Pathophysiology of Sodium Channelopathies

STUDIES OF SODIUM CHANNEL EXPRESSION BY QUANTITATIVE MULTIPLEX FLUORESCENCE POLYMERASE CHAIN REACTION*

(Received for publication, November 3, 1993, and in revised form, April 18, 1994)

Jianhua Zhou and Eric P. Hoffman
From the Departments of Molecular Genetics and Biochemistry, Human Genetics and Pediatrics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

The inherited diseases hyperkalemic periodic paralysis and paramyotonia congenita are caused by mutations in the adult skeletal muscle sodium channel gene. To determine if differences in the expression patterns of the adult and cardiac/fetal sodium channel genes could explain some clinical features of these disorders, we developed a novel mRNA quantitation strategy called quantitative multiplex fluorescent polymerase chain reaction (QMF-PCR). This assay tests the relative levels of multiple mRNA species simultaneously using automated sequencers. We show validation of this method by competitive-PCR and RNase protection. Developmental studies of sodium channel mRNAs in humans and mice by QMF-PCR showed that the adult sodium channel mRNA quickly increased, while the cardiac/fetal sodium channel mRNA slowly decreased similarly in both limb and diaphragm muscle. We find that the adult sodium channel gene expression is predominant in fetal and neonatal muscle of both humans and mice: adult isoform mRNA concentration in fetal muscle was $8.4 \times 10^{-6}$ pg/μg of total RNA; cardiac/fetal isoform mRNA was $2.0 \times 10^{-6}$ pg/μg; and actin mRNA was $3.4 \times 10^{-6}$ pg/μg. Our results suggest that differential sodium channel gene expression correlates with age of onset of disease, but not with diaphragm involvement, in patients with hyperkalemic periodic paralysis.

Dominantly inherited single amino acid changes of the human and horse adult skeletal muscle sodium channel cause both potassium-induced paralytic attacks (hyperkalemic periodic paralysis) and cold-induced muscle contraction (paramyotonia congenita) (1–6). Efforts to understand the pathophysiology of these disorders have been focused on the correlation of results from molecular genetic and electrophysiological experiments with clinical data (7–9). These data have addressed some features of the pathophysiology, such as the molecular basis for disease expression as a heterozygote, and have begun to identify functional abnormalities of the mutant channels. However, other characteristic clinical features of the disorders have yet to be explained. Why is the diaphragm refractory to paralytic attacks in periodic paralysis? Why is the age of onset generally in late childhood? Why are the disorders clinically variable even within families having the same mutation?

There are two well described skeletal muscle sodium channel genes, and the differential expression patterns of these two genes could explain age of onset and diaphragmatic involvement. The gene harboring the disease-causing mutations is the "adult" isoform of the voltage-sensitive sodium channel, and has been mapped to chromosome 17q in humans (10–12). A different gene is expressed in cardiac muscle, fetal skeletal muscle, and in denervated skeletal muscle, and has been localized to chromosome 3 (11, 13, 14).

Previous studies have investigated the developmental expression of the two sodium channel genes in rat skeletal muscle. Some of these studies used Northern analysis or RNase protection assays to determine RNA levels (13, 15). Others studied protein production or sodium channel functions by toxin binding or patch clamp (15–20). These different experimental systems generated different conclusions: RNA studies suggested that cardiac/fetal sodium channel gene expression predominates in fetal muscle, while protein studies suggested that there may be relatively high levels of adult, tetrodotoxin-sensitive channels in fetal muscle. One possible explanation of the differences is the previous inability to accurately determine relative RNA levels from the two genes in a single sample.

To address questions of pathophysiology of HyperPP1 and paramyotonia congenita, we studied sodium channel gene expression in human and mouse skeletal muscle and diaphragm using a novel RT-PCR method. Many protocols have been designed to use PCR to quantitate both DNA and RNA (21–26). One of the most popular, competitive PCR, determines the concentration of a target by using a range of co-amplified, exogenously added competitor of known concentration (21, 22). Disadvantages of this and most other quantitative PCR techniques are that they measure levels of a single target sequence, and require numerous PCR reactions for a single sample. In many applications, it is important to measure the relative levels of multiple target sequences. We have recently described a PCR assay for genomic DNA which measures relative levels of multiple templates using fluorescently labeled primers in multiplex reactions analyzed on automated sequencers (27). Here, we extend this assay to studies of multiple RNA templates by measuring the relative mRNA levels of the two sodium channel genes in a single PCR reaction. We show that our assay yields results similar to competitive PCR and RNase protection, yet is much simpler to perform. We find that adult sodium channel mRNA predominates in all skeletal muscle, including both mouse and human fetal muscle. The relative levels of sodium channel mRNAs were similar in the diaphragm and skeletal muscle, suggesting that other factors are important in the sparing of the diaphragm during attacks.

MATERIALS AND METHODS

Muscle Samples—Human skeletal muscle samples were pre-existing diagnostic biopsy specimens which had been flash-frozen in liquid ni-

1 The abbreviations used are: HyperPP, hyperkalemic periodic paralysis; PCR, polymerase chain reaction; QMF-PCR, quantitative multiplex fluorescent-PCR; RT-PCR, reverse transcriptase-PCR; bp, base pair(s); ss cDNA, single-strand cDNA.

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* This work was supported by National Institutes of Health Grant AR41025. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Developmental Expression of Sodium Channels

trigged and sent to Pittsburgh for dystrophin protein analyses (29). Few muscle samples were from pregnancies terminated due to risk for Duchenne muscular dystrophy. Diaphragm samples were from autopsies.

C57BL/10J mice of different ages were sacrificed by chloroform inhalation and the gastrocnemius muscles and diaphragms were removed and frozen in liquid nitrogen.

RNA Isolation and Synthesis of ssDNA—Isolation of total RNA was done as described previously (29). Single-strand cDNA was synthesized by incubation of 0.5–2 µg of total RNA at 42 °C for 1 h in 12.5 µl of 50 mM Tris-HCl buffer (pH 8.3) containing 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, and 0.5 µg of oligo(dT) primer with 15 units of avian myeloblastosis virus reverse transcriptase (BM), 1 µm each dNTPs, and 5 units of placentals rachinose inhibitor (RNasin, BM). The synthesized cDNA was diluted to 125 µl with water for PCR reactions. Control genomic DNA samples of C57BL/10J mice are kindly provided by Dr. Stephen Phillips. Human genomic DNA samples are prepared from peripheral blood as described previously (30).

Cloning and Sequencing of Mouse Sodium Channel Genes—Cloning and sequencing of mouse cardiac/fetal and adult sodium channel genes was done by cross-species PCR, as described previously (12). PCR primers were synthesized from the cDNA sequence for the human and/or rat adult and cardiac/fetal skeletal muscle a subunit genes (10, 12–14) with either a BamHI site (forward primers) or a HindIII site (reverse primers) at the 5' end.

PCR for CA Repeats in Dystrophin Gene—PCR primers and methods have been described previously (27, 31–33).

Quantitative Multiplex Fluorescence PCR (QMF-PCR)—Primers were designed for a actin, cardiac/fetal, and adult skeletal muscle sodium channel genes of both human and mice. The sequence for each primer follows: human cardiac/fetal sodium channel primers 5775F: 5'-GCCCTCTTCCTCCTCTCCACAGCAGGC-3, 5916R: 5'-TGGAGGAGGAGGAGCTGGA-3'; human adult sodium channel primers S6(17)F: 5'-GGTGACTGTGGCAACCCCTCC-3', S6(21)R: 5'-GGGAAGCTTCTCTTACATGTACCGCCACAGC-3'; mouse cardiac/fetal sodium channel primers 5765F: 5'-AAGGATGGCTCTCCCTCTCCCTCCG-3', 5918R: 5'-TGGAGAAGATGAGGAGAGGCTGGA-3'; human adult sodium channel primers S6(21)F: 5'-GCTTCTCAAAGTCATCTTCACCAAGC-3'; mouse adult sodium channel primers MouseF: 5'-TCCCTCAACTAGTATGCCCGACAGC-3', MouseR: 5'-CTCCCTGGTGGGTGGCCAGCTCCATCC-3'; human and mouse a actin primers 576F: 5'-ATCCCTATCTGCTGTAATCAACG-3')); mouse cDNA template (BanII site deleted). The cardiac/fetal sodium channel gene plasmid was digested with BanII. The cardiadfetal sodium channel gene was cloned by cross-species RT-PCR and sequenced (Fig. 1).

Quantitation of Sodium Channel mRNA—The PCR products for adult and cardiadfetal sodium channel genes were performed separately for 20 cycles (adult sodium channel gene) or 22 cycles (cardiadfetal sodium channel gene). The 12.5-µl reaction contained cDNA templates, 25 ng of each primer, 40 µM each of dCTP, dGTP, dATP, and dCCP in a 12.5-µl reaction under the conditions of the QMF-PCR. Reactions for adult and cardiac/fetal sodium channel genes were performed separately for 20 cycles (adult sodium channel gene) or 22 cycles (cardiadfetal sodium channel gene). The 12.5-µl reaction contained cDNA templates, 25 ng of each primer, 40 µM each of dCTP, dGTP, dATP, and dCCP, and 0.3 unit of Taq polymerase.

The PCR products for adult and cardiadfetal sodium channel gene were combined and analyzed on a 6% sequencing gel by an automated sequenator. As a comparison, competitive PCR with 30 cycles was also done using (α-32P) dATP in a reaction and PCR bands resolved on a 6% sequencing gel and exposed to x-ray film.

RESULTS

Development of Quantitative Multiplex Fluorescent-PCR (QMF-PCR)—QMF-PCR was developed to determine the relative levels of adult and cardiac/fetal sodium channel mRNAs in human and mouse skeletal muscle and diaphragm. As sequences for neither the mouse adult nor cardiac/fetal sodium channel genes have been reported, mouse adult sodium channel gene cDNA sequence corresponding to 4828–5517 bp of the human sequence, and the mouse cardiac/fetal sodium channel gene cDNA sequence corresponding to 5769–5916 bp was cloned by cross-species RT-PCR and sequenced (Fig. 1). PCR primers specific for adult sodium channel, cardiac/fetal sodium channel, and α actin genes of both human and mouse were designed and synthesized. Primers were chosen near the 5' end of each cloning sequence to minimize the effect of possible variations in reverse transcription using oligo(dT) (Fig. 1C). All three PCR products for both human and mouse were designed to lie in a narrow size window (mouse adult sodium channel = 176 bp, cardiac/fetal sodium channel = 148 bp, actin = 118 bp; human adult sodium channel = 167 bp, cardiac/fetal sodium channel = 142 bp, actin = 118 bp) and minimize preferential amplification of multiplexed target sequences due to template size differences. PCR primers were selected which amplified both DNA and RNA (within a single exon) so that genomic DNA would be used as a control (1:1 ratio of gene copy number).

The primary goal of this study was to determine the relative levels of the sodium channel gene mRNAs in different tissues. We also studied α actin mRNA levels as an unrelated mRNA control. We found the level of α actin mRNA to be approximately 1,000-fold higher than sodium channel mRNAs. This made co-amplification of all three genes impossible. We therefore multiplexed the sodium channel mRNAs in QMF-PCR, and single-plexed the actin mRNA using 1/10,000 (mouse) or 1/5,000 (human) dilution of cDNA as template. This control was then processed in parallel to the multiplexed sodium channel PCR. The PCR products were then mixed and loaded on the automated sequenator. The heights and areas of photomultiplier tube voltage peaks were used to quantitate the relative amounts of PCR products, and hence of original mRNA. Some RT-PCR products showed multiple peaks, however, multiple peaks were eliminated if T4 polymerase was used (data not shown). This result suggested that the extra peaks were caused by the presence of contaminating DNA in the cDNA preparations.
Panel relative to mature transcripts. hnRNA transcripts are relatively common (37), additional exons (5769 to 5916 bp) were cloned by cross-species RT-PCR and sequenced. mRNA for both human and mouse. By addition of extra nontemplated bases by Taq polymerase. Channel gene sequence. For the adult and cardiac fetal sodium channel mRNA, and the actin sequence; was from the RNA preparation without reverse transcription (27) shows the expected polymorphic output (amount of PCR product) as a function of input DNA as expected (Panel A), single-strand cDNA (Panel B), and RNA (Panel C) were used as templates. All four CA repeats were amplified from genomic DNA as expected (Panel A), while no PCR amplification was found in the RNA preparation without reverse transcription (Panel C), indicating no genomic DNA contamination in the RNA preparation protocol used. Multiplex amplification of reversed transcribed RNA showed the expected amplification of the 3′ CA repeat which is localized within an exon, and the absence of 5′ DYS II which is amplified by intronic primers. Intronic STR 45 and STR 49 repeats were amplified probably from unspliced RNA (hnRNA).

We then conducted an extensive series of controls to validate QMF-PCR as an accurate measure of relative mRNA quantities in small samples. Descriptions of each set of control experiments follows.

RNA Contamination Control—Because primers for QMF-PCR were designed within exons, it was important to rule out genomic DNA contamination of RNA preparations. Four different polymorphic CA repeats in the dystrophin gene were used for PCR amplification (25 cycles) from human genomic DNA, ss cDNA, and RNA preparations. Among these four CA repeats, only the 3′ CA repeat (32) is located within an exon of the dystrophin gene, while the other three (31, 33) are within introns. Multiplex fluorescent PCR and electrophoresis on an automated sequenator (27) shows the expected polymorphic alleles when genomic DNA is used as a template (Fig. 2A) (note that females are usually heterozygous at each CA repeat locus, and different individuals show different alleles). None of the loci were amplified from RNA (Fig. 2C), indicating that there was no genomic DNA contamination. Three of the four loci were amplified from ss cDNA of the same RNA sample (Fig. 2B). It is likely that amplification of STR 45 and STR 49 intronic locus was from ss cDNA of unspliced dystrophin hnRNA rather than genomic DNA because the intronic locus 5′ DYS II (outside of transcription) was not amplified, and non-reversed transcribed RNA as a template showed no amplification products. While hnRNA transcripts are relatively common (37), additional experiments are being planned to determine if the 79-exon, 2.5-mega-base dystrophin gene shows a high proportion of hnRNA relative to mature transcripts.

Quantitative Controls—To validate the quantitative method of QMF-PCR and to provide correction factors for efficiency of PCR for each loci, genomic DNA with known loci ratio of actin:

adult sodium channel:fetal sodium channel of 1:1:1, was used as a template for QMF-PCR over a range of 7.5–240 ng (Fig. 3A). Two separate QMF-PCR reactions were done for each concentration of input DNA and the results of peak voltage were plotted as a function of input DNA (Fig. 3B). These data show that there is a good linearity of the assay as input DNA is increased. Since it is known that the ratio of target actin, adult, and cardiac/fetal sodium channel gene copies in genomic DNA is 1:1:1, the ratio of PCR product signal from genomic DNA (actin/adult/fetal = 4:1:1.8) was used to correct sodium channel mRNA levels in RNA samples. To validate the correction factor for the two sodium channel genes in mouse, we performed QMF-PCR using equal amounts of cloned adult and fetal sodium channel gene plasmid DNA. The correction factor obtained using genomic DNA (adult:cardiac/fetal = 1:1.8) was similar to that obtained using plasmid DNA (adult:cardiac/fetal = 1:1.5). In human, the correction factor obtained from genomic DNA is 1:8 (adult:cardiac/fetal = 1:8) (data not shown). These correction factors permitted normalization of preferential PCR amplification, and have been previously shown to be an accurate method of quantitation within an error of ±10% (27, 38). All subsequent data took into account these correction factors.

Linearity between the amount of input ss cDNA and the fluorescent signal of output PCR products was also determined using a range of 6–200 ng (0.25–8 μl) of ss cDNA as templates (Fig. 3C). The amount of ss cDNA used for all subsequent gene expression studies by QMF-PCR was clearly in the linear range of the assay (2 μl).

Efficiency of reverse transcription could be a major source of variability for any quantitative assay based on cDNA (RT-PCR). To test if reverse transcription had any effect on our QMF-PCR assay, ss cDNA was synthesized from a large range of target mRNA (0.75–24 μg of total RNA) and QMF-PCR was performed. Plotted results (Fig. 3D) show that there was a simple linear relationship between amount of input RNA tem-
FIG. 3. QMF-PCR analyses are linear over a range of genomic DNA, cDNA, and RNA templates. Shown are examples of automated sequenator raw data (left panels) and plotted data of duplicate experiments (right panels). The amount of genomic DNA template was varied from 240 to 7.5 ng for both α actin gene and sodium channel genes. QMF-PCR performed and PCR products were loaded on an automated sequenator with examples of photomultiplier tube voltage as a function of time shown in Panel A. Pairs of peaks can be seen in the fetal channel PCR product due to template-independent addition of an extra base at 3′ end by Taq polymerase. Each sample was done in duplicate, and the peak voltage of each PCR product plotted versus template concentration (Panel B). The lines connect the average of the two independent determinations for each point, and the standard deviation is shown. This analysis shows a good linearity between template concentration and voltage signal given a known, equal copy number of target sequences. The same protocol was used to test linearity of the assay when varying input cDNA in the PCR reactions (Panel C, 6–200 ng, shown as µl of cDNA reaction), and RNA in the cDNA reaction (Panel D, 0.75–24 µg) (only sodium channel results shown). All showed good linearity, with maintenance of the relative ratios of adult and cardiac/fetal isoforms at any given concentration of RNA, cDNA, and DNA.

Comprehensive PCR Control—To test if the ratio of cardiac/fetal and adult sodium channel mRNAs generated from QMF-PCR reflects the real ratio of the specific amounts of input templates, competitive PCR was used to determine the abundance of both cardiac/fetal and adult sodium channel genes from a subset of cDNA samples. The regions of the mouse adult and cardiac/fetal sodium channel genes used for QMF-PCR were cloned into the pT7T3BM vector, and competitor RNAs with 4 bp deletion (adult) or 4 bp insertion (cardiac/fetal) were produced as described under “Materials and Methods.”

cDNA synthesis was performed on the combined constant tissue RNA (1 µg) and varying amounts of competitor RNAs using sequence–specific sodium channel primers. Competitive PCR reactions were done separately for the adult and cardiac/fetal sodium channel genes using the same PCR primers used in QMF-PCR with fluorescein at the 5′ ends, then mixed post-PCR for analysis on the automated sequenator (Fig. 4A). The ratios of competitors to targets against the concentration of competitor were plotted. Zero of (log competitor/target) indicates the amount of adult (−6.65, 2.24 × 10^-7 µg) or cardiac/fetal sodium channel (−7.28; 5.25 × 10^-8 µg) mRNA in the reaction (Fig. 4B).

The competitive PCR method permits the calculation of the precise amount of a particular mRNA in a given amount of total RNA. Considering the molecular weight (size) of in vitro transcribed RNA competitor (240 bp) and sodium channel mRNAs (9 kilobases of single-strand RNA or cDNA), we calculated the amount of adult and cardiac/fetal mRNAs in total RNA from fetal muscle. To normalize for this, the ratio of in vitro RNA length (240 bp) and target mRNA length (9,000 bp) was multiplied by the amount of competitor RNA required to give equal signal in 1 µg of RNA. We found the amount of adult sodium channel mRNA to be 8.4 × 10^-6 µg/µg total RNA (2.24 × 10^-8 µg (37.5) = 8.4 × 10^-6 µg), and the cardiac/fetal sodium channel to be 2.0 × 10^-8 µg/µg total RNA (5.25 × 10^-8 µg (37.5) = 2.0 × 10^-6 µg).

We also calculated the concentration of actin mRNA per microgram of tissue RNA by combining the competitive PCR data with our QMF-PCR data. The QMF-PCR data for mouse fetal skeletal muscle shows the voltages of actin and the adult so-
Developmental Expression of Sodium Channels

**FIG. 4.** Quantitation of mouse adult and cardiac/fetal sodium channel mRNAs by competitive PCR and RNase protection. Shown are automated sequenator traces of quantitative competitive PCR using in vitro transcribed RNA as the competitor (Panel A), a graph showing linearity of competitive PCR (Panel B), and results from RNase protection experiments (Panel C). Panel A, the amount of fetal skeletal muscle RNA (1 μg) was held constant while the amount of competitor used was varied as indicated. Graph of peak areas as zero of log (competitor/target) indicates the amount of adult (-6.65) or cardiac/fetal sodium channel(-7.28) competitors in the reactions. Correcting for the different sizes of in vitro transcribed RNA and sodium channel mRNA, the ratio of adult and cardiac/fetal sodium channel mRNAs in fetal skeletal muscle is about 4.2:1, with a quantity of 8.4 x 10⁴ μg of adult sodium channel mRNA and 2.0 x 10⁴ μg of fetal sodium channel mRNA per microgram of total tissue RNA. Panel C shows RNase protection experiments done to corroborate the results seen by the two PCR-based methods (QMF-PCR and competitive PCR). The adult (A) and fetal (F) antisense RNA probes show the expected protected fragments after hybridization with sense RNA produced from plasmids in vitro (in vitro RNA A+F), and with muscle RNA, whether used singly (A or F) or together (A+F). Fetal skeletal muscle RNA shows protection of both adult and cardiac/fetal sodium channel probes with the adult isoform predominating.

**FIG. 5.** Comparison of oligo(dT)-primed and random-primed cDNA on relative expression of sodium channels determined by QMF-PCR. Shown are the results of QMF RT-PCR from the same mouse fetal skeletal muscle RNA (E17) used for competitive PCR (Fig. 4). Single-strand cDNA was synthesized from the RNA using oligo(dT) or random hexamers as primers and QMF-PCR was performed in triplicate. The corrected ratios with standard errors are shown. By both methods, the adult sodium channel isoform mRNA predominates in fetal muscle.

To determine the effect of different cDNA primers on the results, single-strand cDNA was synthesized with oligo(dT) primers or random primers and QMF-PCR performed from the same RNA sample. After using the correction factor of 1:1.8 derived from genomic DNA studies, the mRNA ratio of adult and cardiac/fetal sodium channel genes is about 1.7 ± 0.3:l for oligo(dT) QMF RT-PCR, and 1.9 ± 0.3:1 for random primer QMF RT-PCR in mouse fetal skeletal muscle (Fig. 5). These results were considered similar, and all subsequent experiments used oligo(dT) as primer for cDNA.
FIG. 6. The adult sodium channel RNA predominates in muscle and diaphragm at all ages. Top, raw data of mouse skeletal muscle QMF RT-PCR reactions analyzed on an automated sequenator. Bottom, summarized data for different developmental stages of mouse skeletal muscle (left panels) and mouse diaphragm muscle (right panels). For each age, three different RNA preparations were prepared from three different mice, and QMF-PCR performed for actin, fetal/cardiac sodium channel, and adult sodium channel mRNAs. Panels A and B show the summed, averaged, and uncorrected peak voltage heights for each mRNA species, with the standard deviation shown (note that actin target was diluted 10,000-fold). Actin mRNA levels are seen to remain relatively constant in all samples, while adult sodium channel mRNA levels rise and cardiac/fetal mRNA levels fall after birth in both limb muscle and diaphragm. Bottom, Panels C and D, the average of the sodium channel mRNA data for all time points was corrected for preferential PCR amplification using factors derived from genomic DNA QMF-PCR, and the values normalized to adult sodium channel mRNA expression in adult skeletal muscle (Panel C, age P60 = 100%). The adult sodium channel mRNA increases while the cardiac/fetal
QMF-PCR methods, we found the ratios of adult to cardiac/fetal sodium channel mRNAs in mouse fetal skeletal muscle to be similar (competitive PCR with cloned DNA and RNA as competitor, 4.2:1; QMF-PCR oligo(dT), 1.7 ± 0.3:1; QMF-PCR random primer, 1.9 ± 0.3:1). These results consistently indicated a high level of adult channel isoform mRNA in fetal muscle. The competitive PCR did not require a correction factor for preferential PCR, however, competitive PCR showed even higher relative levels of adult mRNA in fetal muscle than QMF-PCR: this is the opposite than one would expect if the correction factors in QMF-PCR were skewing the results.

We also tested the effect of using peak height versus peak area for quantitation of PCR products. The DuPont automated sequenators do not have the capability to measure peak area, and for this reason, peak height was used. To test if different results were obtained with peak area, we ran identical samples on an ABI model 373A automated sequenator, which permits determination of both peak height and peak area. QMF-PCR products of mouse neonatal skeletal muscle were found to have the corrected ratio of adult/cardiac/fetal sodium channel mRNA of 3:1 based on peak height on the DuPont sequenator. The same sample analyzed on an ABI sequenator was found to have a peak height ratio of 1,510:307 = 4.3:1, and a peak area ratio of 13,000:2,600 = 5.1. This was not considered a significant difference, and all subsequent results are presented as peak heights on the DuPont sequenator.

RNase Protection Assay—Both PCR-based methods (QMF-PCR and competitive PCR) gave similar results and showed that the adult sodium channel isoform predominates in fetal skeletal muscle. To ensure that this unexpected result was not in some way an artifact of PCR, we repeated quantitation of sodium channel isoforms in mouse fetal muscle using a non-PCR based method, RNase protection.

Mouse plasmid constructs similar to those for competitive PCR were used, except that no internal deletions or additions of sequence were present. Radiolabeled antisense RNA synthesized in vitro from restricted plasmids (adult and cardiac/fetal specific) was hybridized with synthesized sense RNA, mouse fetal skeletal muscle RNA (30 µg), and mouse neonatal skeletal muscle RNA (15 µg). Hybridized samples were then digested with RNases A and T. The adult and cardiac/fetal specific probes were used either singly, or in combination. The expected protected fragments were observed in all samples (Fig. 4C). This analysis showed the adult isoform mRNA to predominate in fetal skeletal muscle, consistent with the two PCR-based methods.

Developmental Expression of Sodium Channel Genes—Biopsies from three different mice for each age of mice (embryonic day 17, postnatal day 1, day 3, week 1, week 2, month 1, month 2, and month 6) were tested by QMF RT-PCR assay and the relative levels of sodium channel RNAs during normal development were determined (Fig. 6, top panels). The height of each signal was measured, corrected for preferential PCR, and averages and standard errors plotted for the triplicate experiments (Fig. 6). The relative levels of α actin, adult sodium channel, and cardiac/fetal sodium channel mRNAs in both skeletal muscle and diaphragm were determined (Fig. 6, bottom A and B). Taking the voltage of adult sodium channel RNA at age M2 as 100%, the relative levels of cardiac and adult skeletal muscle mRNAs at different ages were plotted using the genomic control (Fig. 6, bottom C and D). Our results show that expression of adult sodium channel gene increases with age while the expression of cardiac/fetal sodium channel decreases in both skeletal muscle and diaphragm. In fetal human and mouse fetal skeletal muscle, the level of adult sodium channel mRNA accounts for about 60% of total sodium channel RNA.

Discussion

Quantitative Multiplex Fluorescent RT-PCR (QMF-PCR)—We have developed a novel method for quantitating the relative levels of mRNA in small tissue samples. This method uses low cycle number (20 cycles) PCR to co-amplify multiple single-strand cDNA templates in a single reaction. We have shown that this assay is quantitative over a relatively large range of input template concentrations in both the reverse transcription reactions (0.7–24 µg of RNA), and the PCR reactions (6–200 ng input cDNA; 7.5–240 ng of genomic DNA), as well as of PCR cycle numbers (data not shown). In all experiments except those controls in Fig. 3, 3 µg of total RNA was used to synthesize cDNA. Volumes in figures were obtained from PCR products of 3 ng of total RNA (cDNA). Because the levels of different RNA are measured in the same reaction, the assay is capable of determining precise relative levels of different RNA species. The results obtained using our assay were consistent with those obtained with competitive-PCR, which is a method independent to PCR cycle numbers (21, 22). The latter assay requires both the fabrication of specific competitor plasmid constructs, and multiple PCR reactions per RNA species to be measured. Our assay requires only one set of PCR primers for each RNA species to be studied, and a single multiplex RT-PCR reaction. Correction factors must be introduced due to preferential PCR of certain templates. However, these correction factors are easily determined using genomic DNA as a template. An advantage of competitive-PCR is that the precise concentration of a single RNA species can be determined, while an advantage of QMF-PCR is that the relative levels of multiple RNAs can be easily measured.

Adult Skeletal Muscle Sodium Channel RNA Predominates in All Skeletal Muscle—We used QMF-PCR to measure the relative levels of the RNAs corresponding to the cardiac/fetal and adult skeletal muscle sodium channel isoforms. We found that the adult skeletal muscle isoform predominates in all skeletal muscle at all ages in both mouse and human: human fetal muscle (18–20 weeks gestational age) showed the adult skeletal muscle isoform to comprise 75% of sodium channel mRNA, and the cardiac/fetal isoform to comprise 25%.

Four types of experiments have been previously done to determine the relative level of adult (tetrodoxin-sensitive) and cardiac/fetal (tetrodoxin-insensitive) sodium channels in muscle: toxin binding, antibody binding, electrophysiological patch-clamp studies, and RNA expression studies. Nearly all toxin, antibody, and electrophysiological studies have been done in cultured rat myogenic cells (18, 19, 39, 40) and the results are difficult to extrapolate to intact muscle. A recent study by Lupa et al. (15) used dissociated rat muscle fibers, and compared the relative levels of sodium channels by antibody binding, patch-clamp, and RNA studies. The developmental patterns of channel mRNA expression we obtained in mouse and human muscle generally agree with the results obtained by Lupa et al. (15) in rat. Lupa et al. (15) found 10–20% of rat neonatal muscle sodium channels to be of the adult, tetrodoxin-sensitive isoform by pharmacological studies, while about 40% of the sodium channel mRNA was the adult isoform by RNase protection. These authors suggested that there is a lag between expression of mRNA and expression of functional channels. Using QMF-PCR, we found about 75% of mouse neonatal sodium channel mRNA decreases with age in both skeletal muscle and diaphragm. In fetal skeletal muscle and diaphragm, the mRNA level of the adult sodium channel gene is 2 times higher than that of cardiac/fetal sodium channel gene. E17, embryonic day 17. N0 = P0 = neonate. N3 = P3 = postnatal day 3. W1 = P7 = postnatal day 7. W2 = P14 = 2 weeks. M1 = P60 = 1 month. M2 = P60 = 2 months. M6 = P180 = 6 months.
A goal in undertaking this study was to investigate the light of the discrepancies between studies of gene expression in both humans and horses. It is clear that altered ratios of bors the point mutations causing HyperPP in both human and more severe phenotype than heterozygous horses and ratios of and function channel levels. It is possible that mutant and normal mRNAs have shown a correlation with clinical expression in adult skeletal muscle biopsy and diaphragm muscle (autopsy specimens). We found that the adult isoform sodium channel mRNA continued to increase in concentration relative to both adult mRNA and cardiac/fetal sodium channel mRNA long after birth. In mice, peak relative levels were reached at about 1 month of age, while in our more limited human data set, levels continued to increase after 5 years of age. Thus, our results suggest that the onset of disease may be correlated with increased expression of the adult sodium channel gene. The persistent expression of the cardiac/fetal channel through childhood may protect patients from attacks at a young age.

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Developmental Expression of Sodium Channels