

Modulation of sodium-channel mRNA levels in rat skeletal muscle

(development/voltage-dependent channels)

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ABSTRACT Action potentials in many types of excitable cells result from changes in permeability to Na ions. Although these permeability changes in nerve and muscle are mediated by voltage-gated Na channels that are functionally similar, we found that the Na-channel gene expressed in skeletal muscle is different from the genes coding for two Na channels (type I and type II) in brain. Despite the structural differences between muscle and brain Na-channel genes, a cDNA clone derived from rat brain hybridizes to skeletal muscle Na-channel mRNA of ≈ 9.5 kilobases. We used this cDNA probe to measure changes in Na-channel mRNA levels in skeletal muscle during development and following denervation. By blot hybridization analysis of electrophoretically fractionated RNA, we found that Na-channel mRNA can be detected as early as embryonic day 17 and that mRNA levels increase 2-fold between birth and postnatal day 35. Denervation of adult muscle causes a further 2- to 3-fold increase in muscle Na-channel mRNA levels, suggesting that expression of Na-channel genes in fast-twitch muscle may be regulated by the state of innervation.

Voltage-gated Na channels control the rapid changes in Na permeability responsible for the generation of an action potential in both nerve and muscle (1). Many functional properties of brain and skeletal muscle Na channels are similar (2), suggesting that the structure of the Na-channel protein is highly conserved in these tissues. Recent biochemical studies of Na channels isolated from rat brain and rat and rabbit skeletal muscle support this concept (3, 4). However, Na channels in brain and muscle also exhibit significant differences. Even within the same species, Na channels are immunologically distinct (5, 6) and exhibit differential sensitivity to several neurotoxins (7, 8).

In addition to differences between tissues, Na channels within a single tissue may also exhibit diversity. Messenger RNAs coding for two different Na channels have been identified in rat brain on the basis of sequence analysis of distinct complementary DNA (cDNA) clones (9). There is also evidence for multiple isotypes of the Na channel in skeletal muscle. Mature muscle expresses Na channels sensitive to nanomolar concentrations of tetrodotoxin (TTx), but denervated and embryonic muscle synthesize a TTx-insensitive form of the channel (10-16). Channels in the surface membrane and channels in the transverse (T)-tubular system of mature muscle are antigenically distinct and exhibit differential sensitivity to TTx derivatives and to polypeptide toxins (17, 18). Unfortunately, no information is currently available concerning the primary sequence of Na channels expressed in mammalian skeletal muscle. Therefore, the structural differences between these muscle channels, and the mechanisms that control their differential expression, remain completely unknown.

In this paper we show that extensor digitorum longus (e.d.l.) muscle does not contain detectable levels of rat brain Na-channel gene I or gene II transcripts. Nonetheless, a cDNA encoding 380 amino acids of rat brain Na-channel type II cross-hybridizes effectively to an mRNA in skeletal muscle of the same size as the rat brain Na-channel mRNAs. This cDNA probe was used to study the regulation of Na-channel mRNA levels during development of skeletal muscle and during the physiological modifications induced by denervation.

MATERIALS AND METHODS

Materials. [α - 32 P]GTP (800 Ci/mmol; 1 Ci = 37 GBq), [α - 32 P]UTP (800 Ci/mmol), and GeneScreen nylon membrane were obtained from New England Nuclear. Gemini and pSP64 plasmid vectors, SP6 and T7 RNA polymerases, RNasin, and ribonucleoside triphosphates were from Promega Biotec (Madison, WI). RNase A was from Sigma; RNase T1, from Calbiochem; and Zetabind nylon membrane, from AMF Cuno, Inc.

Isolation of Na-Channel Clones. We have previously isolated a 2300-base-pair (bp) cDNA clone encoding part of the eel (*Electrophorus electricus*) electroplax voltage-gated Na channel. The eel cDNA spans the fourth repeated homology unit within the full-length sequence (19) and also includes ≈ 1000 bp of 3' untranslated sequence (Fig. 1). To obtain a cDNA clone encoding the rat Na channel, we used this eel cDNA as a probe to screen a bacteriophage λ gt11 cDNA library prepared from rat hypothalamic polyadenylated mRNA (20). The λ gt11 library was screened by the technique of Benton and Davis (21), and one positive phage, of 10^5 phage screened, was identified. The cDNA insert in the phage was subcloned and determined by sequence analysis (22) to represent a portion of the type II Na channel in brain, extending from amino acid 1528 to 1912.

To isolate clones containing sequences that would distinguish between Na-channel mRNA type I and type II, a rat genomic library (provided by J. Bonner, California Institute of Technology) was screened with an end-labeled 30-nucleotide oligonucleotide primer. The oligonucleotide primer was complementary to sequences encoding amino acids 5-14 of both type I and type II Na channels, adjacent to the divergent 5' untranslated regions. Two positive phage were identified (of the 10^6 phage screened) and the positive phage were confirmed by rescreening with a second oligonucleotide primer, also corresponding to a sequence within the coding region. The rat sequences were subcloned into the plasmid vector Gemini. Sequence analysis indicated that one of the subclones contained 5' untranslated and coding sequences identical to those for Na-channel type I and that the other

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Abbreviations: e.d.l., extensor digitorum longus; TTx, tetrodotoxin. ‡To whom reprint requests should be addressed.

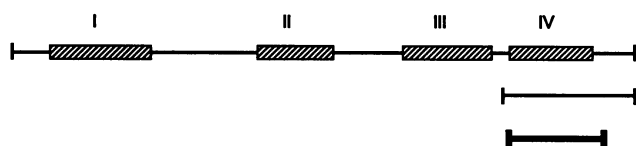


FIG. 1. Schematic representation of the eel (*E. electricus*) Na-channel amino acid sequence. Hatched boxes I-IV depict regions of internal amino acid homology predicted by the cDNA sequence (9). Both the eel cDNA probe (narrow line) and the rat brain cDNA type II probe (wide line) span repeat unit IV.

subclone contained a portion of the 5' untranslated and coding sequences identical to those for Na-channel type II.

RNA Isolation and Analysis. Total cellular RNA was purified from adult rat brain and skeletal muscle by the following modification of the procedure of Chirgwin *et al.* (23). After homogenization of the tissue in guanidinium thiocyanate, the ethanol precipitate was redissolved in a solution containing 6 M guanidine hydrochloride, 0.1 M potassium acetate (pH 5), and 0.025 M EDTA. The pellet was dispersed by shearing 15 times with a 22-gauge needle and the nucleic acids were precipitated by the addition of 0.5 volume of ethanol. The pellet was redissolved in the above guanidine hydrochloride solution and ethanol-precipitated. After phenol extraction and an additional ethanol precipitation, the amount of total RNA purified from a tissue sample was normalized for amount of rRNA. The quantity of rRNA was determined by electrophoretic fractionation of total RNA in nondenaturing 1% agarose gels and subsequent densitometry of photographs of the ethidium bromide-stained gel. Poly(A)⁺ RNA was obtained by oligo(dT)-cellulose chromatography. It has been shown (24) that the poly(A)⁺ RNA levels are equivalent in innervated and denervated muscle. To determine whether the ratio of poly(A)⁺ RNA to rRNA differed in innervated and denervated muscle, a dot blot analysis was performed. Total RNA was dissolved after quantitation in 1.1% formaldehyde, heated to 65°C for 5 min, and cooled on ice. Two microliters of RNA was dotted onto a sheet of GeneScreen (New England Nuclear) at several different concentrations. The membrane was rinsed in 2× standard saline citrate (SSC; 1× is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), baked at 80°C for 2 hr, and hybridized and washed according to the manufacturer's instructions. The membrane was probed with poly(dT) at a concentration of 10⁷ cpm/ml (25).

Na-channel mRNA was detected either by RNA gel blot analysis (26) or by RNase-protection assay (see below), using ³²P-labeled antisense probes. The antisense molecules were synthesized according to conditions specified by the supplier (Promega Biotec), using [α -³²P]GTP for the gel blot assays and [α -³²P]UTP for the RNase-protection analyses.

For the gel blot assays, RNA was electrophoretically transferred from denaturing 0.8% agarose gels to a nylon membrane (Zetabind), using a Hoefer electrotransfer apparatus. Transfer was effected at 80 volts after 4 hr. Blots were hybridized under relatively stringent conditions [68°C, 50% (vol/vol) formamide/5× SSC/0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin] and washed at 68°C (0.2× SSC/0.1% NaDodSO₄). Na-channel mRNA levels were quantified by densitometry after autoradiography.

For the RNase-protection assays, antisense ³²P-labeled Na-channel RNA was purified by electrophoresis in a denaturing 6% polyacrylamide gel and eluted overnight by crush-soaking in 10 mM sodium acetate, pH 5.4/1 mM EDTA/0.5% NaDodSO₄ containing yeast tRNA at 10 μ g/ml. The antisense RNA (0.1 pmol) was coprecipitated with tRNA (10 μ g) and poly(A)⁺-selected RNA (10 μ g) isolated from brain, skeletal muscle, or liver. The RNA pellets were initially resuspended in 20 μ l of deionized formamide and heated at

65°C for at least 1 hr to ensure dissolution of the RNA. Subsequently, hybridization and RNase digestion were performed according to the technique of Zinn *et al.* (27). The reaction products were analyzed by electrophoresis in a denaturing 6% polyacrylamide gel.

Denervation of Rat e.d.l. Muscle. Male Wistar rats (200–300 g) were subjected to unilateral distal hindlimb denervation under anesthesia with ketamine and xylazine. The sciatic nerve was exposed in the upper thigh and transected, and a 0.5-cm section was removed to reduce the possibility of reinnervation. Five or ten days after denervation, animals were decapitated and the e.d.l. muscle was removed on each side. For each animal, the contralateral unoperated limb served as a paired control. Muscle was immediately frozen in liquid nitrogen and maintained at –70°C until extraction of RNA.

RESULTS

Isolation of a cDNA Encoding One of the Homology Units of Rat Brain Na Channel II. By biochemical and immunological criteria, the structures of the α subunits of vertebrate Na channels appear to be highly conserved. We took advantage of this homology by using a cDNA encoding 430 amino acids of the eel electroplax Na channel to clone a portion of a Na-channel cDNA from a rat hypothalamic λ gt11 library. This strategy resulted in the isolation of an 1134-bp rat brain cDNA clone. The rat cDNA contains only coding sequences and includes regions of 100% identity with the amino acid sequence of the eel channel. Nucleotide sequence analysis of the cloned cDNA revealed that it represents a segment of the brain type II Na channel (9), spanning the fourth repeated homology unit within the α subunit, between amino acids 1577 and 1960 (Fig. 1). This clone was designated pRB-211.

Brain Na-Channel Antisense Probe Hybridizes to a Related mRNA in Rat Skeletal Muscle. The pRB-211 cDNA was subcloned into the Riboprobe expression vector pSP64, in reverse orientation (3' to 5') with respect to the bacteriophage SP6 promoter. Single-stranded radiolabeled antisense Na-channel transcripts were used to compare the sizes of presumptive Na-channel mRNAs in brain and skeletal muscle. Gel blot analysis of 5 μ g of total cellular RNA from both rat brain and skeletal muscle detected a hybridizing mRNA species of 9.5 kilobases (Fig. 2). This length corresponds to the size of the brain Na-channel mRNA type II reported previously (9, 28). The antisense probe did not hybridize to rat liver mRNA (Fig. 2).

To determine whether the hybridizing mRNA in skeletal muscle was identical to the brain Na channels or represented a related mRNA, we performed RNase-protection analyses. For these experiments, probes specific for the rat brain channel mRNAs were generated from genomic clones containing sequences unique to either gene I or gene II. Since the sequences for these channel isoforms are highly homologous in their 5' translated regions, but divergent in the adjacent 5' untranslated regions of their respective messages, genomic clones including these 5' junctional regions were sought. Two such clones were isolated from a rat genomic library, sequenced, and identified as corresponding to segments of the terminal 5' translated and contiguous 5' untranslated regions of the rat brain I and II channels. Antisense transcripts of these probes were used in RNase-protection assays with total RNA preparations from brain, muscle, and liver.

Authentic rat brain Na-channel mRNA I protected 317 bases of the 430-base antisense RNA probe specific for gene I (Fig. 3A). This protected fragment corresponds to 52 bases of 5' untranslated and 265 bases of coding region. The unprotected 87 bases represent an intervening genomic sequence (intron) not present in the processed mRNA. Authentic brain

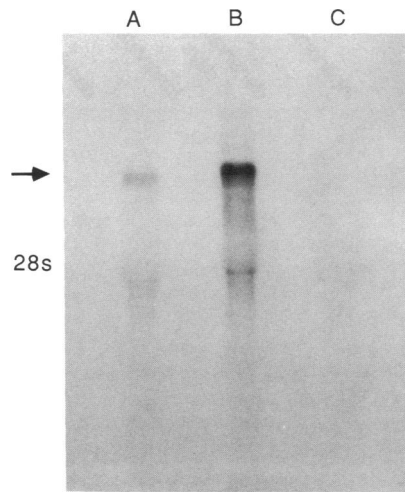


FIG. 2. Detection of Na-channel mRNA in rat skeletal muscle. Total RNA samples (5 μ g) from skeletal muscle (lane A), brain (lane B), and liver (lane C) were electrophoresed in 1% agarose/6% formaldehyde gel and transferred to nylon membrane. The hybridization probe was a 900-base 32 P-labeled antisense strand of rat brain cDNA type II. The \approx 9.5-kilobase hybridizing band is indicated by an arrow. Size markers were λ phage DNA digested with *Hind*III and 5'-end-labeled with 32 P (not shown) and the endogenous 28S rRNA.

Na-channel mRNA II protected a 128-base fragment of the gene II antisense probe, corresponding to 54 bases of 5' untranslated and 74 bases of coding region (Fig. 3B). Neither of the Na-channel antisense probes were protected by poly(A)⁺ mRNA purified from rat muscle or liver (Fig. 3), although sufficient Na-channel mRNA was present in these

assays as demonstrated by gel blot analysis using the probe encoding the conserved fourth repeated domain. These results indicate that the rat muscle Na channel is structurally related to, but not identical to, rat brain type I and II Na channels.

Na-Channel mRNA Levels Increase During Postnatal Development of Rat Skeletal Muscle. Total RNA was purified from the lower hindleg muscles of rats ranging in age from newborn to adult, and equal amounts of RNA were analyzed by gel blot hybridization. The probe used for these studies was 32 P-labeled antisense RNA generated from the pRB-211 cDNA clone. Densitometry of the autoradiographs indicated an \approx 2-fold increase in the levels of Na-channel mRNA between day 0 and postnatal day 35 (Fig. 4A). To determine whether Na-channel mRNA could also be detected in embryonic muscle, RNA was purified from the leg muscles of embryonic day-17 rats. Blot analysis of this RNA indicated that embryonic day-17 muscle contains a hybridizing mRNA of the same size as that present in neonatal and adult skeletal muscle (Fig. 4B).

Na-Channel mRNA Levels in Muscle Increase Following Denervation. To determine whether the state of innervation regulates Na-channel gene expression, we compared Na-channel mRNA levels in innervated and denervated fast-twitch muscle of rats. After either 5 or 10 days, total cellular RNA was prepared from paired control and denervated e.d.l. muscles. Equal amounts of total RNA from the innervated and denervated muscles were probed with radiolabeled Na-channel antisense transcripts generated from the pRB-211 clone. The samples were normalized to the amount of 28S rRNA, because we found that the ratio of poly(A)⁺ RNA to rRNA was the same in innervated and denervated muscle. An autoradiograph showing the results of two gel blot hybridization experiments is shown in Fig. 5. The amount of Na-

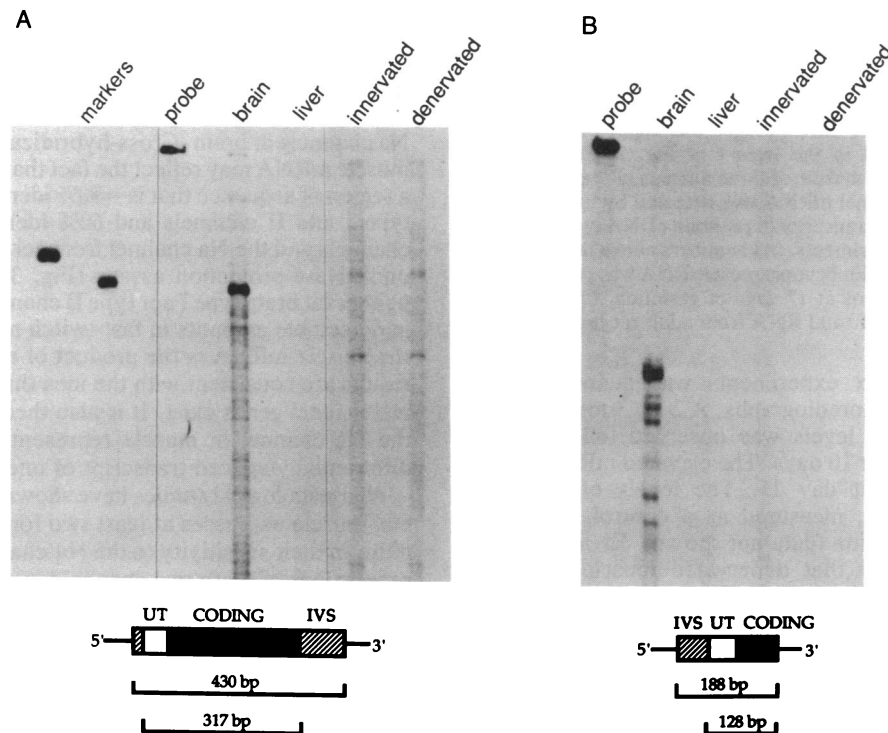


FIG. 3. Fast-twitch (e.d.l.) muscle does not express detectable levels of brain Na-channel mRNA type I or type II. Ten micrograms of poly(A)⁺ mRNA from brain, liver, and innervated and denervated e.d.l. muscle was hybridized in solution with 32 P-labeled antisense transcripts for gene I (A) or gene II (B) and digested with a combination of RNases A and T1. RNase-resistant fragments were resolved in denaturing 6% polyacrylamide gels. A and B are autoradiographs of two separate experiments. The size markers are 320- and 312-base 32 P-labeled synthetic RNAs in A and 32 P-labeled *Dde* I cleavage products of plasmid pUC DNA in B (not shown). The 32 P-labeled antisense probes used and the lengths of the RNase-resistant fragments are shown below the autoradiographs. UT, untranslated sequence; IVS, intervening sequence (intron); coding, sequence encoding amino acids. Exposure time for both autoradiographs was 5 hr with an intensifying screen.

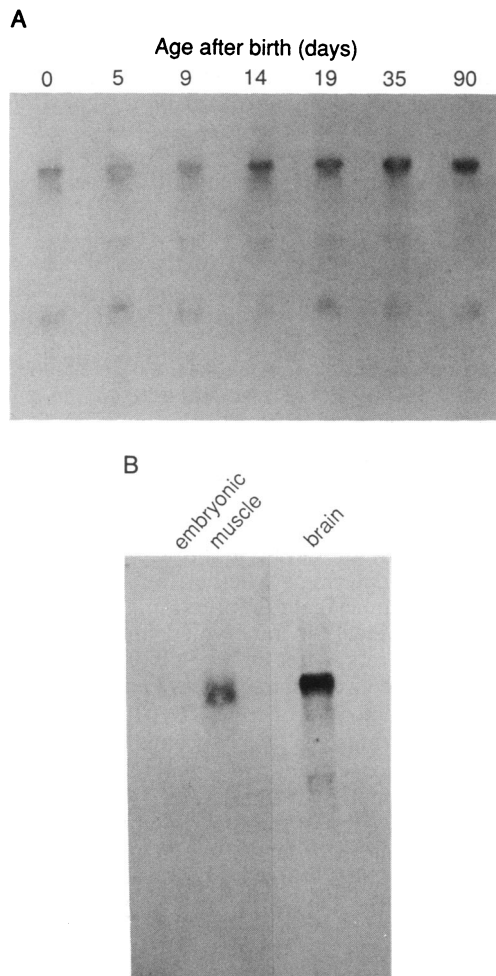


FIG. 4. Detection of Na-channel mRNA in skeletal muscle of developing rats. Forty micrograms of total RNA from the lower hindlimb muscles of rats of different ages was applied to each lane and blotted as described in the legend of Fig. 2. Each sample represents RNA from the muscle of three animals at the same stage of development. Na-channel mRNA was detected by hybridization to a ^{32}P -labeled antisense transcript of rat brain cDNA type II. *A* and *B* represent separate experiments. (*A*) Numbers above lanes refer to postnatal ages of rats. (*B*) Embryonic muscle RNA was purified from the hindlegs of rat embryos at 17 days of gestation. Lane marked "brain" contained 5 μg of total RNA from adult rat brain.

channel mRNA in six experiments was quantitated by densitometry of the autoradiographs. A 2- to 3-fold increase in Na-channel mRNA levels was observed following denervation for either 5 or 10 days. The elevated mRNA levels persisted until at least day 15. The levels of *c-Ha-ras* protooncogene mRNA, measured as a control, were not influenced by denervation (data not shown). RNase-protection analysis indicated that denervated muscle, like the innervated muscle, does not contain detectable levels of rat brain Na-channel type I or type II mRNA (Fig. 3).

DISCUSSION

Voltage-gated Na channels in adult skeletal muscle and nerve are distinguishable by both pharmacological and functional criteria. For example, antibodies raised against purified brain or muscle Na-channel protein often do not crossreact (5, 6). A recently described polypeptide toxin (μ -conotoxin) specifically blocks Na channels in frog and rat skeletal muscle but does not affect Na-channel activity in nerve (8). Finally, mRNA from brain and skeletal muscle produces Na channels

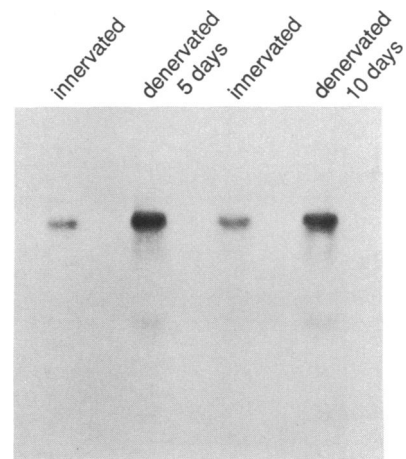


FIG. 5. Na-channel mRNA levels in fast-twitch (e.d.l.) muscle are elevated following denervation. Forty micrograms of total RNA from paired innervated and denervated e.d.l. muscle was analyzed by blot hybridization as described in the legend of Fig. 2. Each lane contains RNA from a single muscle. The probe was a ^{32}P -labeled antisense transcript of rat brain cDNA type II. Denervation was for 5 days or 10 days. Autoradiograph was exposed for 16 hr with an intensifying screen.

with slightly different functional properties when microinjected into *Xenopus* oocytes (29, 30). These observations suggest that the brain and skeletal muscle Na channels may be encoded by separate genes that are highly homologous in some regions but poorly conserved in others.

Although cDNA clones encoding two distinct α subunits in rat brain have been sequenced (9), previous reports indicated that these brain cDNAs do not cross-hybridize with Na-channel mRNA in skeletal muscle (9, 28). The cDNA clone that we have isolated, encoding a portion of the rat brain type II Na channel, hybridizes efficiently to a rat skeletal muscle mRNA of 9.5 kilobases. We interpret this result to indicate that the Na channel in skeletal muscle is homologous to the Na channels in brain. Cross-hybridization of our cDNA with muscle mRNA may reflect the fact that the probe used covers a region of sequence that is $\approx 90\%$ identical between rat brain type I and II channels and 60% identical between type II channels and the Na channel from eel electroplax. However, our RNase-protection assays (Fig. 3) clearly indicate that neither rat brain type I nor type II channel mRNA is produced in detectable amounts in fast-twitch muscle, suggesting that the muscle mRNA is the product of a separate gene. These results are consistent with the idea that multiple mammalian Na channel genes exist. It is also theoretically possible that the Na channel in muscle represents the expression of a differentially spliced transcript of one of the brain genes.

Pharmacological studies have shown that mammalian skeletal muscle expresses at least two forms of Na channel that differ in their sensitivity to the Na-channel blocker TTx. The proportions of these two channel types change during development and after denervation (10–16). Na channels in adult muscle are predominantly TTx-sensitive, whereas those synthesized in embryonic and denervated muscle are relatively resistant to blockade by TTx. Our cDNA probe does not appear to distinguish between TTx-sensitive and TTx-insensitive forms of the Na channel. The antisense probe hybridizes effectively to Na-channel mRNA from embryonic day-17 muscle (Fig. 4), in which the majority of channels present have been shown to be TTx-insensitive (10), but also hybridizes effectively to mRNA expressed in innervated adult muscle, in which only TTx-sensitive channels are present (10). Thus, the probe appears to be an effective

measure of total Na-channel mRNA rather than an indicator of one particular mRNA type.

After denervation, the density of TTX-sensitive Na channels in skeletal muscle decreases by more than 40% (31) while new, TTX-insensitive Na channels with slightly different kinetic properties appear (10, 12–15). The increase in total Na-channel mRNA detected with the probe encoding the fourth repeated domain may well represent the expression of the new TTX-insensitive channel. Although other explanations are certainly possible, it is clear from RNase-protection assays that neither the rat brain I nor the rat brain II channel transcripts are expressed in detectable amounts in denervated muscle. Thus, mRNAs coding for at least two channel isoforms distinct from those in brain may be expressed in rat skeletal muscle. These findings are consistent with the concept evolving from biochemical and electrophysiological studies of multiple related forms of the channel in both brain and muscle (32).

Our data suggest a role for developmental factors and innervation in the regulation of Na-channel gene expression in muscle that was not apparent from previous studies. It will now be important to isolate cDNAs that differentiate among the related Na-channel genes. Such clones should allow these changes in total Na-channel mRNA levels to be dissected into contributions from functionally and pharmacologically distinct muscle Na-channel types.

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