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Cloning and Distribution of a Putative Tetrodotoxin-Resistant Na^+ Channel in Newt Retina

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ABSTRACT—Overlapping cDNA clones spanning the entire coding region of a Na^+ channel were isolated from newt retina. The coding region predicts a 2,007 amino acid protein, designated nRNaCh (newt retina sodium channel), which is homologous to other Na^+ channels. *In situ* hybridization indicated that nRNaCh is expressed exclusively in spiking neurons, where a tetrodotoxin (TTX)-resistant Na^+ current has been recorded. Therefore, nRNaCh cDNA is sure to encode the TTX-resistant Na^+ channel of newt retina. Sequence comparisons show that nRNaCh is more homologous to TTX-sensitive Na^+ channels than to TTX-resistant Na^+ channels. The length of the S5-S6 loop of repeat I of nRNaCh is similar to that of TTX-sensitive channels, whereas TTX-resistant Na^+ channels have a deletion. The 3rd position in the SS2 region of repeat I of nRNaCh is a non-aromatic amino acid (Ala), which is a common feature of TTX-resistant channels. These findings suggest that whether the amino acid at the 3rd position in the SS2 region of repeat I is aromatic or non-aromatic determines the TTX sensitivity of Na^+ channels, not the overall structure of the channel.

INTRODUCTION

Voltage-dependent Na^+ channels are transmembrane proteins responsible for the early increase in Na^+ influx underlying the initial depolarization of action potentials in excitable cells (Hille, 1992). Molecular cloning has revealed the primary structure of several Na^+ channels (Noda *et al*., 1984; Noda *et al*., 1986; Kayano *et al*., 1988; Rogart *et al*., 1989; Trimmer *et al*., 1989; Kallen *et al*., 1990; Ahmed *et al*., 1992; Gellens *et al*., 1992; George *et al*., 1992; Klugbauer *et al*., 1995; Akopian *et al*., 1996; Sangameswaran *et al*., 1996; Chen J. *et al*., 1997). They all contain 4 homologous repeating units (I, II, III, IV), each of which has six putative transmembrane helices (S1-S6). The region between S5-S6, the S5-S6 loop, is thought to contain 2 short regions, SS1 and SS2, which may partly span the membrane from the extracellular side and are postulated to form part of the channel lining (Catterall, 1993).

These Na^+ channels have been classified into two subtypes by their sensitivity to TTX: TTX-sensitive and TTX-resistant Na^+ channels. TTX-sensitive Na^+ channels are blocked by nanomolar concentrations of TTX, whereas TTX-resistant channels require 1–100 μM TTX before they are blocked (Roy and Narahashi, 1992; Yoshida, 1994; Smith and Goldin, 1998). The TTX-binding sites of Na^+ channels are thought to be located near the extracellular mouth of the channel, and to contain negatively charged functional groups, because of the inhibition of TTX-binding by carboxyl-modifying reagents (Baker and Rubinson, 1975), some monovalent cations, divalent metal ions, and protons (Henderson *et al*., 1974). Photoaffinity labeling experiments suggested that the S5-S6 loops of repeats III and IV jointly form part of the TTX-binding site (Nakayama *et al*., 1992). In site directed mutagenesis experiments, the negatively charged residues at the 2nd and 5th positions in the SS2 region of repeats were identified as a major determinant of TTX sensitivity, because single amino acid mutations at these positions strongly reduce the TTX sensitivity (Terlau *et al*., 1991). These results suggest that the SS2 regions of each repeat are TTX-binding sites and that electrostatic interactions between the positively charged guanidinium groups of TTX and negatively charged residues in the SS2 regions have a critical role in TTX binding (Lipkind and Fozzard, 1994). The residues at these positions do not decide the TTX sensitivity of naturally occurring Na^+ channels, however, because they are conserved in both TTX-sensitive and TTX-resistant Na^+ channels. There are two differences in the primary structure of the two types. TTX-resistant channels have (i) a large deletion (13–26 amino acid residues) in the N-terminal part of the S5-S6 loop of repeat I compared with TTX-sensitive Na^+ channels (Rogart *et al*., 1989), and (ii) a non-aromatic amino acid (Cys or Ser) at the 3rd position in SS2 region of repeat I, whereas TTX-sensitive channels have an aromatic amino acid (Tyr or Phe). Site-directed mutagenesis experiments revealed that substitution of Tyr for Cys at the 3rd position in SS2 region of repeat I in mutant TTX-resistant channels confers TTX-sensitive properties (Chen *et al*., 1992; Satin *et al*., 1992). Conversely,

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mutants of TTX-sensitive channels with Cys substituted for Phe/Tyr at this position confer TTX-resistant properties (Backx *et al.*, 1992; Chen *et al.*, 1992; Heineman *et al.*, 1992). Thus, as far as already cloned Na^+ channels are concerned, TTX resistivity is decided primarily by whether the amino acid at this position is aromatic or non-aromatic, and secondarily by the electrostatic interactions between TTX and negatively charged residues in the SS2 region mentioned above.

Some species carry TTX in their tissue, and they have TTX-resistant Na^+ channels (Kidokoro *et al.*, 1974). It is very interesting to study the differences in the amino acid sequences between TTX-resistant Na^+ channels of TTX-carrying species and other Na^+ channels. The newt is one animal that carries TTX in its tissue (Wakely *et al.*, 1966), and it was shown electrophysiologically that Na^+ channels in the newt retina are TTX-resistant (Kaneko and Saito, 1992; Kaneko *et al.*, 1997). Na^+ channels in the newt retina are also important for the investigation of newt retinal regeneration (Hasegawa, 1958). During regeneration, the appearance of Na^+ channels is coordinated with cell differentiation (Cheon *et al.*, 1998), thus Na^+ channels would serve as probe for manifestation of the transdifferentiation of pigment epithelial cells into neuron. Partial sequences of Na^+ channel were determined by RT-PCR from normal newt retina (Kaneko *et al.*, 1997). However, the revealed sequence is restricted to the vicinity of the SS2 region of each repeat, and the complete structure remains to be determined. Therefore, we cloned the Na^+ channel from newt retina and analyzed the expression of the channel by *in situ* hybridization. The cDNA, designated nRNaCh (newt retina sodium channel) predicts a 2,007 amino acid protein that is homologous to other Na^+ channels. *In situ* hybridization analysis demonstrated that nRNaCh is expressed in spiking neurons, where a TTX-resistant Na^+ current has been recorded electrophysiologically (Kaneko *et al.*, 1997). Therefore, nRNaCh cDNA is likely to encode the TTX-resistant Na^+ channel of the newt retina. Sequence comparisons showed that nRNaCh is more closely related to TTX-sensitive Na^+ channels than to TTX-resistant channels. There is also no deletion in the N-terminal part of the nRNaCh S5-S6 loop of repeat I. At the 3rd position in SS2 region of repeat I, nRNaCh has a non-aromatic amino acid (Ala). These findings suggest that TTX sensitivity is decided by whether the amino acid at this position is aromatic or non-aromatic, and does not depend on the overall structure of the channel.

MATERIALS AND METHODS

RNA isolation, cDNA library construction, and screening

Total RNA was extracted from the retina of adult newts (*Cynops pyrrhogaster*) using the guanidinium isothiocyanate method (RNA Extraction Kit, Pharmacia Biotech, Piscataway, NJ). Poly(A)$^+$ RNA was isolated by performing oligo (dT)-cellulose chromatography twice (mRNA Purification Kit, Pharmacia Biotech). The cDNAs were synthesized from either oligo(dT) or a random primer, ligated into λZAP (Stratagene, La Jolla, CA), and transfected into XL-1 Blue cells. The transfected cells were plated over plastic culture dishes to form plaques and transferred onto nylon membrane filters (Hybond-N; Amersham, Arlington Heights, IL). Hybridization was performed at 65°C for 16 hr in 6×SSC, 5×Denhardt's solution, 0.5% SDS, 20 μg/ml Salmon sperm DNA, and ^{32}P-labeled probe. After hybridization, the filters were washed in 0.2×SSC at 65°C for 20 min, and analyzed using a bio-image analyzer (BAS2000; Fuji Photo Film, Japan). Plaque-purified clones were rescued as pBluescript phagemids and sequenced using an ABI 377 automated DNA sequencer (Perkin-Elmer, Foster City, CA).

The oligo(dT)-primed library was screened with a cDNA probe encoding a highly conserved region of the repeat IV (P_{IV}), and the random-primed library was screened with a probe of the repeat I (P_I). The two probes were obtained by RT-PCR using the following primer pairs designed from the nucleotide sequence of the rat brain Na^+ channel; P_I, 5'-CC[A/T]CTGGAGGACCTGGACCCCTACTAT-3' (IS) corresponding to the peptide $_{76}$PLEDLDPYY and 5'-TGTGGCCTG-GTTCTGTTCCTC[A/G]TAGGC-3' (IAS) corresponding to the peptide $_{425}$AYEEQNQAT; P_{IV}, 5'-CTGGTGTTCATTGTGCTGTTCACCGGC-3' (IVS) corresponding to the peptide $_{1578}$LVFIVLFTG and 5'-GAC-AGCGATGTACATGTTCACCACCAC-3' (IVAS) corresponding to the peptide $_{1776}$VVVNMYIAV of the rat brain type I Na^+ channel (Noda *et al.*, 1986). After the reverse transcriptase step was carried out at 42°C for 15 min, a PCR reaction containing 200 nM of each primer was performed using a *GeneAmp* RNA PCR Kit (Perkin-Elmer, Norwalk, CT) under the conditions recommended by the manufacturer. The PCR amplification consisted of 105 sec at 95°C, 35 cycles of 15 sec at 95°C, 30 sec at 60°C and a final 7 min at 72°C. The PCR products were cloned into pCR™II vector using a TA-cloning Kit (Invitrogen, San Diego, CA), and sequenced.

RT-PCR amplification and cloning

cDNA spanning the blank region between the clone from the random-primed library and the clone from the oligo(dT)-primed library was generated by RT-PCR using *PfuTurbo* DNA polymerase (Stratagene), which has 3'–5' exonuclease activity (proofreading activity). First strand cDNA was synthesized from poly(A) RNA with oligo(dT) primer at 42°C for 15 min using a *GeneAmp* RNA PCR Kit (Perkin Elmer). The PCR reaction was performed in a 100 μl reaction solution with 200 nM each primer under the conditions recommended by the manufacturer (Stratagene). The amplification consisted of 1 min at 95°C, 30 cycles of 1 min at 95°C, 1 min at 60°C, 8 min 72°C, and a final 10 min at 72°C. The second PCR reaction was performed using 1 μl of the first PCR product for 20 cycles under the same thermal conditions. The sense primer (PS) was 5'-GGCACAGA-AACAGAAATGAAGAAG-3', corresponding to nt 2,244–2,267, and the antisense primer (PAS) was 5'-ATGCCATAAATCGTTCTTC-CATTG-3', complementary to nt 5866-5889. Agarose gel electrophoresis of the PCR product showed a single band of the expected size. Then, the PCR product was excised from the agarose gel, purified, cloned into pPCR-Script vector using a PCR-Script Amp Cloning Kit (Stratagene), and sequenced.

In situ hybridization

The cDNA fragment corresponding to nt 118–1,197 was obtained by RT-PCR, cloned into pCR™II vector using a TA-cloning Kit (Invitrogen), and confirmed by sequencing. The plasmids were linearized and transcribed with T7 or SP6 RNA polymerase separately, to obtain both antisense and sense probes. The probes were labeled with digoxigenin-labeled nucleotides using a DIG RNA Labeling Kit (Boehringer Mannheim, Indianapolis IN). To prepare newt retina cryosections, after removing the cornea and lens, the eye cups were fixed with 4% paraformaldehyde in PBS overnight at 4°C, cryoprotected by equilibration with 30% sucrose in PBS, and embedded in OCT compound (Tissue-Tek; Miles Inc., Elkhart, IN). 15 μm sections were cut on a cryostat at –20°C and mounted on gelatin-coated slides. The sections were treated with 10 μg/ml proteinase K (Boehringer Mannheim) for 10 min at 37°C, and then fixed with 4% paraformaldehyde in PBS for 30 min. Hybridization was carried out

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by incubation with probe (0.7 μg/ml) overnight at 50°C. Then, the sections were washed several times and treated with 20 μg/ml RNase A (QIAGEN Inc., Chatsworth, CA) in 500 mM NaCl, 10 mM Tris-HCl (pH8.0) for 30 min at 37°C, with final washes in 1×SSC, 50% formamide for 30 min at 45°C and for 30 min at room temperature. Digoxigenin was detected with alkaline phosphatase-labelled anti-digoxigenin antibodies using a Digoxigenin Detection Kit (Boehringer Mannheim). The specimens were examined and photographed using a transmitted light microscope (DMR; Leica Instruments, Germany).

RESULTS

Isolation and primary structure of the newt retina Na^+ channel

The cDNAs encoding highly conserved regions of repeats I and IV were amplified by RT-PCR with specific primer sets, [IS, IAS] and [IVS, IVAS], respectively. PCR products of the expected sizes were seen as single bands in 1% agarose gels. These were excised from the gels, and cloned into vector. Sequencing the DNA revealed a high sequence similarity to known Na^+ channels. Specifically, P_I, the product from primer pair [IS, IAS], had a deduced amino acid sequence similar (83%) to that in the repeat I of the rat brain type I Na^+ channel, and P_{IV}, the product from primer pair (IVS, IVAS), was similar (93%) to the repeat IV. These were used as probes for library screening.

One cDNA clone (R1), which had an identical sequence to the probe (P_I) in their overlapping region, was isolated from the random-primed cDNA library using P_I. One clone (T1) isolated from the oligo(dT)-primed cDNA library using P_{IV} also had an identical nucleotide sequence to the probe in their overlapping region. The rest of the coding region of the Na^+ channel between R1 and T1 was obtained by RT-PCR using *PfuTurbo* DNA polymerase (Stratagene), which has high 3'–5' exonuclease activity. The PCR product (P1) shares 419 and 798 bp overlapping sequences with R1 and T1, respectively. This shows that all the clones (R1, P1, and T1) are derived from a transcript of the same type of Na^+ channel. These clones collectively encompassed 8,378 bp of cDNA, which was designated nRNaCh. Fig. 1 depicts the positions of these overlapping clones, which constitute nRNaCh.

The complete nucleotide sequence of nRNaCh consists of a 227 bp 5'-untranslated sequence, an open reading frame of 6,024 bp, and an 2,127 bp 3'-untranslated region. The Met codon at nucleotide residues 228–230 was deemed the translation initiation site, because this is the first in-frame ATG triplet that appears downstream from the nonsense codon TAA (residues 207–209). This assignment is supported by the fact that the sequence around the ATG triplet agrees with the favored sequence for eukaryotic initiation sites, that is A/GXXAUGG, where X can be any nucleotide (Kozak, 1981). The 3'-untranslated region contains a potential polyadenylation signal (AATAAA) 21 bp before the poly(dA) region. The continuous open reading frame of 6,024 bp encodes 2,007 amino acid residues with a calculated molecular weight of 228, 397. Fig. 2 shows the nucleotide sequence and deduced amino acid sequence of nRNaCh. The amino acid sequence of nRNaCh predicts general structural features shared with other cloned Na^+ channels, 4 homologous repeating units with six putative transmembrane helices in each repeat. In general, the transmembrane segments are well conserved, whereas the putative cytoplasmic and extracellular loops are more divergent and contain a number of insertions or deletions. However, the cytoplasmic loop between S6 of repeat III and S1 of repeat IV(III-IV linker) is well conserved and contain IFM tripeptide motif (residues 1489–1491) that is essential for fast inactivation. The nRNaCh contains seven potential cAMP-dependent protein kinase (PKA) sites, nine potential N-linked glycosylation sites and one potential protein kinase C phosphorylation site in the III-IV linker (West, 1991). The length of the nRNaCh S5–S6 loop of repeat I is similar to that of TTX-sensitive channels, while there is a deletion in this region of TTX-resistant Na^+ channels. There is a non-aromatic amino acid (Ala) at the 3rd position in the SS2 region of repeat I of nRNaCh, the 386th residue of nRNaCh.

In situ hybridization analysis of Na^+ channel expression

We performed *in situ* hybridization using a digoxigenin-labeled cRNA probe to examine expression of nRNaCh within

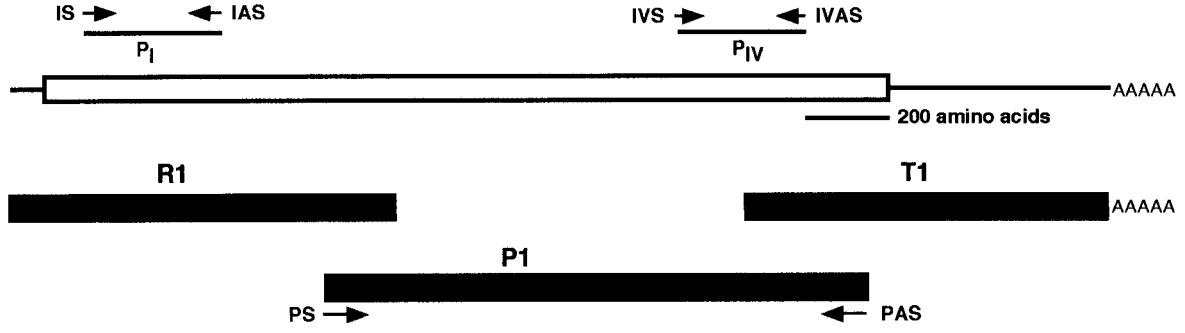

Fig. 1. The overlapping cDNA clones that constitute nRNaCh. The open box indicates the protein-coding region. Closed boxes represent each cDNA clone. The clone R1 (nt 1-2,686) was isolated from the random-primed library using the probe P_I and the clone T1 (nt 5,068-8,378) was isolated from the oligo(dT)-primed library with the probe P_{IV}. The solid lines indicate the probes. P_I was obtained by RT-PCR using primers IS and IAS, P_{IV} was obtained using primers IVS and IVAS. The cDNA corresponding to the remaining portion of nRNaCh between R1 and T1 was isolated by RT-PCR (P1, nt 2,268–5,865) using primers PS and PAS.

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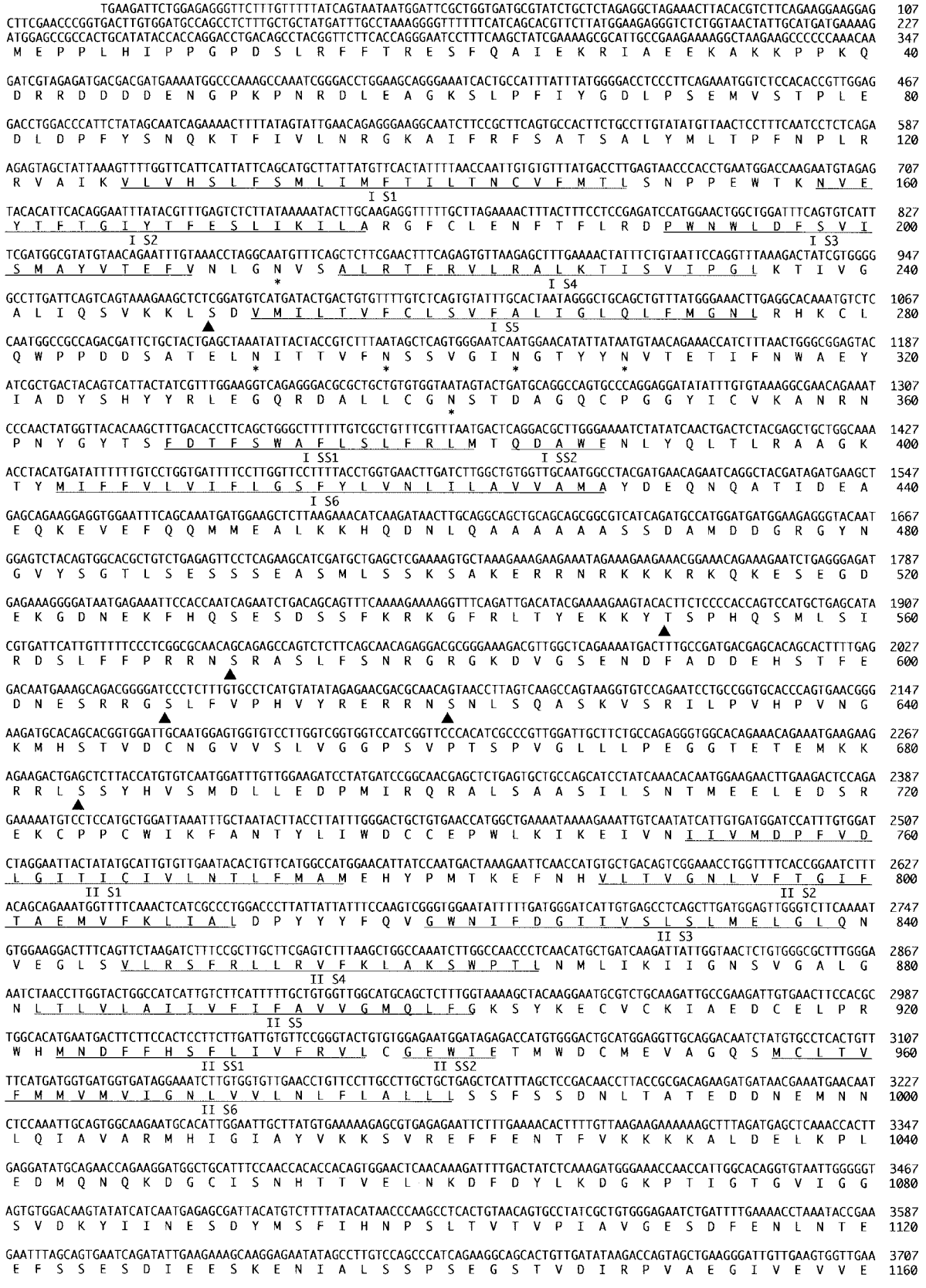

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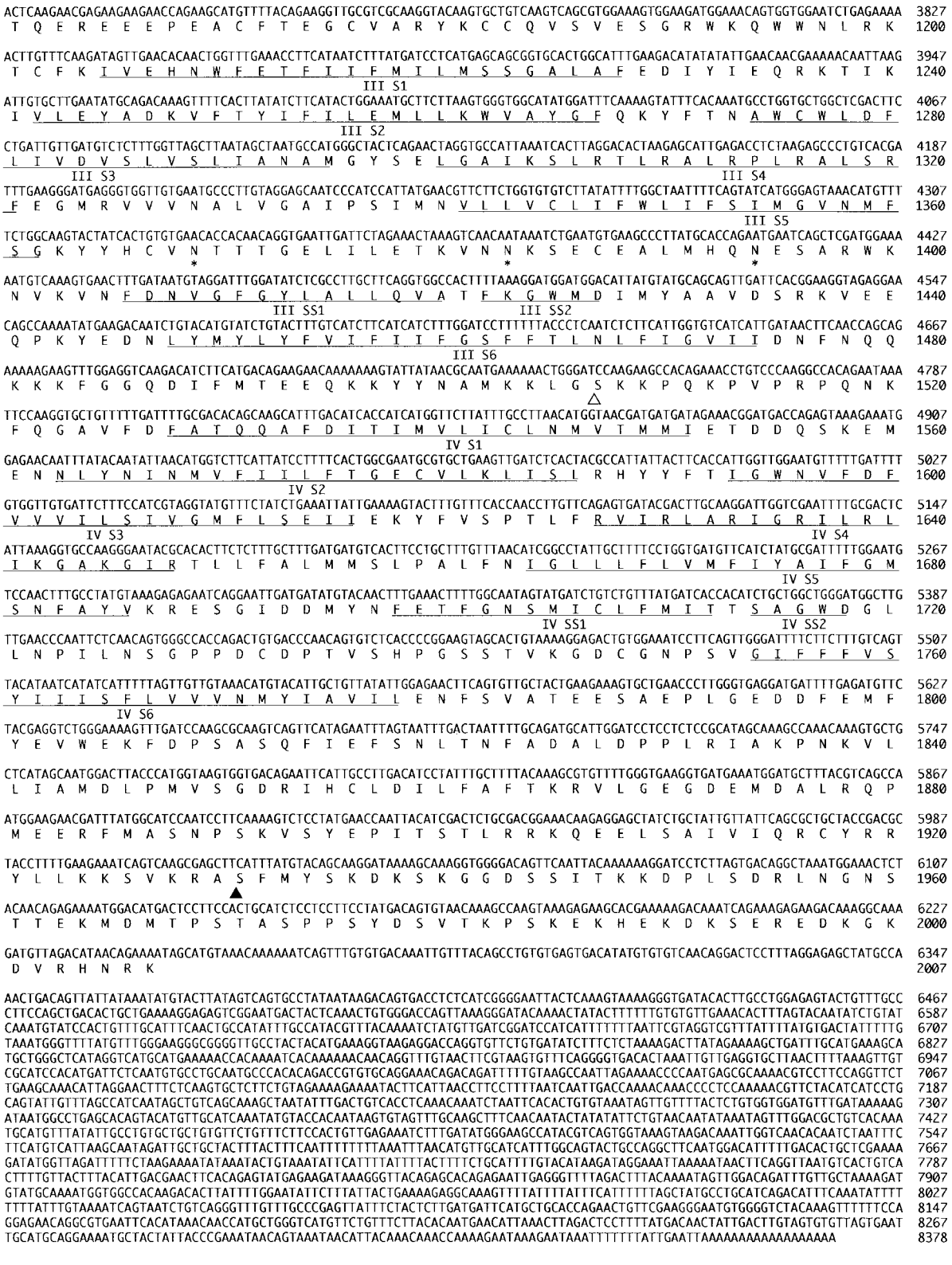

Fig. 2. Nucleotide sequence of the cDNA encoding the putative newt retina sodium channel (above) and its deduced amino acid sequence (below). The complete 8,378 nucleotide sequence has been deposited in the GenBank database with the accession number AF123593. The sequence was determined from the clones R1, P1, and T1 (Fig. 1). The suggested location of six transmembrane helices and SS1-SS2 region within each repeat are designated by horizontal lines (Noda *et al.*, 1986; Guy and Conti, 1990). Predicted intracellular cAMP-dependent protein kinase (PKA) sites are indicated with closed triangles (▲), predicted extracellular N-glycosylation sites are indicated with asterisks (*) and the protein kinase C site involved with modulation of channel activity (West *et al.*, 1991) is indicated with an open triangle (△).

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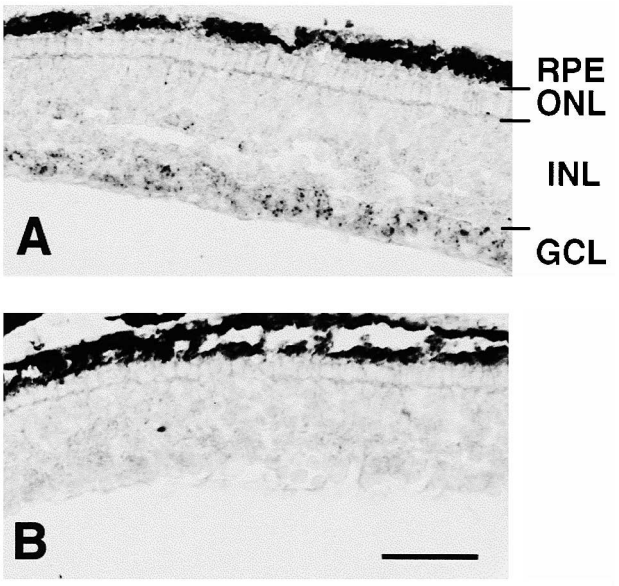

Fig. 3. *In situ* hybridization showing nRNaCh expression in the newt retina. (**A**) nRNaCh expression is observed in the ganglion cell layer of the retina using DIG-labeled antisense nRNaCh probe. (**B**) The control is not stained with the sense probe. Abbreviations: RPE, retinal pigment epithelial cell layer; ONL, outer nuclear cell layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar, 100 μm.

the retina (Fig. 3). In sections of newt eye, antisense probe distinctly hybridized to the ganglion cell layer throughout the retina, from the central to peripheral regions. It was reported that the ganglion cell layer consists of spiking neurons; ganglion cells and displaced amacrine cells (Ball and Dickson, 1983). Thus, nRNaCh is expressed primarily in ganglion cells and displaced amacrine cells. Weak signals were detected on the vitreal side of the inner nuclear layer, where amacrine cells and displaced ganglion cells are located (Ball and Dickson, 1983). Therefore, nRNaCh is expressed exclusively in spiking neurons throughout the newt retina.

Comparison with other Na^+ channels

The sequences of vertebrate Na^+ channels, for which TTX sensitivity is known, were aligned using the program GENETYX (Software Development Co., Ltd. Japan). Using the UPGMA method, the simple relationship shown in Fig. 4 was obtained from the alignment. The sequences fall into two obvious groups, the TTX-R group, which are the TTX-resistant channels less nRNaCh, and the TTX-S group, which are the TTX-sensitive channels plus nRNaCh. Although nRNaCh is TTX resistant, it is more homologous to channels in the TTX-S group.

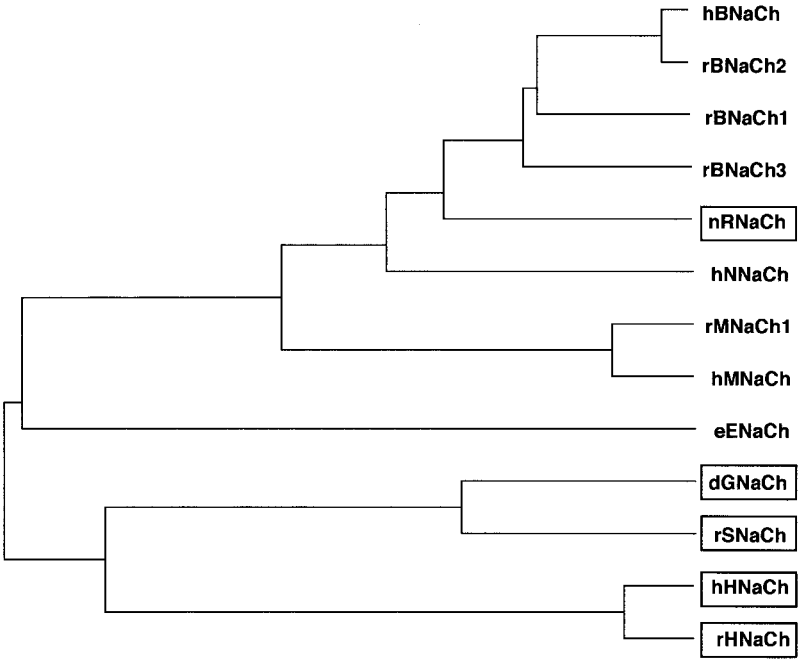

Fig. 4. A comparison of vertebrate Na^+ channel sequences with known TTX sensitivity. The amino acid sequences were aligned using the program GENETYX. The genetic distances between the sequences were estimated using the UPGMA method and are reflected in the length of the horizontal lines. Sequences: hBNaCh, human brain TTX-sensitive channel (Ahmed *et al.*, 1992); rBNaCh2, rat brain type II TTX-sensitive channel (Noda *et al.*, 1986); rBNaCh1, rat brain type I TTX-sensitive channel (Noda *et al.*, 1986); rBNaCh3, rat brain type III TTX-sensitive channel (Kayano *et al.*, 1988); nRNaCh, newt retina TTX-resistant channel; hNNaCh, human neuroendocrine cell TTX-sensitive channel (Klugbauer *et al.*, 1995); rMNaCh1, rat skeletal muscle TTX-sensitive channel (Trimmer *et al.*, 1989); hMNaCh, human skeletal muscle TTX-sensitive channel (George *et al.*, 1992); eENaCh, eel electroplax TTX-sensitive channel (Noda *et al.*, 1984); dGNaCh, dog nodose ganglion neuron TTX-resistant channel (Chen *et al.*, 1997); rSNaCh, rat sensory neuron TTX-resistant channel (Akopian *et al.*, 1996; Sangameswaran *et al.*, 1996); hHNaCh, human heart TTX-resistant channel (Gellens *et al.*, 1992); rHNaCh, rat heart TTX-resistant channel (Rogart *et al.*, 1989; Kallen *et al.*, 1990);. The TTX-resistant channels are in boxes.

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DISCUSSION

The newt is one of the animals that has TTX in its tissue, and it has been shown electrophysiologically that the Na^+ channels in the newt retina are TTX-resistant (Kaneko and Saito, 1992; Kaneko *et al*., 1997). In the partial sequences of the newt retina Na^+ channel determined by RT-PCR, (i) the 2nd and 5th amino acids in each SS2 region are identical to those in other known Na^+ channels and (ii) the amino acid (Ala) at the 3rd position in the SS2 region of repeat I is non-aromatic (Kaneko *et al*., 1997). Therefore, it seems likely that the Na^+ channels in the TTX-carrying newt acquired TTX resistance that has the same mechanism as that of other TTX-resistant channels. Initially, the entire primary structure of nRNaCh was not known (the sequence was limited to the SS2 region of each repeat), however, so we could not determine whether the deletion within the S5-S6 loop of repeat I (characteristic of TTX-resistant channels) or the overall structural features affected the TTX sensitivity. Therefore, we cloned the Na^+ channel from the newt retina, deduced its primary structure, and analyzed the expression of the channel by *in situ* hybridization.

cDNA clones spanning the entire coding region of a Na^+ channel were isolated from the newt retina cDNA library with the use of RT-PCR. The coding region predicts a 2,007 amino acid protein, designated nRNaCh, which is homologous to other Na^+ channels. *In situ* hybridization analysis demonstrated that nRNaCh is expressed exclusively in spiking neurons throughout the newt retina (Fig. 3). It was reported that the voltage-dependent Na^+ current in newt retina spiking neurons is TTX-resistant electrophysiologically (Kaneko *et al*., 1997). Therefore, nRNaCh cDNA is sure to encode the TTX-resistant Na^+ channel of spiking neurons. Fig. 4 shows the relationship between the amino acid sequences of vertebrate Na^+ channels with known TTX sensitivity. With the exception of nRNaCh, these Na^+ channels can be classified into two groups based on the similarity between sequences. One group consists of the TTX-sensitive channels, while the other consists of the TTX-resistant channels. This implies that the overall structure of the Na^+ channel has an effect on TTX sensitivity. This is not the case, however, because nRNaCh is more homologous to the TTX-sensitive Na^+ channels and the length of its S5–S6 loop of repeat I is similar to that of TTX-sensitive channels, although it is TTX-resistant. These results suggest that TTX sensitivity does not depend on the overall structural features of the channel or on the deletion within the S5-S6 loop of repeat I. In nRNaCh, the 3rd residue within the SS2 region of repeat I, located at position 386, has a non-aromatic amino acid (Ala), which is a common characteristic of TTX-resistant channels. Therefore, it seems as though TTX resistance is determined by whether the amino acid at this position is aromatic or non-aromatic. The two clusters of similar sequences (Fig. 4) suggest that vertebrate Na^+ channels diverged into two groups in the course of evolution. Both groups include TTX-resistant channels, so it is likely that TTX resistance was acquired independently after the two groups of Na^+ channels diverged.

The partial sequences of the Na^+ channels from the brain of the puffer fish (a TTX-carrying animal) have been reported (Shahjahan *et al*., 1993). The amino acid at the 3rd residue within the SS2 region of repeat I of these channels is aromatic (Phe), which contradicts the principle mentioned above. However, it is not certain whether the Na^+ channels of the puffer brain are indeed TTX resistant. If this is the case, there might be different ways for Na^+ channels to acquire TTX resistance, other than by having a non-aromatic amino acid at the 3rd residue within the SS2 region of repeat I.

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