytes (Fig. 3B, E, and H; compare with C and F). Desmin staining and the staining of GFP-NMHC II-A using an antibody to GFP confirmed the presence of NMHC II-A in the cardiac myocytes of B\(^{**}\)/B\(^{**}\) hearts (Fig. 3, H and I). Note that wild-type hearts (B\(^{+}/B^{+}\)) do not contain NMHC II-A in the cardiac myocytes (Fig. 3, A and C; and Refs. 24, 34, and 35) but that it is present in the vasculature and other non-myocytes. Replacement of NMHC II-B by II-A was also seen in the developing brain (Fig. 3, J–M) as well as the lungs and skin,\(^5\) organs that normally express both NMHC II-A and II-B. As shown in Fig. 3J, the B\(^{+}/B^{+}\) 4-day-old brain was enriched for NMHC II-B with NMHC II-A mostly confined to the vasculature (Fig. 3L). In B\(^{**}/B^{**}\) mice, NMHC II-B was absent from the brain (Fig. 3K) and was replaced by NMHC II-A (Fig. 3M). Taken together, these results confirm that NMHC II-A expression is under the control of the endogenous NMHC II-B promoter in the B\(^{**}/B^{**}\) mice.

Some F2 B\(^{++}/B^{+}\) Mice Survive Embryonic Lethality—Our previous studies showed that 100% of the NMHC II-B knockout mice (B\(^{-}/B^{-}\)) died before E14.5 and birth (23, 24). Table 1 summarizes the genotypes found following the F1 cross from heterozygous matings. Of note is that heterozygous (B\(^{GFP}/B^{GFP}\)) mice and B\(^{+}/B^{+}\) mice were no different from B\(^{-}/B^{-}\) mice with respect to survival and phenotype. We also examined the hearts from B\(^{+}/B^{**}\) mice at ages 3, 6, and 12 months by echocardiography and histology (\(n \geq 4\) for each). We found no evidence for abnormalities in any of these hearts. Whereas most of the B\(^{**}/B^{**}\) mice generated from F1 generation matings of B\(^{+}/B^{**}\) mice died during embryonic development, 15 B\(^{**}/B^{**}\) mice of the 172 mice born survived to adulthood. This indicates that substitution of NMHC II-A for NMHC II-B could rescue embryonic lethality in at least some of the NMHC II-B ablated mice. However, following the F2 and F3 crosses, no live B\(^{**}/B^{**}\) mice were generated. We reasoned that this was due to changes in the background strain (see “Discussion”). Table 1 also shows that most

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\(^{5}\)X. Ma and R. S. Adelstein, unpublished observation.
of the Ba*/Ba* mice that did not survive died by E16.5. As described below, all of these mice had cardiac defects. To compensate for the effects of strain dependence on phenotype, the present studies compared mice born from the same litters.

**Cardiac Findings in Ba*/Ba* Mice**—In contrast to the Ba*/Ba* mice that survived embryonic lethality, seven of seven of the Ba*/Ba* mice analyzed between E13.5 and E16.5 following the F2 cross had a VSD (Fig. 4, right panel, yellow arrow), and five had dextroposition of the aorta resulting in a DORV. In the other two cases, the aorta straddled the two ventricles. These findings were similar to those reported for B/H11002/B/H11002 mice (23, 24).

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**TABLE 1**
Frequency analysis of genotypes of offspring from F1 generation heterozygous (+/a*) matings

DNA from adult and fetal mice was genotyped by Southern blotting as detailed in Fig. 1, and the numbers of +/+ , +/a*, and a*/a* mice were tabulated.

| Age          | Total | +/+  | +/a* | a*/a* |
|--------------|-------|------|------|-------|
| Adult*       | 172   | 59   | 98   | 15    |
| E13.5–E16.5  | 89    | 28   | 45   | 16    |
| E11.5–E12.5  | 47    | 10   | 27   | 10    |

* Older than 3 weeks.
Previous work from this laboratory demonstrated a 70% decrease in the number of cardiac myocytes in B-/-B- mice at E14.5 but no change in the number of non-myocytes in the heart (24). This was attributed to a defect in cytokinesis in the cardiac myocytes that was manifested by increased binucleation in the embryonic heart. Because the cardiac myocytes normally lack NM II-A, but non-myocytes do not (see Fig. 3A), we attributed the defect in cytokinesis in the myocytes of B-/-B- mouse hearts to the absence of both NM IIs (II-A and II-B), which was compensated for in the non-myocytes by the presence of NM II-A. Therefore, it was of particular interest to whether the expression of NM II-A in cardiac myocytes could rescue this defect.

Fig. 4B (right panel) shows that increased binucleation was still present in the B+*/B+* cardiac myocytes at E13.5 (yellow arrows). The percentage of binucleated cardiac myocytes was 10.9 ± 4.4% (mean ± S.E., n = 8 mice) in B+*/B+* mice compared with 1% found in control littermates. Flow cytometric analysis of cardiac myocytes from E16.5 hearts using sarcomeric α-actinin to separate the cardiac myocytes from the non-myocytes confirmed the decrease in the percentage of cardiac myocytes versus non-myocytes in B+*/B+* mice as compared with B+*/B- mice (38% in B+*/B+* mice, n = 6, and 65% in B+*/B- mice, n = 4; Fig. 4C). Comparison of the total numbers of cardiac myocytes indicated that the cardiac myocytes in B+*/B+* mice were decreased by 70% compared with B+*/B- mice. In contrast, there was no difference in the numbers of non-myocytes in the hearts of these mice. We confirmed by terminal deoxynucleotidyltransferase dUTP nick-end labeling assay that the decreased number of cardiac myocytes was not due to apoptosis.

**Dilated Cardiomyopathy in Surviving B+*/B+* Mice**—The B+*/B+* mice, which are born and survive to become adults, did not show any structural cardiac defects such as a VSD or DORV. However, beginning at 3 months, the B+*/B+* mice showed signs of a dilated cardiomyopathy. Fig. 5, A and B, compare the heart of a B+*/B+* mouse with that of a B+*/B+* mouse from the same litter at 3 months. Cardiac function was quantified using echocardiography, which showed a decrease in fractional shortening in the B+*/B+* hearts and an increase in the end systolic and diastolic dimensions (Table 2). Fig. 5, C–F, shows an increase in the size of B+*/B+* cardiac myocytes compared with control littermates following H&E staining or fluorescently labeled wheat germ agglutinin staining (cardiac myocyte area: B+*/B+* myocytes, 107 ± 4 μm²; B+*/B+* myocytes, 221 ± 15 μm²; n = 3 mice for each, 100 cells measured/mouse).

Consistent with a dilated cardiomyopathy, Fig. 6 shows the presence of interstitial fibrosis in a B+*/B+* heart at 3 months (Fig. 6B) compared with a control (Fig. 6A) as detected with Masson’s trichrome stain. As expected from the hypertrophic morphology, the B+*/B+* mice at 3 months of age exhibited a higher heart/body weight ratio (mg/g) than B+*/B+* mice (Fig. 6C). They also showed increased expression of hypertrophy-related genes (31), including atrial natriuretic factor and the β-cardiac myosin heavy chain as examined by quantitative reverse transcription-PCR (Fig. 6D).

**Rescue of Hydrocephalus in B+*/B+* Mouse Brains**—Of particular interest is our observation that none of the B+*/B+* mice suffered from the severe hydrocephalus that was found in all B-/-B- mice (25). Recently we have demonstrated that the cause of hydrocephalus in B-/-B- mice is due to a defect in cell-cell adhesion in the cells lining the spinal canal. This defect allows the underlying neuroepithelial cells to protrude into and obstruct the spinal canal (36). Fig. 7A compares the brains from wild-type, B-/-B-, and B+*/B+* mice, showing the presence of hydrocephalus in the B-/-B- mice (middle panel) and its absence in both B-+/-B- and B+*/B+* mice during the perinatal period (top and bottom panels). Fig. 7B shows that unlike B-/-B- mice, which have an obstructed spinal canal due to the protrusion of neuroepithelial cells (middle panel), the spinal

![FIGURE 5. Dilated cardiomyopathy in surviving B+*/B+* mice. A, H&E-stained cross-sections of hearts from 3-month-old mice (A and B). Scale bar, 1.3 mm. B, B+*/B- (C and E) and B+*/B+* (D and F) heart sections were stained with H&E (C and D) and Alexa Fluor 594-conjugated wheat germ agglutinin to visualize the cell membrane (E and F). The blue in E and F is 4,6-diamidino-2-phenylindole staining of nuclei. Scale bars, 50 μm (D) and 20 μm (F). RV, right ventricle; LV, left ventricle.](image)

**TABLE 2**

| Echocardiography parameters of B+*/B- and B+*/B+* mice at 3 months |
|-------------------------|------------------------|
|                         | B+*/B- (n = 6)          | B+*/B+* (n = 6) |
| HR (bpm)                | 509 ± 43               | 441 ± 82       |
| LVEDD (mm)              | 4.10 ± 0.15            | 4.57 ± 0.43*   |
| LVESD (mm)              | 2.32 ± 0.19            | 3.05 ± 0.43*   |
| IVS (mm)                | 0.59 ± 0.06            | 0.57 ± 0.03    |
| PW (mm)                 | 0.55 ± 0.04            | 0.54 ± 0.02    |
| % FS                    | 43.57 ± 3.75           | 33.50 ± 5.14*  |

*p < 0.05 between genetic groups.

*p < 0.01 between genetic groups.
Expression levels of atrial natriuretic factor (ANF) and brain natriuretic factor (BNF) at postnatal day 4 (P4) in B***/B** mice. Note that in the B**/B** mouse brain, the lateral ventricles are enlarged due to severe hydrocephalus. Scale bar, 100 μm.

Previously for the NMHC II-B ablated and NMHC II-B mutated mice (25, 26). Thus, unlike the restoration of cell adhesion in IV, neuronal cell migration, which was not rescued in the cells of Ba*/Ba* mice when stained with antibodies to N-cadherin, and NM II-A in Ba*/Ba* mice. Note that in the B**/B** mouse brain, the lateral ventricles are obstructed in B**/B** mice. In contrast, the spinal canal is obstructed in B**/B** mice. The middle panel in B is reprinted with permission from Ma et al. (36). Scale bar, 100 μm.

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FIGURE 6. Evidence for dilated cardiomyopathy in B***/B** mice. A and B, Masson’s trichrome staining of heart sections at 3 months of age to detect interstitial fibrosis (blue) in a B***/B** mouse (B) but not in a B**/B** littermate (A). Scale bar, 50 μm. C, quantification of heart/body weight ratio from 3-month-old B**/B** and B***/B** littermate mice. The heart/body weight ratio is 4.62 ± 0.31 in B**/B** mice and 6.07 ± 0.89 in B***/B** mice (mean ± S.E.; n = 6; **, p < 0.01). D, quantitative reverse transcription-PCR analysis of mRNA expression levels of atrial natriuretic factor (ANF), brain natriuretic factor (BNF), α-cardiac myosin heavy chain (αMHC), and β-cardiac myosin heavy chain (βMHC) (mean ± S.E.; n = 6; **, p < 0.01).

FIGURE 7. Rescue of hydrocephalus in B***/B** mice. A, coronal sections of brains from B**/B**, B**/B** (postnatal day 0 (P0)), and B***/B** (postnatal day 4 (P4)) mice. Note that in the B**/B** mouse brain, the lateral ventricles are enlarged due to severe hydrocephalus. Scale bar, 100 μm. B, cross-sections of spinal cords at the thoracic level of B**/B** and B***/B** mice (E12.5). Note that the spinal canal is patent in B**/B** and B***/B** mice. In contrast, the spinal canal is obstructed in B**/B** mice. The middle panel in B is reprinted with permission from Ma et al. (36). Scale bar, 100 μm.

Note that a similar structure was also present in the cells of B***/B** mice when stained with antibodies to NMHC II-A or antibodies to GFP (Fig. 8A, panels d and f). As expected, no NMHC II-B was detected in B***/B** mice (Fig. 8A, panel e). These results indicate that NMHC II-B can be replaced by NMHC II-A with respect to both the expression and localization at the apical border of the neuroepithelial cells lining the spinal cord.

FIGURE 8. Rescue of hydrocephalus in B***/B** mice. A, coronal sections of brains from B**/B**, B**/B** (postnatal day 0 (P0)), and B***/B** (postnatal day 4 (P4)) mice. Note that in the B**/B** mouse brain, the lateral ventricles are enlarged due to severe hydrocephalus. Scale bar, 100 μm. B, cross-sections of spinal cords at the thoracic level of B**/B** and B***/B** mice (E12.5). Note that the spinal canal is patent in B**/B** and B***/B** mice. In contrast, the spinal canal is obstructed in B**/B** mice. The middle panel in B is reprinted with permission from Ma et al. (36). Scale bar, 100 μm.

The ability of GFP-NMHC II-A to replace II-B restored cell-cell adhesion and the integrity of the cells lining the canal, thereby preventing the neuroepithelial cells from blocking the canal (Fig. 7B, middle panel). This explains the rescue of the hydrocephalus in B***/B** mice.

On the other hand, NMHC II-A did not rescue all of the brain defects found in B***/B** mice. For example, the facial and pontine neurons migrated abnormally, and the former protruded into the fourth ventricle. Thus, unlike the restoration of cell adhesion in B***/B** mice, which appears to depend on a scaffolding property of myosin II, neuronal cell migration, which was not rescued in...
these mice, appears to depend on the specific motor properties of NM II-B (see “Discussion”).

Interestingly all the B+/B+ mice that survived embryonic lethality had a normal gait and could right themselves without difficulty, suggesting that, unlike hypomorphic NMHC II-B mutant mice (26), they have a normal functioning cerebellum. This was confirmed by analysis of serial sections of the cerebellum that showed only minor changes in foliation (Fig. 9, compare D with C). This is in contrast to the hypomorphic NMHC II-B mutant mice, which showed marked abnormalities in their cerebellar foliation pattern (26).

DISCUSSION

In this study, we addressed the question of functional redundancy of two of the three nonmuscle myosin II isoforms in vivo. Previous work demonstrated that ablation of NM II-A results in a distinct phenotype causing lethality by E6.5 due to a failure in cell-cell adhesion and visceral endoderm formation (27). As noted above, ablation or a severe reduction of NM II-B results in lethality beginning at E14.5 with defects in the heart and nervous system (23, 37). In this report we show that NM II-A rescued hydrocephalus but did not rescue defects in neuronal cell migration or in cardiac myocyte cytokinesis or cardiac structural abnormalities.

How do we explain the ability of NMHC II-A to rescue some, but not all, of the phenotypes found in the B+/B+ mice? One reason could be that the expression of NMHC II-A is not identical to that of NMHC II-B. Because our cDNA construct uses the SV40 3’ untranslated region rather than the native 3’ untranslated region and because the inserted cDNA could displace downstream transcriptional regulatory elements, we were concerned about whether it would faithfully recapitulate the different levels of expression of the endogenous II-B during development. We cannot rule out the possibility that there could be subtle differences, which we did not detect. However, the following findings are in favor of GFP-II-A expression generally mimicking the endogenous II-B expression. In the brain, GFP-II-A could replace II-B in the meshlike structure in cells lining the spinal canal (see Fig. 8). This structure is only transiently present in the brain (between E9.5 and E12.5; Ref. 36), and the expression of GFP-NM II-A in this structure followed the II-B pattern of expression. With respect to the heart, we found that the overall expression of GFP-NM II-A in the cardiac myocytes decreased following birth of the B+/B+ mice that survived, similar to NM II-B expression in B+/B+ mice.

A second approach is to consider the functional roles played by NM II-B in vivo and whether these roles involve the enzymatic or structural properties of the myosin molecule. Work from a number of laboratories (14–17) has demonstrated major differences in the kinetic properties of the various NM-II isoforms. For example, NM II-B has the highest duty ratio (portion of the kinetic cycle spent in a state strongly bound to actin) of any myosin II and is particularly suitable for bearing sustained tension on actin filaments. These unique kinetic properties may not be replaceable by other NM-II isoforms and could be responsible for a specific role for NM II-B during cytokinesis in the heart. The defects in cytokinesis are at least partially responsible for the 70% reduction in cardiac myocytes and may

FIGURE 8. Localization of NMHC II-A and II-B in the neuroepithelial cells and staining of the adhesion structure lining the spinal canal at E11.5. A, cross-sections of the spinal cord were stained with anti-NMHC II-A (panels a and d; green) or anti-NMHC II-B (panels b, c, e, and f; red) or with anti-GFP (panels c and f; green) and 4’6’-diamidino-2-phenylindole (DAPI) (panels c and f; blue). Note that NMHC II-A is correctly localized to the apical border of the neuroepithelial cells in B+/B+ mice (panel d), similar to the localization of NMHC II-B in B+/B+ mice (panel b). B, paraffin-embedded cross-sections of E11.5 mouse spinal canals stained for cell-cell adhesion structures in neuroepithelial cells lining the spinal canal in B+/B+ (panel a), B+/B− (panel b), and B−/B− mice (panel c). Green, NMHC II-B staining (panels a and b) and NMHC II-A staining (panel c); red, β-catenin staining (panels a–c); blue, 4’6’-diamidino-2-phenylindole staining of nuclei. Scale bar, 8 μm.

FIGURE 9. Abnormal migration of facial and pontine neuronal cells but normal cerebellar architecture in B+/B+ mice. A and B, sagittal sections from E16.5 B+/B+ and B+/B− mice. Note that the pontine neurons (arrow in A) are absent from their normal destination in B+/B− mice (arrow in B). Double arrows in B show the abnormal protrusion of facial neurons into the fourth ventricle of B+/B− mice. Scale bar, 100 μm. C and D, sagittal sections from 7-month-old B+/B− (C) and B+/B+ (D) mouse cerebellums showing only minor alterations in the foliation pattern in the B+/B− brain. The arrowhead in C indicates a groove that is not present in the B+/B+ brain. The arrow in the lower part of D points to abnormally protruding facial neurons. Scale bar, 500 μm.
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also explain the presence of the VSD and DORV because the reduced number of these cells could translate into defects in cardiac looping resulting in structural defects. The failure of NMHC II-A to rescue the defect in cytokinesis is also consistent with our previous results using cultured cardiac myocytes wherein expression of NMHC II-A also failed to rescue this defect (24). The abnormal migration of specific groups of neurons (facial and pontine) may be attributed to the failure of NM II-A to replace II-B due to a difference in the motor activity of the two myosins and a specific role for NM II-B in the normal migration of these particular cells.

Dilated cardiomyopathy is a leading cause of heart failure and is believed to result from abnormal remodeling of cardiac tissues in response to a variety of stresses (38, 39). Mutations in cytoskeletal proteins are presumed to disrupt the myocyte cytoarchitecture, thus impairing the transmission of contractile force generated by the sarcomere to the extracellular matrix, leading to dilated cardiomyopathy. Here we report a mouse model in which NM-II, a cytoskeletal protein, is involved in diluted cardiomyopathy. Cardiac myocytes appear to be unique in that they do not contain NMHC II-A after E9.5. Whereas both NMHC II-B and II-C are expressed in cardiac myocytes before birth, their expression is markedly decreased after birth, and they appear to be confined to the intercalated disks (40). The onset of cardiac myocyte hypertrophy, thinning of the ventricular walls, and interstitial fibrosis seen in B+/B− hearts may reflect a response of these cells to the presence of the “foreign” NMHC II-A isoform as well as a decrease in the number of cardiac myocytes found in B+/B− mouse hearts.

In contrast to the cardiac and brain abnormalities not rescued by the II-A isoform is our finding that NMHC II-A rescued the severe and persistent hydrocephalus found in B−/B− mice. One plausible explanation for the rescue of this abnormality is that the ability of II-A to replace II-B in the brain is independent of the differences in the motor function and duty ratio of the two isoforms and resides in the structural or scaffolding properties present in the myosin molecule, properties that are common to both II-A and II-B. Recently we reported evidence for the existence of a meshlike adhesion complex present at the apical border of the cells lining the spinal canal during development (36). The proper configuration of this complex is dependent on the presence of NM-II because in its absence the complex collapses, and the neuroepithelial cells lining the border no longer adhere to each other (Fig. 7B, middle panel, and Ref. 36). Thus, similar to the role that NM II-A plays in maintaining cell-cell adhesion in ES cells and the early mouse embryo (27), NM II-B is required to maintain adhesion of the cells lining the spinal canal. Here we show that expression of II-A in place of II-B was sufficient to restore the normal meshlike structure of the adhesion complex (see Fig. 7B, panel c), which is consistent with the ability of II-A to function in cell adhesion. Moreover our recent study demonstrates that a mutant NM II-B with an impaired motor activity can also function to maintain the adhesion complex (36). Thus, the ability of GFP-NM II-A to localize to the apical border adhesion plaques present in the neuroepithelial cells appears to depend on a structural feature of myosin II common to both myosin isoforms.

The ability of NM II-A to rescue the migratory defects found in the cerebellar granule cells and not the defects found in the facial and pontine neurons might be attributed to the different mechanisms used by these cells for migration (41). Whereas the migration of cerebellar granule cells (which is rescued by expression of NM II-A) is glial cell-dependent, the migration of pontine and facial neurons is tangential and depends on neural cell guidance. This suggests that the interaction between cerebellar granule cells and glial cells, which are enriched for endogenous NM II-A (42), also reflects a structural property of the myosin that is common to both isoforms. The restoration of normal cerebellar architecture in the B+/B− mice was accompanied by normal gait and balance, which are compromised in the hypomorphic mutant NM II-B mice (26).

Finally it is worth noting that, prior to the F2 crosses, 15 of the B+/B− mice survived the embryonic lethality that affects all B−/B− mice. The surviving mice did not suffer from any major cardiac defects (VSD or DORV) that affect the other B+/B− mice, nor did any other organ system appear to be compromised. They did, however, manifest abnormal migration of pontine and facial neurons and as noted developed a cardiomyopathy. We attribute the survival of these mice to the effects of modifying genes in the different genetic backgrounds. The identity of these modifying genes is a subject for further study. On the other hand, the survival of some B+/B− mice to adulthood is allowing us to study the development of a diluted form of cardiomyopathy in a mouse model of the human disease.

Acknowledgments—We thank Qize Wei and Sachio Kawamoto for help with the design and construction of the targeting vector and Antoine Smith for technical assistance. We thank Charles Birdsell and Stanislav Sidenko from the Cardiovascular Branch, NHLBI, as well as Brenda Klausenber from the Mouse Imaging Facility, NINDS, National Institutes of Health, for echocardiography. We thank Georgina F. Miller (Division of Veterinary Resources, National Institutes of Health) and Zu-Xi Yu (Pathological Core Facility, NHLBI) for help in characterizing the cardiac defects. We also acknowledge the professional skills and advice of Philip McCoy, Jr. (Flow Cytometry Core Facility, NHLBI) and Christian A. Combs (Light Microscopy Core Facility, NHLBI). We thank Catherine Magruder for editorial assistance and Mary Anne Conti, Sachio Kawamoto, James Sellers, and Neal Epstein for kindly reading the manuscript.

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