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Functional expression of cloned cDNA encoding sodium channel III

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mRNA synthesized by transcription in vitro of the cloned cDNA encoding rat brain sodium channel III directs the formation of a functional sodium channel in *Xenopus* oocytes. The tissue distribution of the mRNAs encoding sodium channels I, II and III has been studied by blot hybridization analysis with specific probes.

Na^+ channel; cDNA expression; Na^+ current; μ-Conotoxin; RNA blot hybridization analysis; (*Xenopus* oocyte)

1. INTRODUCTION

The voltage-gated sodium channel is a transmembrane protein essential for the generation of action potentials in excitable cells [1]. The complete amino acid sequences of the sodium channel from the electric organ of *Electrophorus electricus* [2] and three distinct sodium channels (designated sodium channels I, II and III) from rat brain [3,4] have been elucidated in this laboratory by cloning and sequencing the cDNAs. Our previous studies [5,6] have shown that mRNAs derived from the cloned cDNAs encoding rat sodium channels I and II direct the formation of functional sodium channels in *Xenopus* oocytes, although the magnitude of the tetrodotoxin-sensitive response induced by the sodium channel I-specific mRNA is small. Here we report the functional expression of the cloned cDNA encoding rat sodium channel III. The properties of sodium channel III expressed in *Xenopus* oocytes are compared with those of sodium channel II. The tissue distribution of the three rat sodium channel mRNAs has also been studied by blot hybridization analysis with probes specific for the respective mRNAs.

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Abbreviation: TTX, tetrodotoxin

2. MATERIALS AND METHODS

2.1. *Construction of recombinant plasmid for cDNA expression*

The pSP65 [7] recombinant carrying the cDNA for rat sodium channel III [4] (designated pRIII) was constructed as follows (see fig.1). The *Mae*II(−26)/*Hha*I(311) fragment from prSCH801, the *Hha*I(311)/*Bgl*II(516) and *Bgl*II(516)/*Pst*I(801) fragments from prSCH722 and the 3039-base-pair (bp) *Pst*I/*Acc*I fragment from pSP65 were ligated to yield pSRIII-1. The *Bgl*II(516)/*Pst*I(801) fragment from prSCH722, the *Pst*I(801)/*Tth*111I(1035) fragment from prSCH705, the *Tth*111I(1035)/*Sac*I(1478) fragment from prSCH616 and the 3040-bp *Sac*I/*Bam*HI fragment from pSP65 were ligated to yield pSRIII-2. The *Pst*I(1393)/*Xba*I(2018) and *Xba*I(2018)/*Bgl*I(2623) fragments from prSCH417, the *Bgl*I(2623)/*Cla*I(3108) fragment from prSCH331, the *Cla*I(3108)/*Hin*dIII-(3351) fragment from prSCH321 and the 3043-bp *Hin*dIII/*Pst*I fragment from pSP65 were ligated to yield pSRIII-3. The *Cla*I(3108)/*Sac*I(3778) and *Sac*I(3778)/*Mae*III(3998) fragments from prSCH321, the *Mae*III(3998)/*Pst*I(4158) fragment from prSCH306 and the 3039-bp *Pst*I/*Acc*I fragment from pSP65 were ligated to yield pSRIII-4. The ~440-bp *Pvu*II(6070)/*Pvu*II(on vector) fragment from prSCH203 was inserted into the *Hin*cII site of pSP65 in the orientation opposite to that of the SP6 promoter to yield pSRIII-5. The *Pst*I(4158)/*Hin*cII-(5922) fragment from prSCH203 and the ~3.2-kilobase-pair (kb) *Hin*cII(6401)/*Pst*I(on vector) fragment from pSRIII-5

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were ligated to yield pSRIII-6. The 806-bp *Eco*RI(on vector)/*Aat*II(750) fragment from pSRIII-1, the 728-bp *Aat*II(750)/*Sac*I(1478) fragment from pSRIII-2 and the 3038-bp *Sac*I/*Eco*RI fragment from pSP65 were ligated to yield pSRIII-7. The 1516-bp *Bam*HI(on vector)/*Sac*I(1478) fragment from pSRIII-7, the 1863-bp *Sac*I(1478)/*Afl*II(3341) fragment from pSRIII-3, the 817-bp *Afl*II(3341)/*Pst*I(4158) fragment from pSRIII-4 and the 3026-bp *Pst*I/*Bam*HI fragment from pSP65 were ligated to yield pSRIII-8. The 1063-bp *Bam*HI(on vector)/*Pst*I(4158) fragment from pSRIII-4, the ~1.9-kb *Pst*I(4158)/*Sac*I(on vector) fragment from pSRIII-6 and the 3040-bp *Sac*I/*Bam*HI fragment from pSP65 were ligated to yield pSRIII-9. The 3397-bp *Eco*RI(on vector)/*Afl*II(3341) fragment from pSRIII-8, the ~2.7-kb *Afl*II(3341)/*Xba*I(on vector) fragment from pSRIII-9 and the 3024-bp *Xba*I/*Eco*RI fragment from pSP65 were ligated to yield pRIII.

2.2. *Expression of sodium channels in* Xenopus *oocytes*

mRNAs specific for rat sodium channels II and III were synthesized in vitro [7], using *Sal*I-cleaved pRII-2 [5] and *Sal*I-cleaved pRIII (see section 2.1), respectively, as templates. Transcription was primed with the cap dinucleotide $m^7G(5')ppp(5')G$ (1 mM) [8]. Total RNA from the whole brain or the hind leg skeletal muscle of male Wistar rats (~200 g body wt) was extracted by the phenol method as in [9], and poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography [10]. *Xenopus laevis* oocytes were injected with the following RNA preparations: sodium channel II-specific mRNA (0.05 μg/μl), sodium channel III-specific mRNA (0.1 μg/μl), brain poly(A)$^+$ RNA (1.0 μg/μl) or skeletal muscle poly(A)$^+$ RNA (1.0 μg/μl); the average volume injected was ~50 nl per oocyte. The injected oocytes were incubated at 19°C for 3–6 days as in [5]. The follicular cell layer was removed from oocytes [11,12] prior to electrophysiological measurements. Whole-cell currents were recorded under the conditions described in [5]. Transient capacitative currents were roughly compensated by a network with three adjustable time constants. Current records were digitized at 20 kHz unless otherwise stated and were stored on a computer for subsequent analysis. μ-Conotoxin GIIIA [13,14] was kindly provided by Dr Teruo Abe (Brain Research Institute, Niigata University, Niigata, Japan).

2.3. *RNA blot hybridization analysis*

For the preparation of RNA probes specific for rat sodium channels I, II and III, three recombinant plasmids were constructed as follows. The *Alu*I(7140)/*Sau*3AI(7563) fragment from prSCH109 [3] and the 3034-bp *Bam*HI/*Hin*cII fragment from pSP65 [7] were ligated to yield pSPNCRI. The *Pst*I(6605)/*Pst*I(7080) fragment from prSCH202 [3] was inserted into the *Pst*I site of pSP65 in the orientation opposite to that of the SP6 promoter to yield pSPNCRII. The *Hin*cII(5922)/*Hin*cII(6401) fragment from prSCH203 [4] was inserted into the *Hin*cII site of pSP65 in the orientation opposite to that of the SP6 promoter to yield pSPNCRIII. For the preparation of an RNA probe common to rat sodium channels I, II and III, the *Fnu*DII(4835)/*Hpa*II(5142) fragment from prSCH202 and the 3186-bp *Acc*I/*Sma*I fragment from pBSM13(+) (Stratagene) were ligated to yield pBSCRII. The RNA probes specific for sodium channels I, II and III were synthesized in vitro with [α-^{32}P]GTP, using pSPNCRI, pSPNCRII and pSPNCRIII linearized with *Hin*dIII, respectively, as templates for transcription with SP6 polymerase (New England Nuclear) [7]. The RNA probe common to the three sodium channels was synthesized in vitro with [α-^{32}P]GTP, using *Eco*RI-cleaved pBSCRII as template for transcription with T_3 polymerase (Stratagene), as recommended by the vendor. RNA blotting analysis was carried out essentially according to [15], except that Biodyne nylon membrane (Pall) was used and that hybridization was performed at 65°C in the presence of 50% formamide and 0.5% SDS. When the blots were hybridized with a specific probe in the experiments shown in fig.5, the two remaining nonradioactive specific RNA probes, each in a 100-fold molar excess, were added to eliminate cross-hybridization; the intensity of hybridization signals obtained in the presence of the nonradioactive probes was indistinguishable from that obtained in their absence. For blot hybridization analysis, total RNA was prepared from tissues of adult rats (see section 2.2) by the guanidinium thiocyanate method [16], and poly(A)$^+$ RNA as in [10]. The yield of poly(A)$^+$ RNA during oligo(dT)-cellulose chromatography was 5–7% for all preparations.

3. RESULTS AND DISCUSSION

The recombinant plasmid pRIII that carries the entire protein-coding region of the cDNA for rat sodium channel III, linked with the bacteriophage SP6 promoter [7], was constructed (fig.1). Plasmid transcription in vitro by SP6 polymerase generated mRNA specific for rat sodium channel III. The size of this mRNA (~6.1 kilobases), estimated by agarose gel electrophoresis [5], agreed with that expected from the structure of the plasmid.

Xenopus oocytes injected with the sodium channel III-specific mRNA showed a transient inward current when the holding membrane potential was shifted from −100 mV to −10 mV under voltage clamp. In Ringer's solution, the maximum inward current ranged up to 19 μA, being comparable in size to that induced by the sodium channel II-specific mRNA [5]. Following application of 0.3 μM TTX, the inward current was progressively diminished and disappeared within 2 min (fig.2A). Similarly, 0.3 μM saxitoxin blocked the inward current completely. The effects of both the toxins were reversible. Fig.2B shows an example of the dose-response curves for TTX. The apparent dissociation constant for TTX (K_{TTX}) was 15.3 ± 5.1 nM (mean ± SD, n = 5), being similar to that of rat sodium channel II expressed in oocytes (14.7 ± 5.3 nM, n = 8) (see also [5]).

When the external Na^+ concentration was lowered by replacement with sucrose or tetraethylammonium ions, the inward current was

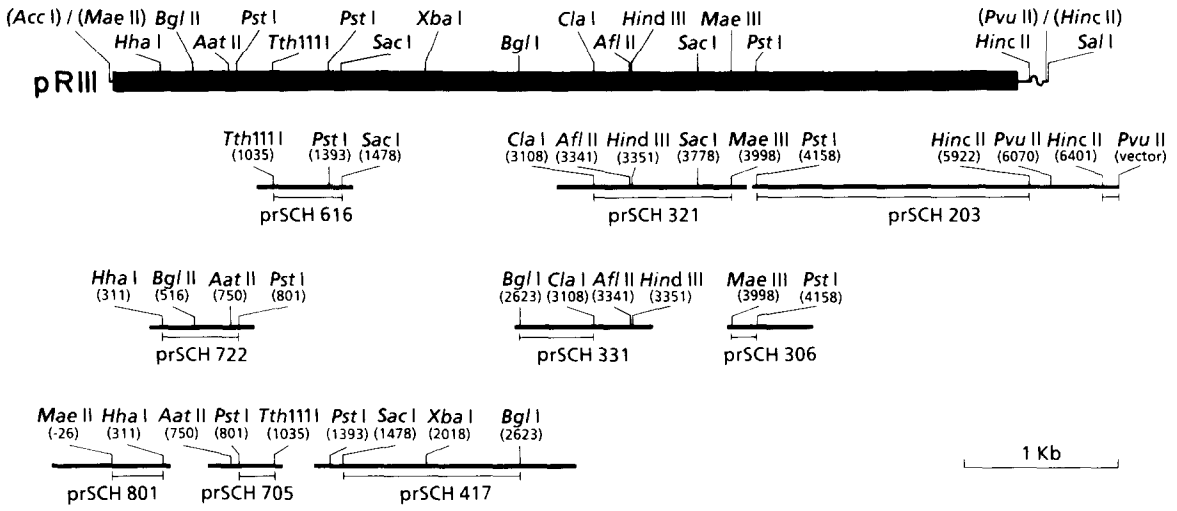

Fig.1. Construction of the recombinant plasmid pRIII used for expression of rat sodium channel III. Only relevant restriction endonuclease sites are shown and identified by numbers indicating the 5'-terminal nucleotide generated by cleavage (for nucleotide numbers, see [4]); endonuclease sites that no longer exist in pRIII are parenthesized. The protein-coding region is represented by a filled box, and the poly(dA)·poly(dT) tract by a wavy line. The cDNA clones [4] used for the construction are indicated by thick lines, and their fragments constituting pRIII by bars beneath. A scale of 1 kb is given.

reduced in a dose-dependent manner and was virtually abolished at a Na^+ concentration of ~3 mM (fig.3A). Fig.3B shows current recordings in Ringer's solution that were made when the oocyte membrane potential was stepped from −100 mV to various levels between −40 mV and +30 mV. Fig.3C exemplifies the peak current-voltage ($I-V$) relationship. The maximum current occurred at a potential of -5.3 ± 5.5 mV ($n = 6$). This value is similar to that observed for rat sodium channel II expressed in oocytes (-7.1 ± 5.6 mV, $n = 7$) (see also [5]).

The TTX-sensitive fast current induced by the sodium channel III-specific mRNA showed inactivation following a depolarizing prepulse in a time- and voltage-dependent manner. In view of the large membrane capacitance, the steady-state inactivation was measured with a test pulse to 0 mV following a long (2 s) prepulse stepped from −100 mV to different potential levels (−90 mV to −10 mV). The potential at which activation of the inward current was 50% [17] was -51.4 ± 3.7 mV ($n = 5$). This value is comparable to that observed for rat sodium channel II expressed in oocytes (-53.8 ± 7.3 mV, $n = 6$) (see also [5]).

It is known that μ-conotoxin GIIIA from *Conus geographus* blocks skeletal muscle sodium channels, but has little or no effect on peripheral nerve and brain sodium channels [14,18]. We tested the effect of this toxin on sodium channels expressed in *Xenopus* oocytes. Following application of 1 μM μ-conotoxin GIIIA, the inward current induced by rat skeletal muscle poly(A)$^+$ RNA virtually disappeared within 2 min (fig.4D), whereas that induced by the mRNA specific for rat sodium channel II or III or by rat brain poly(A)$^+$ RNA was unaffected (fig.4A, B and C, respectively). These data suggest that sodium channels II and III are distinct from sodium channels in skeletal muscle.

The results described above show that the mRNA derived from the cDNA encoding rat sodium channel III directs the formation of a functional sodium channel in *Xenopus* oocytes. Using whole-cell current measurements, we detected no significant difference in functional properties between rat sodium channels II and III expressed in oocytes.

In order to examine the tissue distribution of the mRNAs encoding sodium channels I, II and III, we prepared RNA probes specific for the respective mRNA species, using divergent cDNA sequences in the 3'-noncoding region as templates (see section 2.3). An RNA probe common to the three mRNA species was also prepared, using a

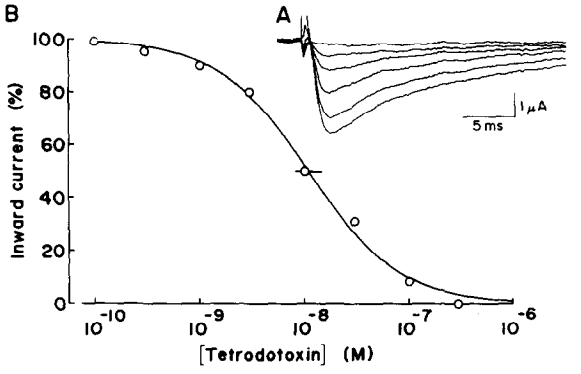

Fig.2. Effect of TTX on depolarization-activated whole-cell inward currents in *Xenopus* oocytes injected with the sodium channel III-specific mRNA. (A) Time course. The inward currents were elicited by a 50-ms pulse stepped from −100 to −10 mV. The six records (from bottom to top) were obtained before and 10, 20, 30, 40 and 60 s after perfusion with 0.3 μM TTX. The response obtained 10 min after washing the TTX solution was 90% of that observed before TTX application. (B) Dose-response curve obtained from another oocyte. The peak inward current evoked by a step from −100 to −10 mV, relative to that observed before TTX application, is plotted against the logarithm of TTX concentration. Each point represents the average of four consecutive records obtained 2 min after exposure to a new dose of TTX. The measurements were made in the direction of increasing TTX concentration. The record obtained with 1 μM TTX (average of four consecutive records) was subtracted from the control and those obtained with the other doses of TTX. The curve represents the dose-response relation expected from a K_{TTX} of 11.0 nM (indicated by a horizontal bar) according to the equation $y = (1 + T/K_{TTX})^{-1}$, where T is the TTX concentration.

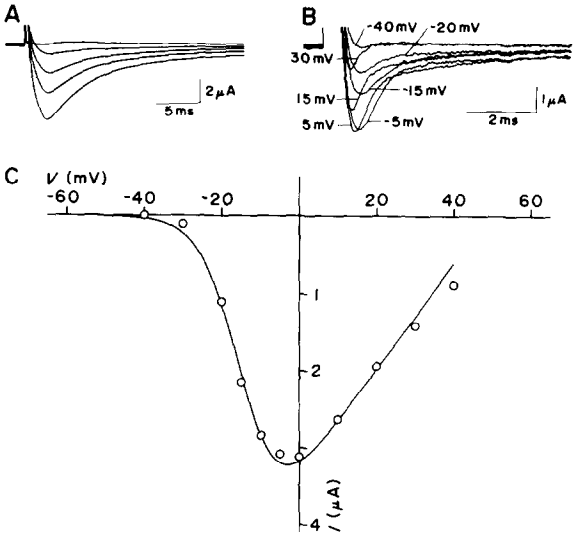

Fig.3. Properties of depolarization-activated whole-cell inward currents in *Xenopus* oocytes injected with the sodium channel III-specific mRNA. (A) Effects of changes in external Na^+ concentration replaced by sucrose. The inward currents were elicited by a 50-ms pulse stepped from −100 to −10 mV. The five records (from bottom to top) were obtained at external Na^+ concentrations of 118, 60, 41, 20 and 2.75 mM, respectively, each trace representing the average of four consecutive records. Each response was recorded 3 min after exposure to a new Na^+ level. (B) Sample records of currents evoked by a voltage step from −100 to −40, −20, −15, −5, 5, 15 and 30 mV in Ringer's solution. Each trace is the average of four consecutive records. (C) Peak current-voltage relationship by step depolarization from a holding potential of −100 mV in Ringer's solution. Each point represents the average of four consecutive records. Data from another oocyte.

conserved cDNA sequence in the protein-coding region as template (see section 2.3). Fig.5A shows blot hybridization analysis of total RNA from adult rat brain, using the sodium channel I-, II- and III-specific probes (lanes 1, 2 and 3, respectively) or the common probe (lane 4). The three mRNA species were allowed to run a sufficiently long distance to resolve their mobilities. The estimated sizes of the major RNA species specific for sodium channels I, II and III were ~9000, ~9500 and ~9000 nucleotides, respectively. Densitometric estimation of the autoradiogram indicated that the content of the sodium channel III mRNA was much lower than those of the sodium channel I and II mRNAs.

In fig.5B, poly(A)$^+$ RNA samples obtained from different excitable tissues and liver (as a control) of adult rats were subjected to blot hybridization analysis, using the sodium channel I-, II- and III-specific probes (panels a, b and c, respectively) or the common probe (panel d). When the autoradiograms were exposed for a shorter time (lane 1), only brain poly(A)$^+$ RNA gave clear hybridization signals (corresponding to the sizes described above) with either of the specific probes (data for other tissues not shown). Upon longer exposure of the autoradiograms (lanes 2–6), poly(A)$^+$ RNA from other excitable tissues also exhibited hybridization signals. With the sodium channel I-specific probe, the ~9000-nucleotide RNA species was detected in heart and skeletal muscle (panel a, lanes 3 and 4). The sodium channel II-specific probe yielded faint signals for the ~9500-nucleotide RNA species with heart and

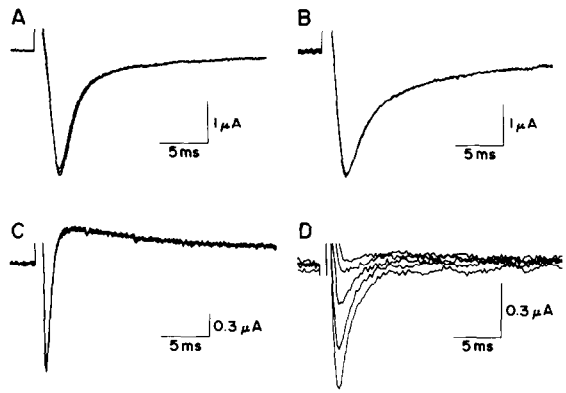

Fig.4. Effect of μ-conotoxin GIIIA on depolarization-activated whole-cell inward currents in *Xenopus* oocytes injected with the sodium channel II-specific mRNA (A), the sodium channel III-specific mRNA (B), rat brain poly(A)$^+$ RNA (C) or rat skeletal muscle poly(A)$^+$ RNA (D). The inward currents were elicited by a 50-ms pulse stepped from -100 to -10 mV. (A–C) The three overlapping records were obtained before and 1 and 2 min after bath application of 1 μM μ-conotoxin GIIIA. (D) The five records (from bottom to top), digitized at 5 kHz, were obtained before and 5, 30, 60 and 90 s after toxin application.

skeletal muscle poly(A)$^+$ RNA (panel b, lanes 3 and 4). The more intense signals corresponding to sizes of ~4500 nucleotides (panel b, lanes 3–6) and ~11 000 nucleotides (panel b, lanes 3 and 4) probably represent unrelated RNA species because they were not observed with the common probe complementary to a conserved coding sequence (panel d, corresponding lanes). With the sodium channel III-specific probe, the ~9000-nucleotide RNA species was detected in heart, skeletal muscle and small intestine (panel c, lanes 3–5). In addition, a minor RNA species of ~7500 nucleotides was found in these tissues as well as in brain (panel c, lanes 2–5). The two mRNA species specific for sodium channel III may arise from polyadenylation at different sites, as discussed in [4]. Similarly, the minor RNA species of ~8600 nucleotides hybridizable with the sodium channel II-specific probe (fig.5A, lane 2; fig.5B, panel b, lane 1) may represent an mRNA species polyadenylated at a site different from that of the major mRNA species (~9500 nucleotides), as suggested by a cDNA clone (prSCH202) carrying a shorter

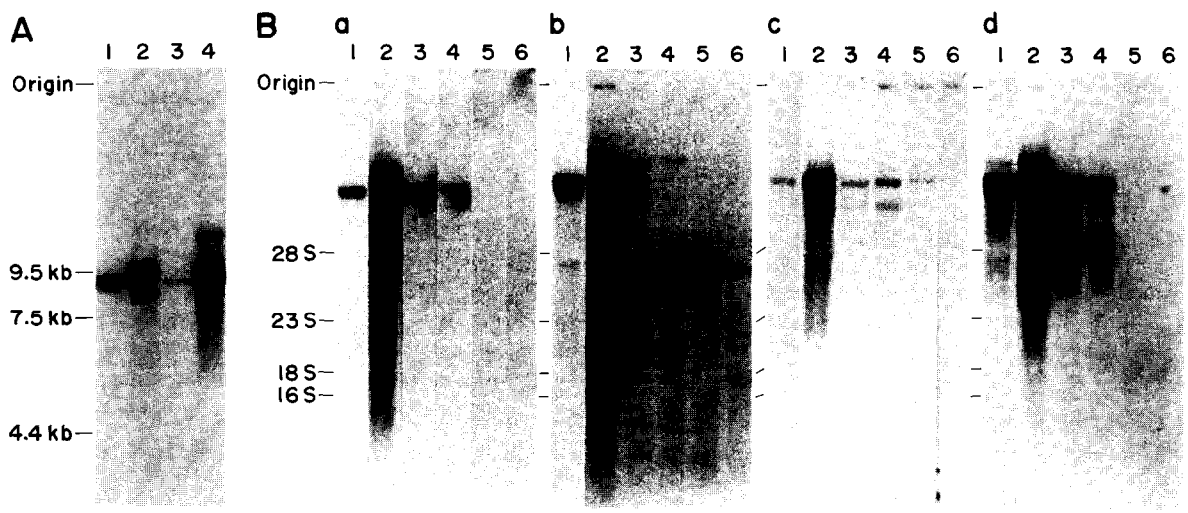

Fig.5. Autoradiograms of blot hybridization analysis of RNA from adult rat tissues. RNA samples were electrophoresed on 1.0% agarose gels. The specific radioactivities of the RNA probes used were $5.6–7.6 \times 10^8$ dpm/μg. (A) Analysis of total RNA (25 μg each) from brain using the RNA probes specific for sodium channels I (lane 1), II (2) and III (3) and the RNA probe common to them (4). Autoradiography was performed at -70°C for 21 h with an intensifying screen. An RNA ladder (Bethesda Research Laboratories) was used as size markers. (B) Analysis of poly(A)$^+$ RNA (10 μg each) from brain (lanes 1 and 2), heart (3), skeletal muscle (4), small intestine (5) and liver (6) using the RNA probes specific for sodium channels I (panel a), II (panel b) and III (panel c) and the RNA probe common to them (panel d). The duration of autoradiography at -70°C with an intensifying screen was 20 h (panels a–d, lane 1), 5 days (panel d, lanes 2–6) or 21 days (panels a–c, lanes 2–6). The size markers used were rat and *Escherichia coli* rRNAs [19], the positions of which are indicated by short lines flanking panels.

3′-noncoding sequence that contains a polyadenylation signal 16 nucleotides upstream of the poly(dA) tract [3].

The weak signals obtained by hybridization with the specific probes of poly(A)$^+$ RNA from heart, skeletal muscle and small intestine may indicate the presence of minute amounts of the sodium channel I, II and/or III mRNAs in these muscular tissues or may alternatively be attributable to the sodium channel mRNAs derived from coexisting neural tissue. The signal intensity resulting from hybridization of heart and skeletal muscle poly(A)$^+$ RNA with the common probe after 5 days' exposure of the autoradiogram (fig.5B, panel d, lanes 3 and 4) was much higher than the sum of the signal intensities observed with the three specific probes after 21 days' exposure (fig.5B, panels a, b and c, lanes 3 and 4). This may suggest the presence of additional types of sodium channel in heart and skeletal muscle. This view is supported in the case of skeletal muscle by the finding that poly(A)$^+$ RNA from this tissue produces μ-conotoxin-sensitive sodium channels in *Xenopus* oocytes, whereas sodium channels II and III are insensitive to the toxin (see above).

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